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Additional Records of *Dactylogyrus* (Monogenea) from Some Cyprinid Fishes from Darbandikhan Lake, Iraq

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Abstract

A total of 48 fresh water fishes, belonging to four species of the family Cyprinidae (*Capoeta trutta*, *Chalcalburnus mossulensis*, *Cyprinion macrostomum* and *Leuciscus lepidus*) were collected from Darbandikhan lake, southwest Sulaimaniya city, Kurdistan region, in the north of Iraq, from March to the end of July 2008. The inspection of gills revealed the infection of these fishes with four species of monogenetic trematoda belonging to genus *Dactylogyrus* namely: *D. alatus*, *D. cyprinioni*, *D. macracanthus* and *D. microcirrus*. The record of these species in the present study is considered as the first record in Iraq.

الملخص

تم جمع 48 سمكة تعود إلى اربعة انواع من الأسماك الشبوطية (*Capoeta trutta* و *Chalcalburnus mossulensis* و *Cyprinion macrostomum* و *Leuciscus lepidus*) من بحيرة دربنديخان في جنوب الغربي من مدينة السليمانية، أقليم كوردستان في شمال العراق، خلال الفترة المحصورة بين شهر أذار الى نهاية شهرتموز 2008. فحصت الأسماك للتعرف على المخرمات أحادية المنشأ من الجنس *Dactylogyrus* التي تصيبها. أظهرت النتائج وجود اربعة انواع من هذا الجنس وهي: *D. alatus* و *D. cyprinioni* و *D. macracanthus* و *D. microcirrus*. ان تسجيل الانواع الاربعة في الدراسة الحالية تعد اول تسجيل في العراق.

Keywords: *Dactylogyrus*; Cyprinid fish; Darbandikhan lake; Iraq

1. Introduction

The Cyprinidae is one of the largest families of teleosts in the world comprising at least 1700 species and over 200 genera. Natural populations of cyprinids are widely distributed in most freshwater rivers, lakes and ponds in world (Hoole *et al.*, 2001). Thirty six species occur in the fresh waters of Iraq (Coad, 2008), and 21 species have been recorded from Darbandikhan lake (Abdullah *et al.*, 2007).

Dactylogyrus spp. (belonging to Class: Monogenea, Phylum: Platyhelminthes) are ectoparasites living on the gills, present the largest group of fish parasites and major importance in the pathology of fishes (Woo, 2006). Young fishes are subjected to the risk of infection with these parasites which might cause diseases and mortalities among fry in hatcheries, and among larger fishes (Amlacher, 1970). For example *D. vastator* caused great damage to the gill filaments of carps and goldfishes in California hatcheries (Hoffman, 1998).

The first information on genus *Dactylogyrus* from the Iraqian freshwater fish was given by Ali *et al.* (1987), who recorded two species from river Tigris in Baghdad, *D. vastator* from *Cyprinion macrostomum* and *D. cornu* from *C. macrostomum*, *Barbus xanthopterus* and *Acanthobrama centisquama*. The checklist of parasites of fishes from Iraq includes 104 species of monogenea belonging to 21 genera, with the highest number (59) of species in *Dactylogyrus* (Mhaisen, 2009).

This is the second paper in a series on trials by the author to known the parasitic fauna of some freshwater fishes collected from Darbandikhan lake. In the first trial, the author surveyed the parasitic fauna of 17 species of fishes, and he recorded 19 species of parasites which included two species of protozoans, nine species of monogenetic trematodes, three species of cestodes, one species of nematode larvae and four species of crustaceans (Abdullah, 2005). In this paper sheds light on monogenea only parasitizing some cyprind fishes obtained from the lake.

2. Materials and Methods

2.1. Study Area:

Darbandikhan lake is located about 76km southwest Sulaimaniya city, Kurdistan region, in the north of Iraq. It is situated between 35º- 36º north latitude and 45º- 46º east longitude. With the altitude of 511 meters above sea's level. The surface area is about 121km² and the lake capacity is 3 million m³.

2.2. Sampling:

The fish specimens taken by gill netting, cast netting, electrofishing, hook or bow-net by local commercial fisherman during the period from March to the end of July 2008.

Fish were put in placed tanks with local river water and immediately transferred to the laboratory as soon as

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possible and were examined within 24 hours after their capture. Fishes were identified according to Froese and Pauly (2008).

In the laboratory, the gill arches from both sides were separated, kept moist in Petri dish, examined under an Olympus dissecting microscope for counting Dactylogyrus on each gill lamella. Worms removed from the water by a small pipette and placed onto a slide, with a very small amount of water, then were stained with aqueous neutral red, and permanent slides were prepared with glycerol-gelatin (Gussev et al., 1993a).

Parasites were identified according to shape of the sclerotized parts of the haptor (median hooks or anchors, connecting bars, supplementary bars and hooklet or marginal hooks) and reproductive organs (copulatory organ and vaginal armament), using light microscope equipped. The measurement of parasites was achieved by ocular micrometer, and the terminology (Fig. 1) was used as recommended by Gussev (1985) and Jarkovskỳ *et al.* (2004). The figures were drawn by using a Camera Lucida (Drawing tube). Parasite identification was done according to Bykhovskaya-Pavlovskaya *et al*. (1962) and Gussev (1985).

Each of scale bars in the figures corresponds to 0.02 mm. Designations in the figures are: cb= connective bar, co= copulatory organ, hl= hooklet, mh= median hook, sb= supplementary bar.

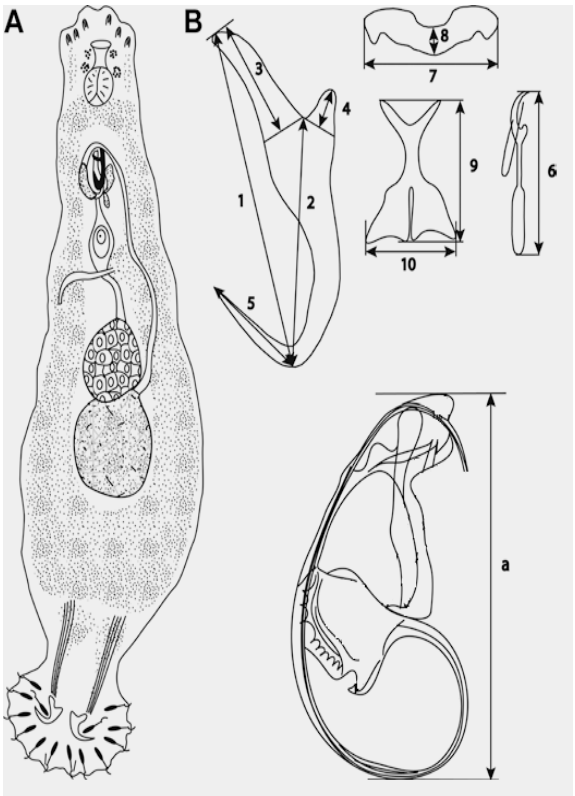

Figure 1 a. *Dactylogyrus* overall view according to Jarkovskỳ *et al.* (2004).

3. Results

A total of 48 fishes, belonged to four species of the family Cyprinidae, were collected from Darbandikhan lake. The inspection of fishes revealed their infection with four species of the genus *Dactylogyrus*. The distribution of these parasites and the prevalence are summarized in Table (1). The following is an account on description and measurements of these parasites.

Table 1. The distribution of *Dactylogyrus* from the gills of fish hosts from Darbandikhan Lake.

Species of the *Dactylogyrus*	Host	No. of fishes examined/ infected	Prevalence %	Mean intensity (range)
D. alatus	*Chalcalburnus mossulensis*	3 / 1	33.33	3
D. cyprinioni	*Cyprimon macrostomum*	20 / 3	15	5 (4-6)
D.macracanthus	*Leuciscus lepidus*	15 / 3	20	4.3 (3-7)
D. microcirrus	*Capoeta trutta*	10 / 2	20	4 (2-6)

The following is an account on description and measurements of these parasites**:**

- *Dactylogyrus alatus* Linstow, 1878
- Host: *Chalcalburnus mossulensis* (Heckel, 1843)
- Site infection: Gill filaments
- Prevalence: 33.33% (Table 1).
- Locality: Darbandikhan lake.
- Description: Large worms, length: 0.8-1.2mm, width: 0.22-0.28mm. Length of hooklet: 0.018-0.03mm. Median hooks massive, with powerful processes, particularly inner, which expand terminally, their length equal to base or larger, external process at least half as large as base. Overall length of median hooks 0.042-0.055mm, shaft 0.014-0.018mm, point 0.008-0.01mm, inner root 0.014-0.016mm, outer root 0.006-0.008mm. Connecting bar: 0.005-0.0055 x 0.03-0.038mm. Supplementary bar: 0.004-0.0048 x 0.02-0.024mm. Copulatory organ: about 0.038-0.045mm, diameter of tube: somewhat over 0.002mm (Fig. 2).

b. Metric parameters of the *Dactylogyrus* attachment apparatus and copulatory organ: 1= total median hook length, 2= shaft of median hook, 3= inner root of median hook, 4= outer root of median hook, 5= point of median hook, 6= length of hooklet, 7= length of connecting bar, 8= width of connecting bar, 9= length of supplementary bar, 10= width of supplementary bar, a= total length of copulatory organ.

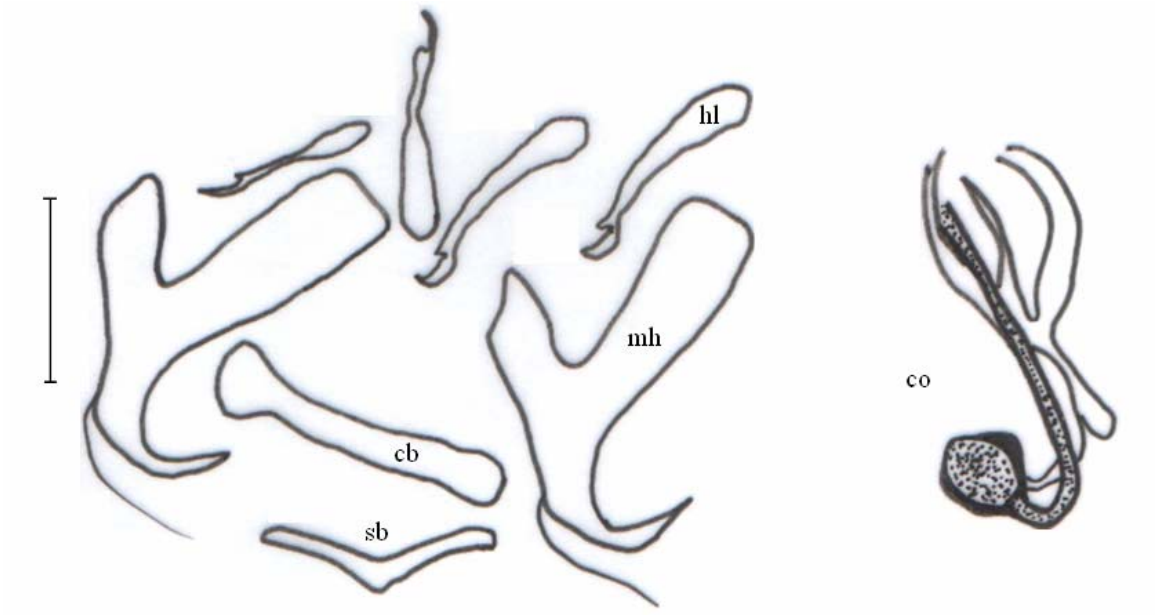

Figure 2. *Dactylogyrus Alatus*.

- *Dactylogyrus cyprinioni* Gussev, Jalali et Molnár, 1993
- Host: *Cyprimon macrostomum* (Heckel, 1843)
- Site infection: Gill filaments
- Prevalence: 15% (Table 1).
- Locality: Darbandikhan lake.
- Description: Worms of medium size with a length of 0.5-0.65mm, and width: 0.12-0.22mm. Length of hooklet of 0.015-0.028mm. Median hook of wunderi-type, with well-developed roots and with a short recurved point. The total length of median hook: 0.030-0.040mm, shaft: 0.025-0.03mm, inner root: 0.010-0.012mm, outer root: 0.003-0.004mm, point: 0.007-0.0075mm. Connecting bar of wunderi-type, but with a small posterior process in the middle and with a membranous tuft on the posterior edge, 0.003-0.004 x 0.025-0.03mm in size. Supplementary bar: sticklike, with enlarged central part, 0.002-0.0025 x 0.016-0.022mm in size. Length of copulatory organ: 0.03-0.04mm (Fig. 3).

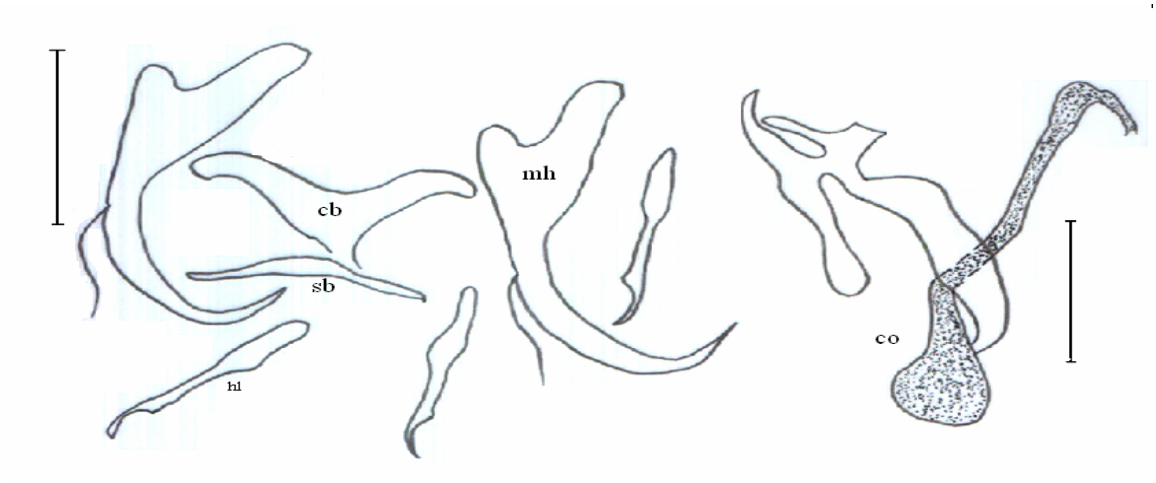

Figure 3. *Dactylogyrus cyprinioni*.

- *Dactylogyrus macracanthus* Wegener, 1909
- Host*: Leuciscus lepidus* (Heckel, 1843)
- Site infection: Gill filaments
- Prevalence: 20% (Table 1).
- Locality: Darbandikhan lake.
- Description: Large worms, length: 0.7-1.2mm, width: 0.2-0.25mm. Length of hooklet: 0.025-0.035mm. Median hooks massive, with powerful processes, particularly inner, which expand terminally, their length equal to base or larger,

external process at least half as large as base. Overall length of median hooks 0.045-0.052mm, shaft: 0.013-0.017mm, point: 0.007-0.01mm, inner root: 0.012-0.015mm, outer root: 0.005-0.007mm. Connecting bar: 0.01-0.012 x 0.03-0.04mm. Supplementary bar: 0.002-0.0025 x 0.02-0.026mm. Copulatory organ: about 0.045-0.055mm (Fig. 4).

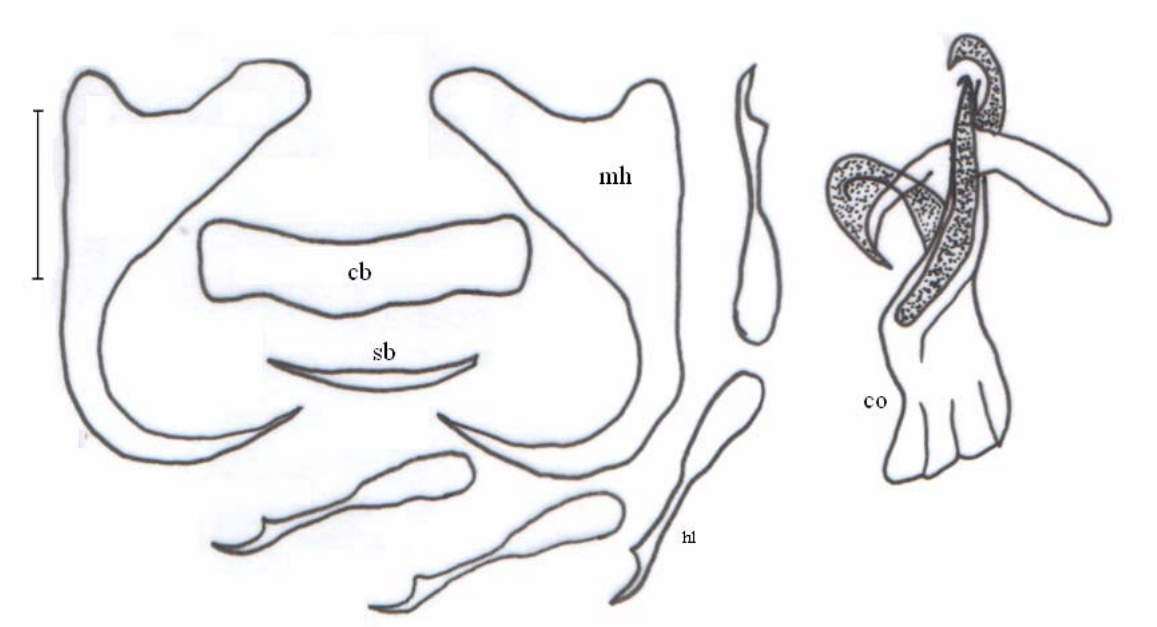

Figure 4. *Dactylogyrus macracanthus*.

- *Dactylogyrus microcirrus* Gussev, Jalali et Molnár, 1993
- Host: *Capoeta trutta* (Heckel, 1843)
- Site infection: Gill filaments
- Prevalence: 25% (Table 1).
- Locality: Darbandikhan Lake.
- Description: Worms of small size with a length of 0.30-0.40mm, and a width of 0.05-0.09mm. Hooklet: long with distinct heel of point and with an enlarged handle of similar size or slightly longer than the pivot. The total length of hooklet: 0.015-0.03mm. The total length of median hook: 0.032-0.048mm, shaft: 0.02-0.025mm, point: 0.008-0.01mm, inner root: 0.001-0.012mm, outer root: 0.002-0.0025mm. Connecting bar: bent, with tuft on the posterior edge. Size: 0.003-0.004 x 0.015-0.018mm. Supplementary bar: rodlike, slightly enlarged in the middle. Size: 0.0015-0.002 x 0.015-0.018mm. Copulatory organ: very small, with an elongated almost cylindrical tube, vesicula-like or triangular at the initial part. Accessory piece: Y-shaped, with thin, approximately parallel-running wings. Length of copulatory organ: 0.012-0.015mm (Fig. 5).

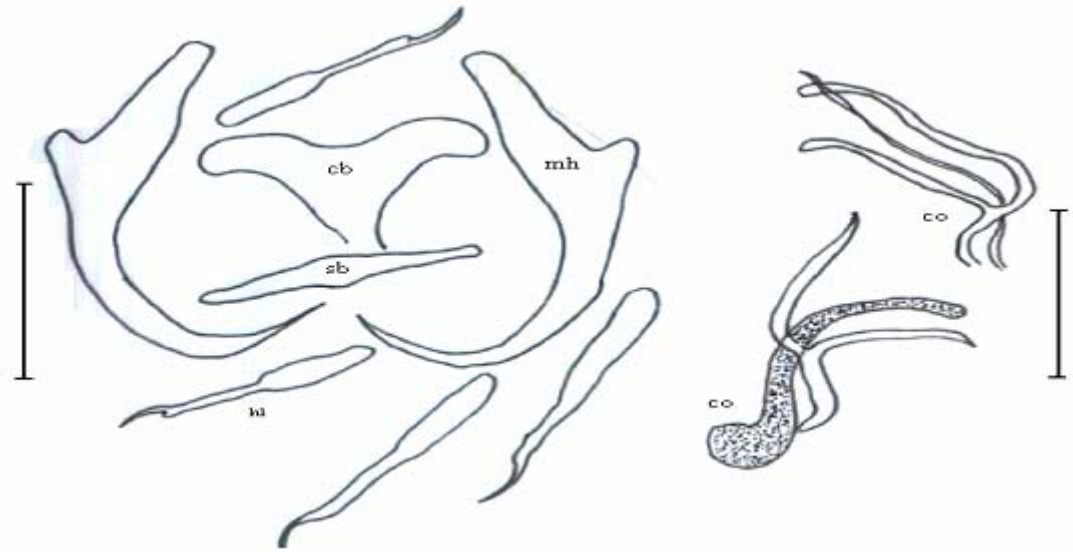

Figure 5. *Dactylogyrus microcirrus*.

4. Discussion

In the previous study conducted on the parasites of fishes from Darbandikhan lake, six species of *Dactylogyrus* were recoded namely: *D. barbuli* from *Barbus barbulus, D. extensus* and *D. vastator* from *Cyprinus carpio*, *D. hypophthalmichthys* from *Hypophthalmichthys molitrix, D. pavlovskyi* from *Barbus grypus* and *D. vistulae* from *Leuciscus lepidus* (Abdullah, 2005). In this study four species of *Dactylogyrus (D. alatus, D. cyprinioni, D. macracanthus and D. microcirrus*) were found on the gills of five fish species in this lake.

The description and measurements of *D. alatus* which recorded in the present study are nearly similar to those reported by Bykhovskaya-Pavlovskaya et al. (1962), who detected from gill filaments of Siberian roach Rutilus rutilus from waters of Kazakh in U.S.S.R..

D. alatus is a widely distributed parasite of cyprinid fishes, has been reported from *Abramis alburnus* in Poland (Prost, 1972), *Alburnoides bipunctatus*, *Alburnus charosinii* and *Chalcalburnus chalcoides* from Iran (Molnar and Jalali, 1992), *Abramis alburnus* from Turkey (Koyun, 2001), *Leuciscus idus* from Central Europe (Ondračkova et al., 2004).

The present specimens of *D. cyprinioni* are similar to those of Gussev et al. (1993b), who described as a new species on gills of the same host from Kharoon River, water system of Tigris River, Iran.

The classification of *D. macracanthus* was confirmed due to the coincidence of the characters described here with those reported by Bykhovskaya-Pavlovskaya et al. (1962), who recorded it for the first time from tench Tinca tinca collected in Europe and U.S.S.R.. This species also was recorded from the gills of Leuciscus cephalus from Karoon River and Dez River in Iran (Shamsi and Jalali, 1997)

The morphological data of the species D. microcirrus fall within the size ranges given by Gussev et al. (1993b), who detected it from gills of the same host from Dez River, water system of Tigris River, Iran. it seems to be the geographical distribution of D. cyprinioni and D. microcirrus is limited, only exist in the Tigris and Euphrates basin.

It has been noticed that the four species which were listed in the current study showed high host specific. Each one was found on a single host. Even in studies which were carried out in the outside of Iraq, were recorded either on the same host or a specific number of hosts. In generally, most species *of Dactylogyrus* are strictly host-specific, which were limited to a single or a few closely related hosts (Kearn, 1994).

So far, more than 59 species belong to the genus Dactylogyrus have been reported from wild and farmed fishes of Iraq, of the 50 species reported from Cyprinid fish (Mhaisen, 2009). Only one species (D. fallax) was recorded on the gills of *Chalcalburnus mossulensis* from Greater Zab river (Abdullah, 2008). Six species (D. *achmerowi*, *D. cornu, D. latituba, D. macrostomi, D. pulcher and D. vastator*) were found on the gills of *Cyprinion macrostomum* (Abdullah and Mhaisen, 2000). Two species (*D. vastator and D. vistulae*) were reported on the gills of *Leuciscus lepidus* (Abdullah and Mhaisen, 2004), and no infection from species *D. alatus, D. cyprinioni, D. macracanthus* and *D. microcirrus* in Iraq, the present record represents the first one in this country.

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Sublethal Effects of Tobacco (*Nicotiana Tobaccum*) Leaf Dust on Enzymatic Activities of *Heteroclarias* (a Hybrid of *Heterobranchus Bidorsalis* and *Clarias Gariepinus*)

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Abstract

Tobacco (*Nicotiana* tobaccum) leaf dust has both piscicidal and fertilizer properties. Thus there is a need to study its effects at sub-lethal concentrations (375.60, 187.80, 93.90 0.0mg/L) on some enzymatic activities of *Heteroclarias* in a static semi-renewable bioassay system with the aim to ascertain its effect on the test fish after the 14 days exposure period. Water quality parameters and physiological parameters were monitored/determined according to standard procedures. Water quality parameters were monitored after 48hours with the exception of temperature which was monitored daily. The monitored water quality parameters such as temperature, free carbon (iv) oxide, pH and dissolved oxygen were significantly decreased while total alkalinity and conductivity increased significantly in the exposed media, compared to the control test. The effects of the plant dust on the test fish was dose-dependent, revealing insignificant difference in alkaline and acid phosphatases, aspartate and alanine aminotransferases and gamma glutamyltransferase while significant difference was observed in lactate dehydrogenase in serum, liver and kidney respectively of the fish exposed to the plant dust, compared to the control after the 14 days exposure period. From the determined enzymatic activities, the effect of the plant dust was most pronounced in the kidney, but less in the liver and in the serum. However, the monitored water quality parameters revealed that the plant dust has effects on primary productivity, and consequently the biodiversity of organisms.

Keywords: Tobacco Leaf Dust, Enzymatic Activities, *Heteroclarias*, Serum, Liver and Kidney.

1. Introduction

Aquaculture is increasingly becoming one of the fastest growing aspect of the agricultural industry worldwide (FAO, 2004). In semi-intensive system of farming, the management of water pond weeds is one of the most important aspects of a successful production system (Noga, 1996). However, the presence of predatory fishes; 'weed' fishes, such as *Chaoborus* larva, tadpoles, frogs and leeches in fish culture ponds is a serious problem in aquaculture, which is due to their faster growth rate, as they share and better utilize cultured habitats and their food (Jhingran 1983). Therefore, removal of predators/weed fishes from pond is necessary before the seed of cultured fish is added. For controlling these predators, fish farmers often use tobacco leaf in controlling these unwanted organisms/pests (Konar, 1970, Tobor, 1990). According to Aleem (1987), the use of tobacco leaf dust is due to its inexpensiveness, local availability and easier degradability. Despite, the effective use of this plant material, eco-toxicologists are interested in the ecotoxic properties of plant origin pesticides/piscicides, such that plant origin pesticides / piscicides cannot be used directly in freshwater bodies unless their toxicity and sublethal long term effect have been studied on non-target animals, sharing the habitat with the target animals.

The most valuable part / active ingredient of the plant used, is the nicotine (Hassal, 1982). Nicotine (C_5H_4N)-CH-$(CH_2)_3$-N-(CH_3) is made up of pyridine and pyrroliding ring. Nearly all the nicotine is produced in the root and transported to the leaves for storage. It is soluble in water, alcohol, chloroform, ether, kerosene and some fixed oils (Vogue, 1984). Tobacco leaf dust has been used in Nigeria as an effective insecticides and treatment of predators/pest in water (pond) since it is completely biodegradable (Aleem, 1987; Tobor, 1990).

Heteroclarias is a hybrid of the African catfish *Clarias gariepinus* and *Heterobranchus bidorsalis.* They are omnivores which are desirable as food valuable species world-wide. They are one of the commercially important species of fish for rapid aquaculture expansion in Nigeria and elsewhere in the developing world.

Omoniyi *et al.,* (2002) and Agbon *et al.,* (2002) had reported the effect of tobacco leaf dust on *Clarias gariepinus*. Control of mollusk in fish pond can be accomplished by using tobacco waste (FAO, 1970). In Taiwan, tobacco waste dust is applied at 1 ton acre as a pesticides and fertilizer in fish ponds (Jhingran, 1975).

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The choice of the test fish *Heteroclarias* is attributed to the report of Rand *et al.,* (1995) that in order to extrapolate meaningful, relevant and ecological significant results from aquatic toxicity tests, not only appropriate test but also appropriate organism should be used, whenever possible, species should be studied if indigenous to, or representative of the ecosystem that may be impacted; thus the choice of the *Heteroclarias* which constitutes one of the main fish species is of economic importance in the Niger Delta as an abundant cultural fish species in Nigeria and is very popular with fish farmers and consumers.

The knowledge of sublethal effects of xenobiotic compounds on enzymatic activities is very important to delineate the health of fish status and to provide a future understanding of ecological impacts (Radhaiah *et al.,* 1987). The activities of enzymatic activities are useful 'markers' physiological of damage to the organs such as the liver and hepatocytes (Chapatwala *et al.,* 1982; Ngaha, 1982; and Akanji *et al.,* 1993), thus are needed to be assayed in the test fish.

The aim of this research is to ascertain the assumption whether tobacco leaf dust (*Nicotina tobaccum*) in a sublethal concentration and in a medium exposure time can influence changes in the values of enzymatic activities (alkaline and acid phosphatases, aspartate and alanine aminotransferases, lactate dehydrogenase and gamma glutamyltransferase) in serum, liver and kidney of the test fish – *Heteroclarias* and which of the examined tissue/organs (serum, liver and kidney) determined enzymatic activities after the sublethal exposure is most affected by the pesticide/piscicides – tobacco leaf dust after the 14-day exposure period.

2. Materials And Methods

2.1. Experimental Fish

Juvenile of the fish *Heteroclarias* of mixed sex and the same brood stock: mean weight (29.07±0.34g) and length (17.35±0.23cm) were obtained from Asaba fish farm in Emede, Isoko Local Government Area of Delta State, Nigeria. They were transported to the Department of Animal and Environmental Biology Research Laboratory, Delta State University at the early hours of the morning (6.00 – 8.00 hour) in a large plastic container. The fishes were acclimatized for 14 days during which they were fed to satiation with commercial fish feed pellets (copens 2.0mm) twice daily. Left-over feed and faeces were siphoned off promptly and dead fish were promptly removed to avoid contamination. The percentage of death recorded during acclimatization was less than 2% as such the fishes were accepted as being adapted to the laboratory conditions. They were then transferred to the experimental plastic aquaria (ten (10) fish/40L aquaria).

2.2. Tobacco Leaf Dust

The leaves of tobacco (*Nicotiana tobaccum*) were obtained from Apinko garden, Lokoja, Kogi State, Nigeria, which was identified by Dr. S. M. Ayodele of the Department of Botany, Delta State University, Abraka, Nigeria. The collected sample leaves where sun dried for 14 days (as has been practiced by the fish farmers) and grounded into powder with the use of laboratory mortar, and pestle, and sieved before been stored in a sealed plastic container until required. The concentrations of tobacco used for the experimentation were calculated as 50% 96 h LC_{50}, 25% 96 h LC_{50} and 12.5% 96 h LC_{50} (96 h LC_{50} of tobacco leaf dust on the *Heteroclarias* obtained from preliminary investigation was 751.20mg/L). Thus 375.60, 187.80 and 93.90mg of tobacco leaf dust were measured and homogenously mixed in 1 liter of water to give 375.60, 187.80, 93.90 0.0mg/L concentration of the tobacco leaf dust. These concentrations were introduced into four (4) sets of aquaria with one replication.

2.3. Experimental Procedure

Forty (40) liters capacity aquaria were maintained throughout the exposure period. Ten (10) juveniles each were placed in the 40L plastic aquarium. Bore-hole water was used during the acclimatization and exposure period. Feeding regime (0.800 and 1800 hours) during exposure period was the same as that of acclimatization period. In order to monitor the toxicant strength, level of dissolved oxygen, the effects of evaporation; ammonia concentration and reduce stress during experimentation, the test media were replaced by 50% prepared – concentrations of the same quantity after removing its equivalent along with undigested food and defaecation every 48 hours to maintain the requisite level and potency of the concentration. The exposure period lasted for fourteen (14) days during which some water quality parameters were monitored after 48 hours with the exception of temperature which was monitored daily by using the method described by APHA (1998). At the start (0 hour) of the experiment, the sum total of ten (10) fishes was sacrificed and analyzed for the enzymatic activities.

2.4. Sampling Techniques

Blood was obtained from randomly selected fifteen (15) fish from the control and the exposed test after the 14 days exposure period, using 2.0ml plastic syringe. This was done as described in Kori-Siakpere, (1998). The blood was transferred into a lithium heparin anticoagulant tube and allowed to clot at room temperature for 30 – 40 minutes (Mahoba, 1987). Serum was thereof obtained by centrifugation, using Hawkley centrifuge for 10 minutes at 3,000rpm (Ogbu and Okechukwu, 2001). The serum was transferred into anticoagulant free test-tube and stored at refrigerator until analyses.

After blood collection, the fishes were sacrificed. The desired organs (liver and kidney) were removed from the fish and pulverized in a laboratory mortar and pestle while extractions were prepared by adding 2ml of 10% sucrose solution before been centrifuged (Mahoba, 1987) and stored in another test-tube in the refrigerator until analyses.

2.5. Data Analysis

2.6. All data were presented as means ± standard error, the data from the 14-day tobacco leaf dust exposure was first analyzed using a one-way (concentration) analysis of variance, after which individual means were compared, using Bonferoni multi-sample correction/test. Control values obtained at the beginning and the end of the 14-day exposure period were not significantly different and were therefore combined as one control. In all cases, differences were considered statistically significant at either $p<0.01$ or $p<0.05$. All statistical analyses were performed, using the software (GraphPad Prism® Software version 5.0, San Diego, CA – Trial version).

3. Results

3.1. Water Quality Parameters

The mean values of the water quality parameters of the different sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust and control media to which the test fish *Heteroclarias* were exposed over the 14 days exposure period is as presented in Table 3.1. The value of temperature and free carbon (iv) oxide, pH and dissolved oxygen were found to significantly (p<0.05) and (p<0.01) decreased as the concentrations of tobacco leaf dust increased. However, the values of total alkalinity and conductivity in the exposed media were found to be significantly (p<0.01) increased as the concentrations of tobacco (*Nicotiana tobaccum*) leaf dust increased, compared to the control test.

3.2. Enzymatic Activities

The changes in enzymatic activities (gamma, lactate dehydrogenase, acid phosphatase, alkaline phosphatase, aspartate aminotransferase glutamyltransferase and alanine aminotransferase) recorded in the test fish *Heteroclarias* following exposure to the various sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust over a 14 days exposure period are as presented herein.

The activities of acid phosphatase in the sampled tissue and organs of the *Heteroclarias* exposed to sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust after the 14-days exposure period are as presented in

Table 3.1. Mean values[#] (SE) of Water Quality Parameters of the different sublethal concentrations of Tobacco (*Nicotiana tobaccum*) leaf dust and control to the test fish *Heteroclarias* during the 14 days exposure period.

CTLD (mg/L)	Water Quality Parameters					
	Temp (^{O}C)	FCO (mg/L)	TAL (mg/L)	pH	DO (mg/L)	Cond (µs/cm)
0.00	30.63 (0.34)	1.85 (0.12)	0.25 (0.02)	7.15 (0.06)	6.62 (0.00)	146.47 (0.37)
93.90	29.60 (0.60)	1.50 (0.13)	0.35 (0.03)	6.60 (0.02)	5.57 (0.08)	150.75 (0.38)*
187.80	28.77 (0.34)*	1.25 (0.17)*	0.47 (0.06)*	6.27 (0.02)**	4.20 (0.06)**	154.80 (0.90)**
375.60	28.77 (0.34)*	0.87 (0.05)*	0.57 0.09)**	6.07 (0.02)**	3.21 (0.08)**	167.42 (1.23)**

- mean value obtained from 14 sampling with replicate; SE- standard error; CTLD- Concentration of Tobacco (*Nicotiana tobaccum*) leaf dust; Temp- Temperature; FCO- Free Carbon IV Oxide; TAL- Total Alkalinity; DO- Dissolved Oxygen; Cond- Conductivity; * P < 0.05 and ** P < 0.01.

Fig 3.2.1. There was an insignificant ($p>0.05$) decrease and increase in serum and (liver and kidney) acid phosphatase as the concentration of tobacco leaf dust increased. Statistically, the activity of acid phosphatase was most pronounced in the liver, less in the serum and least in the kidney of the exposed fish after 14 day exposure period.

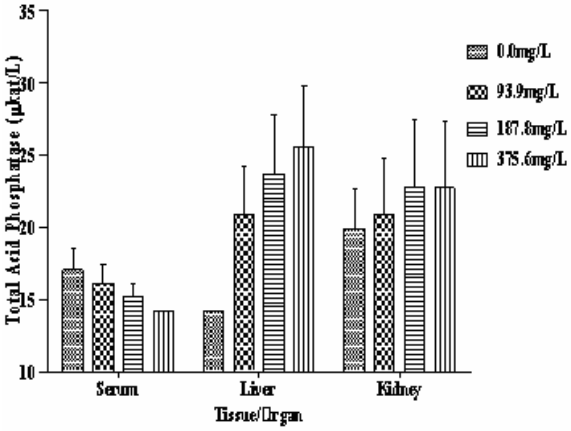

Fig 3.2.1. Acid Phosphatase activities of test fish *Heteroclarias* following 14-days exposure to various sublethal concentrations of Tobacco (*Nicotiana tobaccum*) leaf dust. Each column represents mean value while the bar represents standard error

The activities of alkaline phosphatase in the sampled tissue and organs of the *Heteroclarias* exposed to sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust after the 14-days exposure period are as presented in Fig 3.2.2. There were insignificant ($p>0.05$) decrease and increase in (serum and liver) and kidney alkaline phosphatase as the concentration of tobacco leaf dust increased. Statistically, the activity of alkaline phosphatase was most pronounced in the liver, less in the kidney and least in the serum of the exposed fish after 14day exposure period.

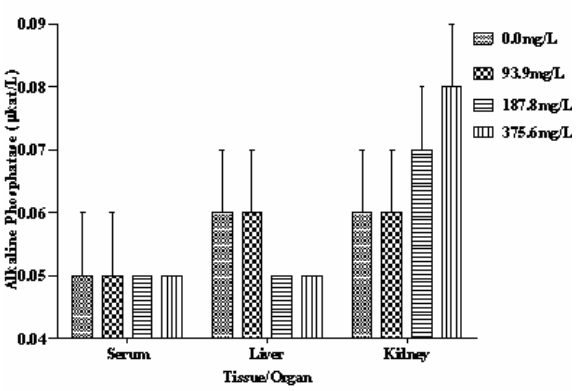

Fig 3.2.2. Alkaline phosphatase activities of test fish *Heteroclarias* following 14-days exposure to various sublethal concentrations of Tobacco (*Nicotiana tobaccum*) leaf dust. Symbols as in Fig. 3.2.1

The activities of alanine aminotransferase in the sampled tissue and organs of the *Heteroclarias* exposed to sublethal concentrations of Tobacco (*Nicotiana tobaccum*) leaf dust after the 14-days exposure period are as presented in Fig 3.2.3. There were insignificant ($p>0.05$) decrease and increase in (serum and liver) and kidney alanine aminotransferase as the concentration of tobacco leaf dust increased. Statistically, the activity of alanine aminotransferase was most pronounced in the kidney, less in the liver and least in the serum of the exposed fish after 14-day exposure period.

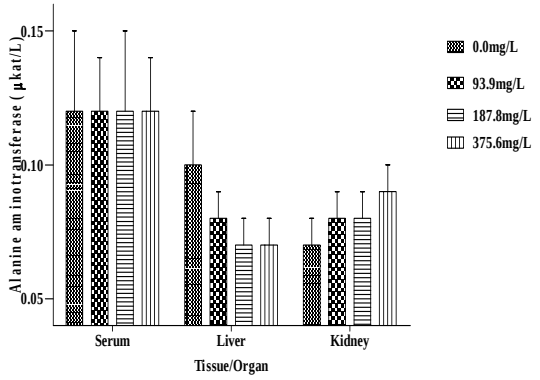

Fig 3.2.3.Alanine aminotransferase activities of test fish *Heteroclarias* following 14-days exposure to various sublethal concentrations of Tobacco (*Nicotiana tobaccum*) leaf dust. Symbols as in Fig. 3.2.1

The activities of aspartate aminotransferase in the sampled tissue and organs of the *Heteroclarias* exposed to sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust after the 14-days exposure period are as presented in Fig 3.2.4. There were insignificant ($p>0.05$) increase and decrease in (serum and kidney) and liver aspartate aminotransferase as the concentration of tobacco leaf dust increased. Statistically, the activity of aspartate aminotransferase was most pronounced in the kidney, less in the serum and least in the liver of the exposed fish after 14-day exposure period.

The activities of lactate dehydrogenase in the sampled tissue and organs of the *Heteroclarias* exposed to sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust

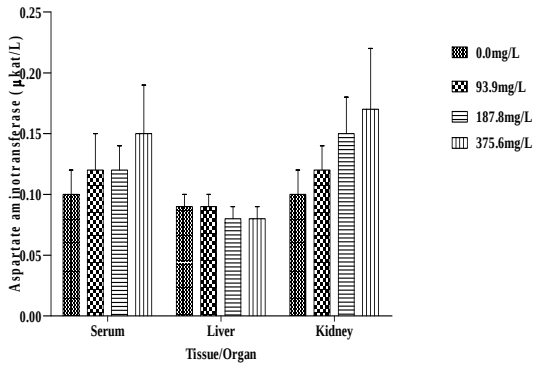

Fig 3.2.4. Aspartate aminotransferase activities of test fish Heteroclarias following 14-days exposure to various sublethal concentrations of Tobacco (Nicotiana tobaccum) leaf dust. Symbols as in Fig. 3.2.1

after the 14-days exposure period are as presented in Fig 3.2.5. The activity of serum lactate dehydrogenase was significantly ($p<0.01$) decreased, while liver and kidney lactate dehydrogenase were noticed to decrease insignificantly ($p>0.05$) when compared to the control. Statistically, the activity of lactate dehydrogenase was most pronounced in the serum, less in the liver and least in the kidney of the exposed fish after 14-day exposure period.

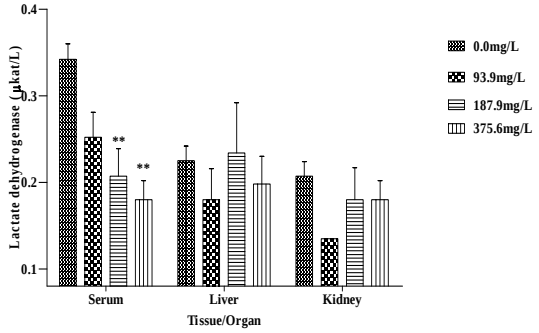

Fig 3.2.5: Lactate dehydrogenase activities of test fish *Heteroclarias* following 14-days exposure to various sublethal concentrations of Tobacco (*Nicotiana tobaccum*) leaf dust. Symbols as in Fig. 3.2.1, ** - $p<0.01$

The activities of gamma glutamyltransferase in the sampled tissue and organs of the *Heteroclarias* exposed to sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust after the 14-days exposure period are as presented in Fig 3.2.6. There was generally insignificant ($p>0.05$) decrease in the activity of gamma glutamyltransferase in serum, liver and kidney of *Heteroclarias* exposed to sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust after the 14-days exposure period. Statistically, the activity of gamma glutamyltransferase was most pronounced in the kidney, less in the serum and least in the liver of the exposed fish after 14-day exposure period.

4. Discussion

Water quality parameters such as temperature, dissolved oxygen, free carbon (iv) oxide, pH, alkalinity and conductivity are parameters that are paramount to the

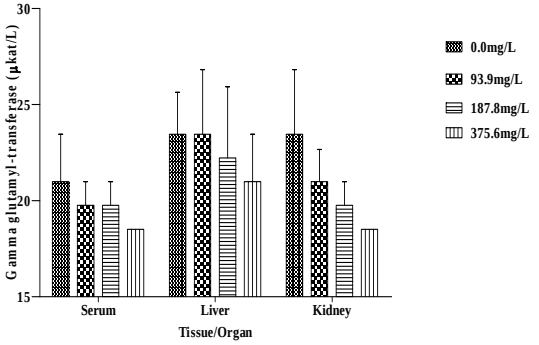

Fig 3.2.6. Gamma glutamyltransferase activities of test fish *Heteroclarias* following 14-days exposure to various sublethal concentrations of Tobacco (*Nicotiana tobaccum*) leaf dust. Symbols as in Fig. 3.2.1

many factors which affect fish health, growth and reproduction (Camus *et al.*, 1998 and Hill, 1955). However, Richards (1977) reported that the main cause of mortality in aquarium fish is the adequate maintenance of the water environment. In this study, the monitored parameters were noted to be significantly different from the control test after the 14-days exposure period which invariably means that tobacco leaf dust has an effect on the water chemistry. The study of Omoniyi *et al.,* (2002) on the sublethal effects of tobacco leaf dust on the haematological parameters of the *Clarias gariepinus* revealed insignificant increase and decrease in the monitored water quality parameters. The variation in the reported result of monitored parameters may be associated to the exposure period and the concentration of tobacco leaf dust used. Noga, (1996) and Richards (1977) have recommended the pH for fresh water fish to be 6.5 to 8.5, the value of pH in the highest concentration of tobacco leaf dust was found to be lower than the recommended value. Thus the significance decrease in pH value as the concentrations of tobacco leaf dust increased revealed that the toxicant resulted in acidic condition. This was supported by the findings of Omoniyi *et al.,* (2002) who reported acidic condition in water of *Clarias gariepinus* exposed to tobacco leaf dust. The decline in pH with time may be due to the production of acidic metabolites (Delyan *et al.,* 1990). Odokuma and Okara (2004) attributed the pH of various treatments to be a function of the chemical composition of the treatment, which was related to the nature of the carbon source and other nutrients present, and thus signifying that tobacco leaf dust as the potential of fertilizing the pond as reported by Aleem (1987). The acidic condition of the water had resulted in the decrease in the level of dissolved oxygen, free carbon (iv) oxide and temperature with a corresponding increase in the values of total alkalinity and conductivity. Omoniyi *et al.,* (2002) had also reported decrease in temperature, dissolved oxygen with increase in conductivity values respectively. The decrease in the available free carbon (iv) oxide may affect the survival of plants, and thus reduction of dissolved oxygen results in hypoxia in animals which is as a result of degradation of the tobacco leaf dust. According to Almeida-Val *et al.*, (1993) low dissolved oxygen environments are found in many tropical plain lakes, ponds, swamps and other eutrophic water xenobiotes when nutrient potential is introduced.

Enzymes catalyze physiological reactions by lowering the activation energy level that the reactant (substrate) must reach for the reaction to occur. The effect of toxicant on enzymatic activity is one of the most important biochemical parameters, which is affected under stress. When an organ is diseased due to the effect of a toxicant, enzymatic activity appears to be increased or inhibited due to the active site being either denatured or distorted. Since some enzymes catalyze some steps in the metabolism of carbohydrate and protein, they are present in most tissues. The increase or decrease in their level may be sufficient to provide information of diagnostic values.

Phosphatases are important enzymes of animal metabolism, which play important roles in the transport of metabolites across the membrane (Vorbrodt, 1959). Alkaline phosphatase diagnosis is important in the bone disease and hepatobiliary disease thus employed to assess the integrity of plasma membrane and endoplasmic reticulum (Akanji *et. al.,* 1993 and Wright and Plummer, 1974) while acid phosphatase diagnosis is intended to detect the carcinoma of the prostate. Verma *et al.,* (1984) had reported an increase in serum acid phosphatase in *Mystus vittatus* (Bloch) exposed to different pesticides. The activity of serum acid phosphatase in the test fish *Heteroclarias*, exposed to sublethal concentrations of tobacco leaf dust, revealed an insignificant decrease which differs from the report of Verma *et al.,* (1984) while the activity of liver and kidney acid phosphatase was found to insignificantly increase. The insignificant difference recorded in acid phosphatase may be an indication of no serious or mild effect on bone and hepatobiliary disease as well as the integrity of the plasma membrane. However, the activity of alkaline phosphatase was found to insignificantly decrease in the serum and liver with a corresponding insignificant increase in the activity of kidney acid phosphatase in *Heteroclarias* exposed to sublethal concentrations of tobacco leaf dust after the 14-days exposure period. The dose-dependent inhibition observed in this investigation is in agreement with the report of many authors. Ogueji and Auta (2007) reported reduced value of serum alkaline phosphatase in African catfish *Clarias gariepinus*, exposed to lambda-cyhalothrin. Sastry and Sharma, (1980) reported alkaline phosphatase inhibition after 96h exposure to diazinon. Goel, *et al.,* (1982), reported serum alkaline phosphatase inhibition by 15% in *Heteropneutes fossilis* resulting from the effect of malathion. Similarly, Das and Mukherjee (2003) reported depletion of alkaline phosphatase due to sublethal exposure of *Labeo rohita* fingerlings to cypermethrin. Rashatwar and Ilyas (1983) had reported significance decrease in alkaline phosphatase activity in fresh water fish *Nemachelius denisonii* (day) exposed to sublethal concentrations of Basalin. Due to the resulting activity values of alkaline phosphatase, it may be assumed that the liver tissue of the experimental fish was not markedly affected by tobacco leaf dust. The inhibition in protein level may also be due to the decrease in alkaline phosphatase activity as it plays an important role in protein synthesis (Pilo *et. al.,* 1972). The insignificant difference recorded in alkaline phosphatase may be an indication of no serious or mild effect on the carcinoma of the prostate.

Aminotransferases are gainfully used in the diagnosis of disease and tissue damage. It functions as a link between carbohydrate and protein metabolism by catalyzing the inter conversion of strategic compounds respectively (Martin *et. al.,* 1983). They are normally localized within the cells of the liver, heart, gill, kidney, muscles and other organs. The enzymes are important in assessing and monitoring liver cytolysis (Wells and Snell, 1962). They are intracellular enzymes which exist in only a small amount of the serum. Their presence in the serum may give information on organ dysfunction (Wells *et al*., 1986). The aminotransferases occupy a central position in the amino acid metabolism as they help in retaining amino group (to form a new amino acid) during the degradation of amino acid; and are also involved in the biochemical regulation of intracellular amino acid pool. They also help in providing necessary intermediates for gluconeogenesis. Alanine aminotransferase is remarkably specific for liver function since aspartate aminotransferase is mostly present in kidney (Witthawaskul *et al*., 2003). The activities of alanine aminotransferase in the test fish *Heteroclarias*, exposed to sublethal concentrations of tobacco leaf dust, were insignificantly decreased and increased in (serum, liver) and kidney. The liver is especially rich in alanine aminotransferase, being the enzyme measurement used primarily as a test for infectious and toxic hepatitis. The reduction of liver alanine aminotransferase may be attributed to reduced rate of synthesis of the liver enzyme. It may also be that the plant dust had caused leakage of the enzyme into the blood where alanine aminotransferase altered membrane permeability (Wroblewski and La Due, 1956). The sublethal effects of tobacco leaf dust on the activity of aspartate aminotransferase of the test fish (*Heteroclarias*) after the 14-days exposure period revealed insignificant increase and decrease in serum, kidney and liver. Jee *et al*., (2005) had reported increase in serum aspartate aminotransferase in Korean rock fish (*Sebastes schlegeli*) exposed to cypermethrin. The increase in serum aspartate aminotransferase may be attributable to the process of either deamination or transamination due to the effect of the plant dust (tobacco leaf dust). The decrease in aspartate and alanine aminotransferases in the liver of experimental fish revealed that the plant extract has an effect on the parenchymatous tissue and skeletal musculature which probably may disturb the permeability and integrity of cell organelles as supported by Adamu and Iloba, (2008). Yakubu *et al.,* (2005) reported significant increase ($p<0.05$) in serum aspartate aminotransferase and liver aspartate aminotransferase, increased significantly ($p<0.05$) in rats exposed to *Khaya senegalensis* during the 18-days exposure period. Oruc and Uner, (1999), reported inhibition in seral aspartate aminotransferase and alanine aminotransferase enzyme activity following 2 and 30 days of exposure to 2, 4-Diamin. Similarly, Sadhu, *et al.,* (1985) had reported decreased aspartate aminotransferase and alanine aminotransferase activities in the serum of *Channa striatus*, following exposure to Malathion for 10 days. The pattern of alanine aminotransferase and aspartate aminotransferase activities observed in this study are biochemical symptoms tending towards liver cytolysis, indicating disturbance in the structure and integrity of cell organelles like endoplasmic reticulum and membrane transport system (Dere and Polat, 2001). Alterations in their activities may have an adverse effect on the amino acid metabolism of the tissues and consequently the intermediates needed for gluconeogenesis. The activities of serum aspartate aminotransferase and alanine aminotransferase did not show any significance change probably due to the low damaging effect of the tobacco leaf dust used as supported by Al-Salahy and Mahmoud (2003).

Lactate dehydrogenase catalyses the conversion of pyruvic acid to lactic acid in aerobic condition; thus acts as an indicator of hepatobiliary disease. The activity of lactate dehydrogenase in the test fish (*Heteroclarias*) exposed to sublethal concentrations of tobacco leaf dust revealed significant decrease and insignificant decrease in serum and liver and kidney lactate dehydrogenase activity. Rashatwar and Ilyas (1983) had reported significant decrease in lactate dehydrogenase activity in fresh water fish *Nemachelius denisonii* (day) exposed to sublethal concentrations of Basalin. The reduction in the activity of liver lactate dehydrogenase, cytosolic enzyme (Wroblewski, 1955) without corresponding increase in the serum enzyme may also be adduced to inhibition of the enzyme activity at the cellular level such a radiation may have consequential effect on the glycolytic pathway thus, affirmed by the low level of dissolved oxygen in the test water. The sluggish movement of the test fish can also be explained by the reduction of the lactate dehydrogenase activity. The reduced lactate dehydrogenase in liver and kidney may have occurred, due to the stress- induced increase in the rate of glycolysis.

Gamma glutamyltransferase catalyses the conversion of pyruvic acid to lactic acid in aerobic condition; thus acts as an indicator of hepatobiliary disease. The activity of gamma glutamyltransferase in serum, liver and kidney of the test fish (*Heteroclarias*) exposed to sublethal concentrations of tobacco leaf dust after the 14-days exposure period was insignificantly decreased which is an indication that affirm the activity of lactate dehydrogenase and the tendency towards hepatobiliary disease as a result of low level of dissolved oxygen in the test water.

5. Conclusion

Tobacco dust is locally available, inexpensive, easily degraded and serves as piscicides and an organic fertilizer. Tobacco leaf dust may be a useful substitute of synthetic piscicides in killing weed fish from culture pond. This is environmentally safe because their toxic effect is reversible within 3 days after application. Its moderate effect and the rapid rate of degradation make it attractive for aquaculture purposes, as a substance to control the pest and subsequently as an organic fertilizer. This study confirms that the extracts of tobacco leaf exert piscicidal and fertilizer properties. The result revealed that tobacco leaf dust at studied concentrations had slight effect on the intermediary metabolism of *Heteroclarias.* It again suggests that sublethal concentrations of tobacco leaf dust at the tested concentrations have some mild effects on some basic function of the serum, liver and kidney of *Heteroclarias.* Therefore, the determined enzymatic activities can be suitably used to determine the effect of toxicant on the physiology of fish under sublethal condition prior to sudden death of the fish. The activities

of determined enzymatic activities showed mild damage in kidney, liver, plasma membrane, endoplasmic reticulum as well as any hepatobiliary and bone disease and carcinoma of the prostate enzymes. However, it has been known to affect the chemistry of the water thus may primarily affect primary productivity and the biodiversity of organisms as a result of decrease in the level of dissolved oxygen, free carbon iv oxide, temperature and increase in total alkalinity and conductivity.

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Polymorphism of Protein Fractions as Biochemical Markers for Identification of Wheat Varieties

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Abstract

The electrophoretic banding patterns of gliadin, albumin and globulin in wheat varieties grown in Sulaimanyah were determined by acid-polyacrylamide gel electrophoresis (Acid-PAGE) and Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The varieties of bread wheat used in the study showed 21 different patterns: six of them were identified to be corresponding to Ω-gliadin, three to the γ-gliadin, six to the β-gliadin, and other six to the α-gliadin. The gliadin patterns of wheat bread varieties greatly differed from the patterns of Italian wheat variety (Costantino), and the variation detected in wheat varieties from Sulaimanyah was limited to forty seven patterns. Only Tammuz showed the presence of gamma 47. The gliadin gamma 47 is only genetic markers for technological properties in wheat. The electrophoretic banding patterns showed variations between the bread wheat varieties. The highest value of similarity was observed between the varieties Cham 4 and Cham 6 for gliadin analysis. The gliadin pattern revealed more polymorphisms than the albumin and globulin pattern. On the other hand, in durum wheat varieties, two types of omega gliadin: omega gliadin 33 and 35 and two types of gamma gliadin were found: gamma gliadin 42 and gamma gliadin 45. A total of 84 band patterns were identified (durum wheat), of which 2 different mobility bands were in the region of omega gliadins, 1 in the region of gamma gliadins, 2 in the region of beta gliadin and 2 in the region of alpha gliadins. Genetic diversity index was highest in Creso Kurde and followed by Ovanto.

الملخص

تتضمن الدراسة تحديد التركيب النموذجى لبروتينات كليادين والبومين وكلوبيولين لستة اصناف من الحنطة الناعمة المزروعة فى السليمانية بوساطة الفصل الكهربائى لهلام الحامض متعدد الاكريلامايد. وتم اكتشاف 21 نموذجاً او تراكيبياً مختلفة منها 6 نماذج لكليادين اوميكا و3 نماذج لكليادين كاما و6 نماذج لكليادين بيتا 6 نماذج لكليادين الفا. ان التركيب النموذجى لكليادين الموجود فى الاصناف الحنطة الناعمة المزروعة فى السليمانية تختلف عن تركيب النموذجى لكليادين الموجودة فى صنف الايطالى. ان بذور الصنف تموز هو الوحيد الذى يحتوى على كليادين كاما 47 التى تعتبر كدليل ايجابى لنوعية الحنطة. ان التركيب النموذجى لبروتين كليادين والبومين وكلوبيولين اظهرت الاختلاف الوراثى بين الاصناف المدروسة.كما لوحظت اوجة التشابة بين بذور الصنفين شام 4 وشام 6. واكدت النتائج ان التحليل النموذجى لكليادين اظهرت اختلافاً واضحاً بين الاصناف المدروسة مقارنة بالتحليل النموذجى لالبومين وكلوبيولين. وفى الحنطة الخشنة, تم اكتشاف نوعين من كليادين اوميكا 33 و 35 ونوعين من كليادين كاما 42 و 45. وجدت 2 نماذج مختلفة لكليادين فى المنطقة اوميكا ونموذج واحد فى منطقة كاما و تركيبين مختلفين فى المنطقة بيتا و تركيبين مختلفين فى المنطقة الفا. ان الاصناف كريزو كردى و اوفانتو من الاصناف الاكثر اختلافا عن باقى الاصناف المدروسة.كما ان تركيب النموذجى لبروتين البومين و كلوبيولين برزت الاختلاف الوراثى بين الاصناف المدروسة.

Keywords: Bread wheat, Durum wheat, Gliadins, Albumin, Globulin, Acid-PAGE, SDS-PAGE.

1. Introduction

The gluten proteins of wheat provide unique attributes to flour. The gluten proteins are classified based on their electrophoretic mobilities into glutenins and gliadins. Gliadins are monomeric proteins of molecular weight ranges between 30, 000 – 75, 000 Daltons. Gliadins are known to have extensive genotypic polymorphism (Branlard *et al.,* 1993). Based on the electrophoretic mobilities, the gliadins are classified into four different group's alpha (α), beta (β), gamma (γ), and omega (Ω) or (ω) gliadins. The genes coding for these proteins are present on the short arm of chromosome 1 and chromosome 6 of wheat. They are tightly linked genes present on the three homologous loci of chromosome 1 as Gli-A1, Gli-B1 and Gli- D1. In chromosome 6, they are present as Gli-A2, Gli-B2 and Gli-D2 (Wrigley and Shepard, 1973; Brown and Flavell, 1981). The Gli-1 genes code for the ω and γ-gliadins and the Gli-2 genes code for α and β-gliadins. The Gli-1 locus is tightly linked to the LMW glutenin loci Glu-3 in chromosome1. The gliadin

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loci are known to be inherited in simple Mendelian inheritance and multiple-allelism has been reported for both the Gli-1 and Gli-2 loci (Metakovsky *et al*., 1984; Metakovsky, 1991).

Gliadin components have high content of glutamine amino acid. For instance ω-gliadins have more than 50% glutamine (Lasztity, 1984). The proline content is also high in gliadins exceeding what found in HMW glutenins. The high proline content plays an important role in the secondary structure of gliadins (Makarenko *et al*., 2002).

However, gliadins are poor in other basic amino acids such as lysine, arginine and histidine (Kasarda *et al*., 1974). The determination of the N-terminal sequence of the gliadins has supported the theory that a large number of gliadins is due to a genetic mutation of a common precursor during natural evolution of bread wheat. Most of α, β-gliadins and some of the γ gliadins have similar N-terminal sequence. The α, β, γ-gliadins have six to eight cysteine residues, as a result, three to four intramolecular disulphide bonds occur (Kasarda *et al.,* 1984). Although the HMW and LMW glutenins form the disulphide cross-linked gluten matrix a small proportion (5-10%) of α and γ-gliadins occupy the matrix cross-link function. The ω gliadins also may take part in the polymer formation (Kukataite *et al.,* 2004). The gliadins, like the LMW glutenin, can also acts as the chain terminators. The gliadins are generally contributed to the viscosity and extensibility of the dough. The glutenin: gliadin ratio is thought to be an important determinant of quality. The higher glutenin to gliadin ratio is shown to contribute to greater dough strength (MacRitchie, 1985). Another study suggests that an increase in the relative gliadin content is associated with increase extensibility and loss of dough strength (Edwards *et al.,* 2001). Glutenin enrichment can circumvent this and increase dough strength. Although gliadins have been associated to certain parameters of bread making quality, they are considered unimportant to dough strength (Gianibelli *et al.,* 2001). Changes in the glutenin to gliadin ratios modify the bread making quality. In the previous studies deletions in the Gli-1 loci were found to exhibit greater dough strength (MacRitchie & Lafiandra 2001). This study aimed to examine the variations of the gliadin, albumin and globulin patterns in wheat varieties.

2. Materials and Methods

2.1. Plant Samples

One hundred gram of grains of each wheat varieties (bread wheat: Costantino, Tammuz, Aras, Rabia, Cham 4, Cham 6 and durum wheat: Creso kurde, Creso Italy, Ovanto, Bakrajo 1, Acsad 65, Cemmitto) were collected from the department of Agriculture of Sulaimanyah. All varieties used in this study are genetically pure.

2.2. Gliadin Extraction and Electrophoresis

Gliadin patterns were determined by A-PAGE. Twenty gram of wheat grains was hammer-milled to a fine powder. Forty mg of flour was mixed with 200 µl of 60% (v/v) aqueous ethanol. The mixture was incubated in a closed microtube for 2h at 60°C and centrifuged for 10 min, at 14000 rpm. The supernatant was transferred to a microtube and diluted with 1 ml of acetone. The mixture was incubated for 10 min at RT. After centrifugation for 10 min at 14000 rpm, the pellet was resuspended in buffer A (30% Glycerol, 6 M Urea, 25 mM acetic acid, 0.01% w/v pyronin) (Sewa *et al.,* 2005).

To polymerize the gel, one drop (about 200 µl) of fresh 30% H_2O_2 was added to 75 ml of cold gel solution (8.3% acrylamide, 0.415% N,N'-methylenebis-acrylamide, 0.1% ascorbic acid, 0.00067% $Fe_2(SO_4)_3$ in 5.1 mM Allactic buffer, pH 3.1). Each sample (30 µl) was loaded onto a gel sized 180 (length) X 140 (width) X 2 (thickness) mm. Electrophoresis from the anode (the upper buffer) to the cathode (the lower buffer) was performed at 500 V for about 4 h until the tracking dye, pyronin, passed to the gel bottom. The gel was immersed into the staining solution containing 0.05% Coomassie Brilliant Blue R-250 (w/v) and 8% trichloroacetic acid for 5–16 h, and distained with distilled water (Metakovsky, 1991).

2.3. Albumin Extraction and Electrophoresis

Twenty gram of wheat grains was hammer-milled to a fine powder. Forty mg of flour was mixed with 400 µl of 0.5 M NaCl. The mixture was incubated in a closed microtube for 2h at 4°C, and centrifuged at 14000 rpm for 10 min; the supernatant was precipitated in 1 ml acetone. The pellet was suspended in solution [28.5% sample buffer (7% SDS, Tris-HCl 0.01 M, pH 6.8, 30% glycerol, 0.001% comassie bleu) and 5% 2-mercaptoethanol]. The samples were incubated at 80°C for 20 min before loading. Samples of 30 µl were loaded each the slots of SDS-PAGE. The gel consisted of a 15% separating gel, pH 8.4, beneath a 3% stacking gel, pH 6.8. Electrophoresis was carried out at room 27°C using a locally-made vertical electrophoresis apparatus. The running was performed at 15 mA/gel for 18 hours. Subsequently, the gels were stained by immersion in a solution consisting of 12.5% (w/v) trichloroacetic acid, 0.01% (w/v) Coomasie Brilliant Blue R250 and distained with distilled water (Tanaka *et al.,* 2003).

3. Data Analysis

Electrophoregrams for each variety of wheat were scored and the presence (1) or absence (0) of each bands noted. Presence and absence of bands were entered in a binary data matrix. Analysis was carried out using a statistical package SPSS-PC, version 15, by using the Dice similarity.

4. Results and Discussion

4.1. Variation in Gliadin pattern

4.1.1. Bread wheat

Electrophoretic separation of gliadin components exists in the bread wheat material (Figure 1). The Acid-PAGE analysis showed alpha, beta, gamma and omega namely three classes of gliadins. According to the nomenclature of Bushuk and Zillman (1978), the reference Costantino (reference variety) possessed omega gliadin Ω-35, 36, 38 (Table 1). Cham 4, Cham 6 and Rabia possessed only omega gliadin Ω-36. Aras possessed omega gliadin Ω-35

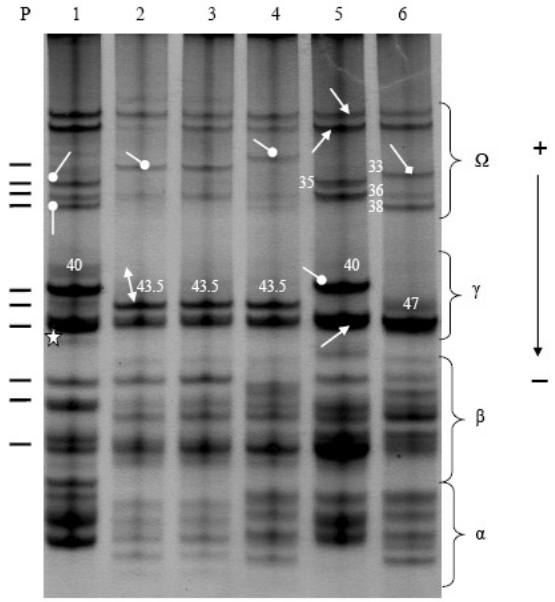

Figure 1. Electrophorogram separation of gliadin components in bread wheat varieties. 1: Costantino; 2: Cham 6; 3: Cham 4; 4: Rabia; 5: Aras; 6: Tammuz. The subunits of gliadins are designed according to Metakovsky, 1991, Bushuk and Zillman, 1978. Arrows correspond to the protein encoded by Gli-D1, oval arrow refers to the protein encoded by Gli-B1, diamond arrow refers to the protein encoded by Gli-A3, star refers to the protein encoded by Gli-A1, double arrows refer to the protein encoded by Gli-B1. P: polymorphic bands.

Table 1. Gliadin subunits composition of six varieties of bread wheat.

Varieties	Omega gliadin (Ω)	Gamma gliadin (γ)
Costantino	35, 36, 38	40
Cham 6	36	43.5
Cham 4	36	43.5
Rabia	36,38	43.5
Aras	35, 36	40
Tammuz	33, 36, 38	47

and 36. On the other hand, Tammuz showed three omega gliadin Ω-33, 36, and 38. According to the nomenclature of Pogna *et al.,* (1995) and Metakovsky (1991), Costantino carries alleles Gli-A1a, Gli-A3a, Gli-B1m, Gli-B3a and Gli-D1k (Table 2). Cham 4 and Cham 6 contain the same alleles in all loci (Table 2). The alleles Gli-B3a are represented in all varieties. The allele composition at the Gli-1 loci of the genotypes analyzed here is reported in Table 1. According to Pagne *et al.,* (1987) the omega gliadins and the D-subunits of glutenin, coded by the Gli-B3 locus, are expressed in low amount and allelic variation is rather limited. Bread wheat Aras and Costantino possess the gamma gliadin component designated 40, whereas Cham 4, Cham 6, and Rabia possess the gamma gliadin 43.5. In addition, Tammuz possesses the gamma gliadin 47 (Table 1). This gamma locus is controlled by the Gli-B1 gene. The studies (Damidaux *et al.,* 1987; Payne, 1984, Joppa *et al.,* 1983) demonstrated the usefulness of gliadin gamma 45 and gamma 42, encoded at Gli-B1 locus as biochemical marker of good and poor pasta quality, respectively. Alpha gliadin patterns varied among the varieties.

Table 2. Allele's composition at the Gli-1 and 3 loci of bread wheat varieties. The alleles are designed According to Pogna *et al*., 1995 and Metakovsky *et al*., 1984.

Varieries	Gli-A1	Gli-A3	Gli-B1	Gli-B3	Gli-D1
Costantino	a	a	m	a	k
Cham 6	m	b	b	a	b
Cham 4	m	b	b	a	b
Rabia	b	b	c	a	b
Aras	b	b	d	a	k
Tammuz	m	a	e	a	k

The viscoelastic properties of gluten in both durum wheat and common wheat are influenced each of gliadin and glutenin patterns. Significant associations have been also detected between wheat quality and specific gliadin components (Damidaux *et al.,* 1987; Pogna *et al.,* 1982; Sozinov and Poperelya, 1980). Pagne *et al.,* (1987) and Pogna et al., (1990) suggested that allelic variation at the Glu-3 loci coding for LMW subunits of glutenin is probably responsible for differences in gluten quality, previously thought to be associated with the closely linked Gli-1 loci coding for gliadins. However, alleles at Gli-1 maintain their interest as genetic markers of quality. Dachevitch T. *et al.,* (1993) showed the genetic background of gliadins coded by the group 1 chromosomes in bread wheat Costantino. The authors identified some alleles coded to gliadins subunits in Costantino.

Result of Acid-PAGE also revealed the variations among the varieties of wheat according to the beta gliadin patterns.

4.1.2. Durum wheat

The Acid-PAGE analysis showed four classes of gliadins namely alpha, beta, gamma and omega (Figure 2). The varieties contain two types of omega: 33 and 35. Only Creso Kurde contained omega 33 and 35 and the rest of varieties possessed omega 35 (Table 3). On the other hand, the acid-PAGE showed one type of gamma gliadin: 45 which is considered as a good marker for pasta making quality (Pogna, 1990). On the other hand, the polymorphism of omega and gamma gliadin is revealed by one and two dimensional gel (Nieto-Taladriz, 1993). Similar studies (Damidaux *et al.,* 1987; Payne, 1984; Joppa *et al*., 1983) demonstrated the usefulness of gliadin gamma 45 and gamma 42, encoded at Gli-B1 locus as biochemical marker of good and poor pasta quality, respectively. The viscoelastic properties of gluten in both durum and common wheat are influenced each of gliadin and glutenin patterns. Significant associations were also detected between wheat quality and specific gliadin components (Damidaux *et al.,* 1987; Pogna *et al.,* 1982; Sozinov and Poperelya, 1980). Pagne *et al*., (1987) and Pogna *et al*., (1990) suggested that allelic variation at the Glu-3 loci coding for LMW subunits of glutenin is probably responsible for differences in gluten quality, previously thought to be associated with the closely linked Gli-1 loci coding for gliadins.

In this study, Acid-PAGE of grain storage proteins gliadin was performed in order to analyze molecular weight of gliadin subunits and investigate genetic diversity

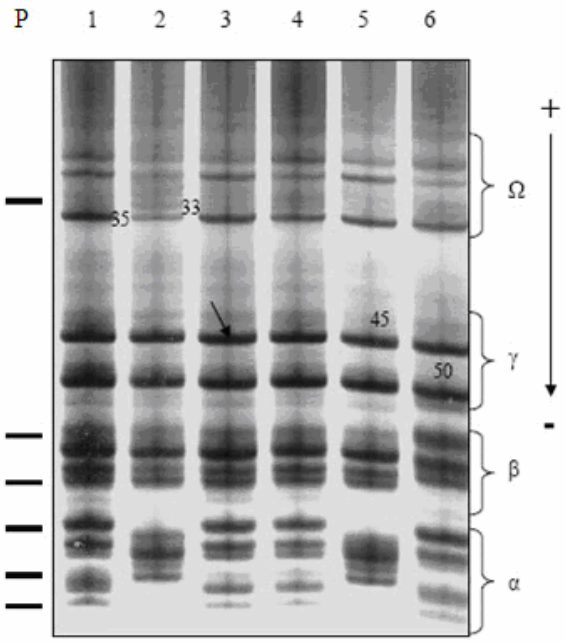

Figure 2. Electrophorogram showing banding pattern of durum wheat protein (Gliadin). 1: Creso Italy; 2: Creso Kurde; 3: Cemmitto; 4: Acsad 65; 5: Ovanto; 6: Bakrajo 1. The subunits of gliadins are designed according to Pogna *et al*. 1990. Arrow corresponds to the protein encoded by locus Gli-B1-2. P: Polymorphic bands.

Table 3. Omega and gamma gliadin subunits composition and locus of gliadin of durum wheat varieties.

Varieties	Omega gliadin	Gamma gliadin	Locus
Creso Italy	35	γ-45 and 50	Gli-B1-2
Creso Kurde	33, 35	γ-45 and 50	Gli-B1-2
Cemmitto	35	γ-45 and 50	Gli-B1-2
Acsad 65	35	γ-45 and 50	Gli-B1-2
Ovanto	35	γ-45 and 50	Gli-B1-2
Bakrajo 1	35	γ-45 and 50	Gli-B1-2

Among different wheat varieties.The electrophorograms showing proteins banding pattern of different wheat varieties are given in figure 2. Eighty four bands were obtained among which 6 bands showed variation, but the other bands were common in all varieties. The results revealed that Ovanto showed only one present band (Figure 2). At low molecular weight of gliadin (alpha gliadin), there are three bands polymorphic, reflecting more diversity.

4.2. Genetic Diversity and Cluster Analysis

4.2.1. Bread wheat

The electrophorogram proteins banding pattern, for seeds of all varieties of wheat detected by Acid-PAGE (Figure 1), showed segregation for gliadin.

Thirty four bands were obtained, and 11 bands were showed variations, but the other bands were common in all varieties. Cluster analysis of wheat grain storage proteins was carried out, depending on the results of Acid-PAGE and using the UPGMA analysis to find the diversity among the given varieties of wheat as shown in the dendrogram (Figure 3). The diagram indicated five main groups: group 1= Cham 6 and Cham 4, group 2= Rabia, group 3= Tammuz, group 4= Costantino, group 5= Aras. At Dice dissimilarity of distance 1, Cham 6 and Cham 4 showed more similarity than others varieties.

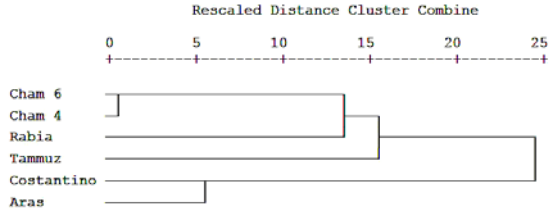

Figure 3. Dendrogram of bread wheat varieties showing the dissimilarity among the varieties based on A-PAGE- Gliadin.

At distance 13, there are 3 groups: group 1= Cham 6, Cham 4, Rabia, group 2= Tammuz, group 3= Costantino and Aras. At distance 17, Cham 6, Cham 4, Rabia and Tammuz are in the group 1 and Costantino and Aras are in the group 2. At dissimilarity distance 24 the diversity divides into two groups: group 1= Cham 6, Cham 4, Rabia and Tammuz and group 2= Costantino and Aras. Costantino and Aras showed more dissimilarity distance with the rest of the varieties.

4.2.2. Durum wheat

Dendrogram cluster analysis of wheat grain storage proteins (Gliadins) resulted from Acid-PAGE (Figure 2), using the UPGMA analysis, showed the diversity among the given varieties on the bases of dissimilarity distance of Dice (Figure 4). The diagram revealed four main groups: group 1= Creso Kurde and Ovanto, group 2= Cemmitto and Ascad 65, group 3= Bakarajo 1, group 4= Creso Italy. At Dice dissimilarity of distance 1, the varieties Creso Kurde and Ovanto and the varieties Cemmitto and Ascad 65 showed more similarity than others varieties.

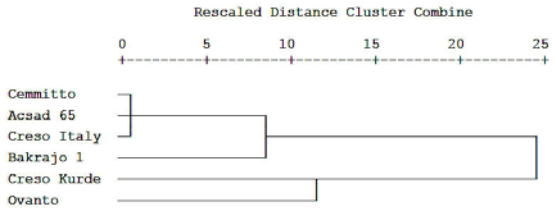

Figure 4. Dendrogram of durum wheat varieties showing the dissimilarity among the varieties based on A-PAGE- Gliadin.

At distance 5, there are 3 groups: group 1= Creso Kurde and Ovanto, group 2= Cemmitto, Ascad 65 and Bakarajo 1, group 3= Creso Italy. At distance 15, the varieties Creso Kurde and Ovanto are in the group 1 and the varieties Cemmitto, Ascad 65, Bakrajo 1 and Creso Italy are in the group 2. At dissimilarity distance 22, the diversity divides into two groups: group 1= Creso Kurde and Ovanto, group 2= Cemmitto, Ascad 65, Bakarajo 1, Creso Italy. The varieties Creso Kurde and Ovanto showed more dissimilarity distance with the rest of the varieties.

The presence of some patterns may correlate with a higher adaptive value of germplasm to the particular environment (Metakovsky *et al.,* 1991; Sewa *et al*., 2005). The association between genotypes of wheat and their

environment has also been reported by Nevo *et al.,* (1988, 1995). However, no significant relationship between genotypes of wheat and their environments was reported (Dreisigacker *et al.,* 2004). Different combinations of gliadin patterns were prevalent in different regions, suggesting the adaptive properties of individual alleles or the chromosome segments, in which these alleles reside (Nevo *et al.,* 1995; Metakovsky and Branlard, 1998). Different gliadins might have some advantage over other gliadins in adaptation to the conditions, prevailing in these zones or these are closely linked with genes having adaptive values to the specific environment, though that needs to be confirmed by genetic analysis (Sewa *et al.* 2005).

4.3. Variation at The Albumin and Globulin Subunit

4.3.1. Bread wheat

Albumins and globulins were characterized by rich protein pattern. The numbers of band varied from 13 to 15, and they were defined by molecular weight 106–2 kDa (Figure 5). The protein pattern of albumins and globulins was divided into two relatively wide areas 66–23 kDa and 16–2 kDa. The electrophoretic pattern of tested varieties showed high similarity. The differentiating areas were located at the four zones with molecular weights of 92–87 kDa, 68–58 kDa, 49–39 kDa and 10-4 kDa for the 1st, 2nd , 3rd and the 4th zone, respectively**.**

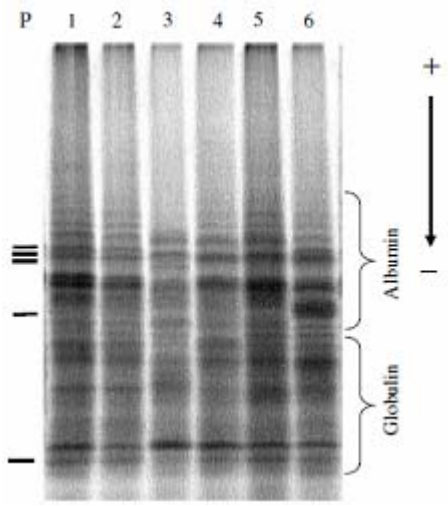

Figure 5. Electrophorogram showing banding patterns of bread wheat protein soluble (Albumin and globulin). 1: Costantino; 2: Cham 6; 3: Cham 4; 4: Rabia; 5: Aras; 6: Tammuz. P: polymorphic bands.

The similarity matrix and the resulting dendrogram (Figure 6) characterized similarity coefficients of the tested varieties. Although the results showed high similarity, the tested varieties were firmly identified. Tammuz possesses the five bands polymorphic, and Cham 4 possesses only one subunit. The highest similarity was found between Rabia and Aras. On the other hand, the lowest value of the similarity coefficient was found between Cham 4 and the rest of varieties**.** The diversity, in high molecular weight protein subunits, is the result of gene silencing in some varieties encoding these proteins (Lawrence and shephred, 1980).

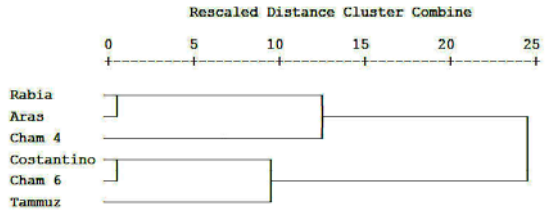

Figure 6. Dendrogram of bread wheat varieties showing the dissimilarity among the varieties based on SDS-PAGE- Albumin and globulin.

4.3.2. Durum wheat

Albumins and globulins were characterized in durum wheat by SDS-PAGE (Figure 7). The differentiating areas were located to zone: 40-60kDa. The SDS-PAGE gel did not show any difference among the varieties at the level globulin. The number of bands present is similar for all varieties.

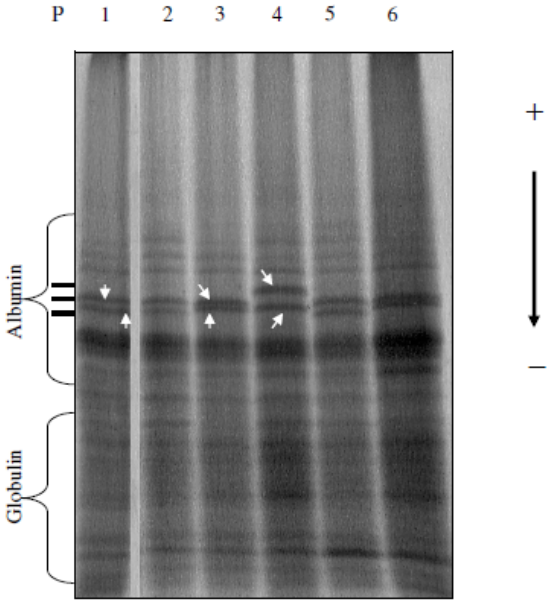

Figure 7. Electrophorogram showing banding pattern of durum wheat protein soluble (Albumin and globulin).1: Bakrajo 1; 2: Acsad 65; 3: Creso Kurde; 4: Creso Italy; 5: Cemmitto; 6: Ovanto, P: Polymorphic bands.

The similarity matrix and the resulting dendrogram (Figure 8) characterised similarity coefficients of the tested varieties. The highest similarity was found between Creso Kurde and Ovanto and also for Acsad 65, Cemmitto and Bakrajo 1 (Figure 8). On the other hand, the lowest value of the similarity coefficient was found between the group which contains Creso Kurde, Ovanto and Creso Italy and the group containing Acsad 65, Cemmitto and Bakrajo 1.

The seed proteins are very suitable and useful genetic markers, because they are not influenced by external conditions, and so they enable to identify tested plant genotypes (Černý and Šašek, 1996). The finding predicates higher level of the intra-varieties polymorphism in these cultivars. Patterns of albumins and globulins among varieties were very similar and their characters corresponded with the detection by American authors, who used water dissolved proteins for identification of two common wheats in non-reduction conditions (Kim and Bushuk, 1995). Apart from the gluten proteins, water-

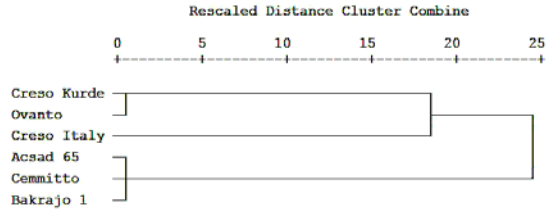

Figure 8. Dendrogram of durum wheat varieties showing the dissimilarity among the varieties based on SDS-PAGE- Albumin and globulin.

soluble albumins and salt-soluble globulins constitute from 10 to 22% of total flour protein (Singh and MacRitchie, 2001a). Albumins such as α-amylase/trypsin inhibitors (Buonocore *et al.,* 1985; Shewry *et al.,* 1984) and purotionins (Garcia- Olmedo *et al.,* 2002) may have dual roles as nutrient reserves for the germinating embryo and as inhibitors of insects and fungal pathogens prior to germination. Puroindolines influence grain hardness (Morris, 2002). Generally, albumins and globulins are not thought to play a critical role in flour quality, although, minor importance on bread-making quality has been reported (Schofield and Booth, 1983). Both protein fractions are important from nutritional point, because of rather high amounts of essential amino acids. This experience is confirmed with the comparison of polymorphism among water soluble protein (albumins and globulins) and seed storage proteins (gliadins) of Dvořáček *et al*. (2001a).

In conclusion, the results showed that the gamma gliadin 47 which is linked to the strong quality of wheat for baking, is less frequent in the varieties tested. The two marker systems were analysed for a complex similarity evaluation of tested varieties. On the other hand, the gliadin pattern revealed more high level of polymorphisms than the albumin and globulin pattern. Identification of gliadins alleles in wheat varieties in Kurdistan may be useful for selection aims in breeding programs to determine the relationship between gluten viscoelastic properties and allelic variations at Gli-1, Glu-3 and Glu-3 in wheat varieties.

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Antinociceptive Effect of Two Flavonoids from *Aloysia Triphylla L.*

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Abstract

Aloysia triphylla, known in Jordan as Mellisa, is a plant that belongs to the Verbenaceae. This plant has been used in herbal medicine as sedative agent and helps to counter depression. Phytochemical analysis of the ground aerial parts of *Aloysia triphylla* resulted in the isolation of two known compounds: artemitin and hesperidin. The two compounds were assessed for antinociceptive activities in mice, using the classical *in vivo* model of pain, the hot plate test. Artemitin and hesperidin (given i.p.) increased significantly ($P<0.05$) the pain latency of nociceptive response in dose dependent manner. The ED50 values were 1.6 $x10^{-3}$ mg/kg for artemitin (n=6) and 3.2 x 10^{-1} mg/kg for hesperidin (n=6). The present data indicate that the two flavonoids (artemitin and hesperidin) possess significant antinociceptive effects in mice which seems to justify the traditional analgesic use of *Aloysia triphylla.*

الملخص

تنتمي نبتة (Aloysia triphylla) المعروفة في الاردن بأسم المليسا الى العائلة الفربينية (المينائية). تستخدم هذة النبتة في الطب الشعبي كعامل مسكن ومقلل للاكتئاب. أدى التحليل الكيميائي للأجزاء العلوية لنبتة المليسه إلى عزل مركبين معروفين تم عزلهم للمرة الأولى من هذه النبتة وهم الأرتيميتين والهيسبيردين. لقد تم دراسة التأثير المسكن لهذه المركبات على الالم النسيجي باستخدام فحص الصفيحة الساخنة، لقد أحدث إعطاء هذين المركبين عن طريق التجويف البطني زيادة في فترة تحمل الألم عند الحيوانات وكانت الزيادة معتمدة على التركيز. ان التراكيز المحدثة لـ 50% من الاستجابة القصوى لمركب الارتيميتين والهيسبيرودين كانت 1.6×10^{-3} ملغم/كغ و 3.2×10^{-1} ملغم/كغ على التوالي. أشارة هذة الدراسة الى ان هذان المركبان يمتلكان تأثيراً مسكناً في الفئران والذي يبرر استخدام هذه النبتة في الطب الشعبي كمادة مسكنة.

Keywords: *Aloysia triphylla;* Artemitin; Hesperidin; Antinociceptive; Rat.

1. Introduction

Aloysia triphylla (Verbenaceae) is a perennial, bushy plant, originally from South America, and cultivated in various areas in the Middle East. *Aloysia triphylla* has long been used in traditional medicine. *Aloysia triphylla* has been reported to have a gentle sedative action and helps to counter depression (Guerrera et al., 1995: Chevallier, 1996; Pascual et al., 2001). An infusion of aerial parts of *Aloysia triphylla* is used as antipyretic, antispasmodic and diuretic agent (Guerrera et al., 1995; Ragone et al., 2007)). The plant has tonic effect upon the nervous system and has reputation for soothing abdominal discomfort (Guerrera et al., 1995). The plant has been found to possess antioxidant effect (Valentão et al., 2002). Phytochemical study of the plant revealed the presence of ganial, neral ,pinene, caryophyllene, limonene, curcumene, camphor, and luteolin 7-diglucuronide (Kim NS and Lee DS; 2004; Carnat, et al., 1995). Large amount of polyphenolic compounds were also isolated (Carnat, et al., 1999).

Despite of the traditional use of *Aloysia triphylla* as an analgesic, no systemic studies concerning the antinociceptive effects are available. In the present study, we are reporting the antinociceptive effects of two flavonoids (artemitin and hesperidin) which were isolated from the *Aloysia triphylla*.

2. Materials and Methods

2.1. Plant

Aerial parts of *Aloysia triphylla* (Verbenaceae) were collected from Hashemite university medicinal plant garden, Zarka, Jordan by (E.Y.Q) in April. The plant material was identified and authenticated taxonomically at the Hashemite university herbarium by a plant taxonomist.

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A voucher specimen (HU # 237) was deposited at the Hashemite university herbarium for future reference.

2.2. Extraction and isolation

The dried and finely powdered whole plant of *Aloysia triphylla* (4.0 kg) was exhaustively extracted with ethanol. The combined ethanol extracts were filtered and evaporated in vacuum to give a residue. The residue was later suspended in H_2O and fractionated with petroleum ether, ethyl acetate and n-butanol. The ethyl acetate extract was applied to a silica gel column with chloroform-ethyl acetate step gradients and finally purified on a Sephadex LH-20 column eluting with chloroform-methanol (1:1,v/v) to afford compound (1) (102 mg). The n-butanol extract was applied to RP-C18 column, using water-methanol step gradients and finally chromatographed repeatedly on a Sephadex LH-20 column eluted with methanol to yield compound (2) (120 mg). The structures of compound 1 and 2 were elucidated as artemitin and hesperidin, respectively (Figure 1). ^{1}H NMR was used to assign the structures of the two compounds.

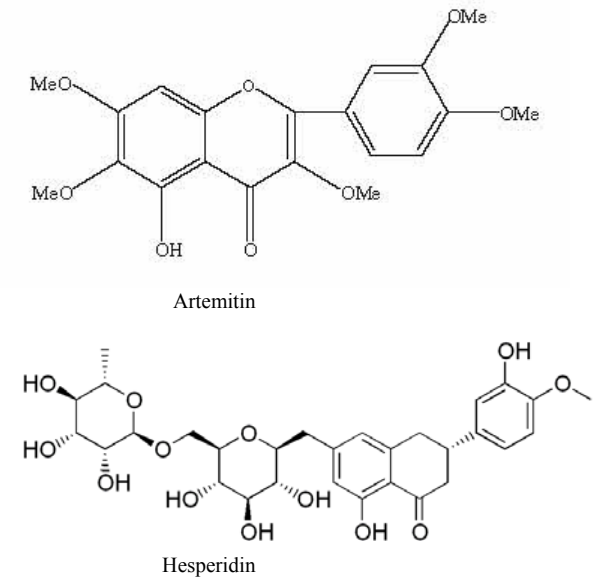

Figure 1. Chemical structure of artemitin and hesperidin.

2.3. Animals

Male mice (29-33 g), housed at 22-25ºC under a 12-h light/12-dark cycle and with access to food and water *ad libitum*, were used in the present study. The experiments were carried out in accordance with the current guidelines for the care of laboratory animals at Hashemite University and in accordance with the Ethical Guidelines for the Investigation of Experimental Pain in Conscious Animals (Zimmermann, 1983).

2.4. Hot-plate test

The hot plate test was assessed by using groups of male mice, each of 6 animals. The temperature of a hot plate was maintained at 50 ± 1ºC. Latency to a discomfort reaction (licking paws) was determined in seconds before and 60 min after intraperitoneal administration artemitin and hesperdin ($3x10^{-4}$- 10^1mg/kg). The cut-off time was 60s. The prolongation of the latency times was compared to the values of the control and used for statistical comparison. Baseline was considered as the mean of three readings of the reaction time obtained before administration of artemitin and hesperdin and was defined as the normal reaction time of animals to this temperature. Change in latency period (% of basal)) was calculated by the formula: (A-B/B) X 100, where A is the mean of three readings of reaction time after treatment taken within 5-7 minutes; B is the mean of three readings of reaction time obtained before treatment.

2.5. Statistical analysis

The values were expressed as the mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. Differences were considered significant when $P < 0.05$. ED_{50} was obtained by the best visual fit from the plot of the individual experiments.

3. Results and discussion

Phytochemical investigation of the aerial parts of *Aloysia triphylla* has led to the isolation of two compounds artemitin (102mg) and hesperidin (120 mg) (figure 1). Identification was on basis of ^{1}H NMR by comparison with data reported previously (Abu Zarga et al., 1995; Garg et al., 2001).

Artemitin and hesperdin significantly increased the time the animals took to raise their hind paw from the hotplate in a dose dependent manner (Figure 2 and 3). The ED50 values were 1.6 $x10^{-3}$ mg/kg and 3.2 x 10^{-1} mg/kg for artemitin and hesperidin, respectively. No mortality was observed during 48hr after drug administration.

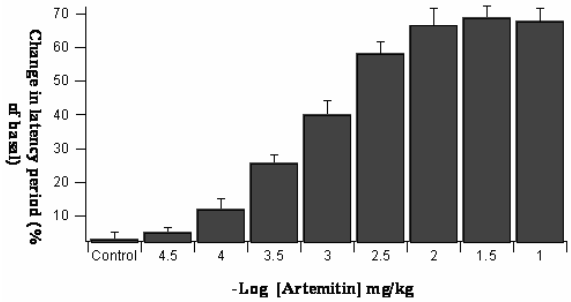

Figure 2. Effect of artemitin on the latency of mice submitted to the hotplate test.

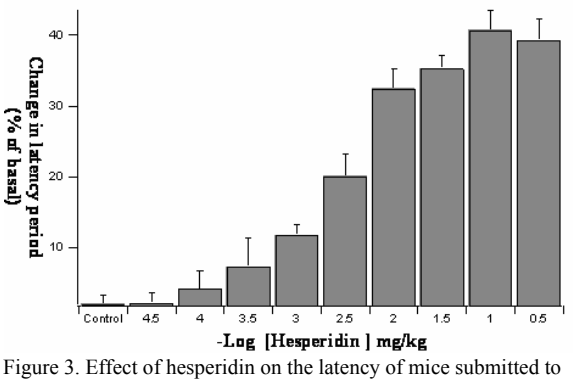

Figure 3. Effect of hesperidin on the latency of mice submitted to the hotplate test.

Artemitin is a bioflavonoid, which has been reported as a potential anticancer (Li et al., 2005) and chemopreventive and chemotherapeutic agent (Ko et al.,

2000). It has been claimed that it has an anti-inflammatory effect (Sertié et al., 1990). However, other studies showed that such claim is not justified (Bayeux et al., 2002). Additionally, artemitin was found to induce relaxation in smooth muscle (Abu Zarga et al., 1995). To the best of our knowledge, this the first report to show that artemitin possess an antinocicpetive activity. The mechanism by which artemitin induced an antinociceptive effects need further studies to be elucidated.

Hesperidin is a bioflavonoid, which has been reported to possess a wide range of pharmacological properties. It has been reported to have significant anti-inflammatory and analgesic effects (Galati et al., 1994). Several mechanisms have been suggested to explain such activity including: inhibition of histamine release (Emim et al., 1994); and inhibition of eicosanoid synthesis (Jean and Bodinier, 1994). Additionally, hesperidin was found to have central nervous system depressant effects (Marder et al., 2003). Recently, Loscalzo et al., (2008) showed that the effects of hesperidin were fully blocked by the nonselective opioid antagonist naltrexone, which may implicate opioid receptors on the antinociceptive effects of hesperidin.

In conclusion, results obtained from the present study indicate that the two flavonoids (artemitin and hesperidin) possess significant antinociceptive effects in mice which seem to justify the traditional analgesic use of *Aloysia triphylla.*

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Effect of Sperm Chromatin Integrity on Parameters of In Vitro Fertilization

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Abstract

Infertility affects 15% of couples seeking children world-wide and male factor is evident in about 30-50% of the cases. To assess the effect of sperm chromatin integrity on the outcomes of intracytoplasmic injection, sperm DNA compactness was evaluated by acridine orange staining in 40 fertile and 44 infertile men attending one infertility clinic. The results showed no significant difference in the percentage of sperm with integrated DNA between the fertile and the infertile groups ($p>0.05$). Also, no significant difference ($p>0.05$) between fertile and infertile men was found in terms of fertilization rate, and pregnancy rate. However, a significant difference was found in developmental rate ($p<0.05$). When the percentage of sperm with DNA fragmentation was ≥38%, there was a decline in embryos quality. The current work concludes that conventional semen analysis is moderately predictive of an individual's infertility. Thus, a need arises for new assays for the evaluation of sperm quality.

الملخص

عالميا، تبلغ نسبة الأزواج الراغبين بالإنجاب و العاجزين عن تحقيق ذلك بسبب إحدى مشاكل العقم 15%،
حيث يكون الرجل مسؤولا عن 30-50% من مثل هذه الحالات. تهدف هذه الدراسة إلى قياس معدل تكسر المادة الوراثية للحيوانات المنوية الموجودة في عينات السائل المنوي لأربع و اربعين رجل عقيم و لأربعين رجل خصيب (بناء على التحليل التقليدي للسائل المنوي)، وذلك بإستخدام طريقة صباغة الحيوانات المنوية بمادة الأكردين البرتقالية. كما أنها تهدف إلى تقييم تأثير التكسر في المادة الوراثية للحيوان المنوي على مخرجات عملية الحقن المجهري للحيوان المنوي داخل الغشاء البلازمي للبويضة، ومقارنة معدل هذه المخرجات بين الأشخاص العقيمين والخصيبين.
توصلت الدراسة إلى أنه لا يوجد فرق معنوي بين الرجال العقيمين والخصيبين من حيث معدل تكسر المادة الوراثية للحيوانات المنوية أو معدل الإخصاب والحمل، و لكن يوجد فرق معنوي بين المجموعتين في معدل التطور الجنيني. كما وجدت الدراسة أيضا أنه في حالة كون نسبة الحيوانات المنوية ذات المادة الوراثية المتكسرة ≤ 38% فإن ذلك يؤثر على نوعية الأجنة الناتجة من الحقن المجهري وذلك بزيادة أعداد الأجنة متوسطة الجودة والرديئة.
إن تحليل السائل المنوي بالطريقة التقليدية قد لا يكون ذا قيمة لإعتماده كمرجع لإختيار الحيوان المنوي المناسب من أجل عملية الحقن المجهري داخل الغشاء البلازمي للبويضة. لذلك فإن تحليل معدل تكسر المادة الوراثية في الحيوان المنوي يعتبر قيمةأخرى فعالة.

Keywords: Compact Chromatin, ICSI, Acridine orange, In Vitro Fertilization, Infertility.

1. Introduction

Infertility is defined as the inability to conceive after one year of regular, unprotected intercourse (WHO, 1999). It affects approximately 15% of all couples seeking to have children (Sharma *et al.*, 2004). Male factor is evident in about 30%-50% of clinical infertility cases worldwide (Larson-Cook *et al*., 2003) and in 35% of infertility cases in Jordan (Tahtamouni *et al.,* 2002). Poor sperm quality is represented by abnormal semen parameters, including low sperm concentration, poor sperm motility, and abnormal sperm morphology (Chen *et al*., 2006).

Over the last decade, the use of assisted reproductive techniques (ART) to overcome the problem of couple infertility has increased, and the use of intracytoplasmic sperm injection (ICSI), initially suggested for cases of severe male factor infertility, has been expanded (Gandini *et al*., 2004). ICSI bypasses all processes of natural selection presented by sperm-oocyte physical interaction, which is still present in conventional in-vitro fertilization (IVF), and relies on the direct injection of sperm into the oocyte cytoplasm. And this increased the concern about sperm genome integrity and the safety of ICSI-born progeny (Gandini *et al*., 2004).

The prominent risks of ICSI come about from forcing fertilization with sperm that might contain hidden DNA damage (Larson-Cook *et al*., 2003). The exact mechanism(s) by which chromatin abnormalities/DNA damage arise in human spermatozoa is not precisely understood, but three main theories have been proposed: namely, defective sperm chromatin packaging, apoptosis and oxidative stress (Lopes *et al*., 1998; Agarwal *et al*., 2003; Sharma *et al*., 2004). Infertile men display higher levels of loosely packaged chromatin and damaged DNA (Virant-Klun *et al.,* 2002).

Sperm DNA instability may have possible negative impact on the outcomes of ART including fertilization, blastocyst development, and pregnancy rates (Seli *et al*., 2004; Huang *et al*., 2005). It has also been reported that sperm concentration, motility and morphology might be affected by sperm DNA fragmentation (Saleh *et al*., 2003).

Rapid advances in reproductive molecular biology in the last two decades have resulted in techniques to assess sperm DNA fragmentation. Acridine orange (AO) staining

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is a simplified microscopic and cytochemical method for determining sperm DNA integrity; allowing the differentiation between normal, double-stranded and abnormal, single-stranded sperm DNA, using the metachromatic properties of the dye (Virant-Klun *et al.,* 2002*)*. The fluorochrome AO intercalates into double-stranded sperm DNA as a monomer and binds to a single stranded sperm DNA as an aggregate. The monomeric AO, bound to normal double-stranded DNA, fluoresces green, whereas the aggregated AO on single-stranded DNA fluoresces yellow to red (Virant-Klun *et al.,* 2002*)*. Increased red fluorescence indicates increased sensitivity to denaturation. Sperm cells having DNA that is sensitive to denaturation also have DNA strand breaks (Gopalkrishnan *et al*., 1999). AO staining is a well-established method to study nucleic acid changes since the early sixties (Bertalanffy, 1963).

Some studies show that sperm single-stranded DNA, detected by AO staining, affects the fertilization process in a classical IVF program negatively (Virant-Klun *et al.,* 2002). In addition, when the level of spermatozoa with single-stranded DNA was increased, there was a significantly lower fertilization rate and a higher percentage of fragmented embryos after ICSI. On the other hand, no correlation was found between the level of spermatozoa with single-stranded NA, pregnancy rate, and live-birth rate achieved by ICSI (Virant-Klun *et al.,* 2002).

The current work aims at assessing the degree of sperm DNA fragmentation, measured by AO staining, in fertile vs. infertile men. Also, it aims at evaluating the effect of sperm single-stranded DNA on ART outcomes.

2. Materials and Methods

2.1. Sample Selection

The study population consisted of 84 chosen couples presented for ICSI at the Infertility Unit of Farah Hospital, Amman-Jordan. All men were informed of the research and all gave their consent for participation.

2.2. Seminal Fluid Collection and Analysis

Semen samples were collected by masturbation into sterile jars after 48 to 72 hours of sexual abstinence. Semen analysis for the 84 men, attending the infertility center, was performed according to the method described in the World Health Organization (WHO) laboratory manual (1992). Analyzed semen parameters were: pH of the semen, concentration of sperm/ml, % motility, and % normal morphology. According to semen sample analyses, 44 couples were diagnosed with male factor infertility, while the remaining 40 men were fertile and were used as the control group.

2.3. Data Collection

Data about the chosen 84 couples were obtained by referring to medical records. The data included type, duration and cause of infertility, patients' age, and ICSI information. The latter also included number of picked up, injected and fertilized oocytes, number and type(s) of developed and transferred embryos, and pregnancy test result confirmed by the determination of serum beta-HCG 15 days after embryo transfer (ET) to the uterus.

In all couples, fertilization rate [no. of fertilized oocytes (presence of two pronculei after 18-20 hr of injection) per all oocytes] was calculated. Forty eight hours after the ICSI procedure (Day 2), the no. of the fertilized oocytes which developed into embryos (at least two cells and preferably three or four cells, cleaving stage) (Larson-Cook *et al*., 2003) was determined as the developmental rate (no. of embryos per fertilized oocyte). According to Farah infertility unit, embryos were classified into four types (I-IV). Type I or excellent embryos had regular, spherical blastomeres of an equal shape and size, with no fragmentation. Type II or good embryos had regular, spherical blastomeres of an equal shape and size, with some fragmentation (<30%). Type III or fair embryos had blastomeres slightly irregular in size and shape with considerable fragmentation (30-50%). Type IV or bad embryos had embryos with barely defined blastomeres with considerable fragmentation (>50%) (Xia,1997).

2.4. Evaluation of Sperm Chromatin Integrity by Acridine Orange Staining

Acridine orange staining was performed according to the method of (Tejada *et al.*, 1984; Virant-Klun *et al.*, 2002). After washing and drying, the slides were immediately observed under a fluorescent microscope (Olympus, Japan; excitation of 450–490 nm). Averages of 200 sperm cell were counted on each slide by the same examiner. Sperm displaying green fluorescence were considered having normal DNA content, whereas sperm displaying a spectrum of yellow–orange to red fluorescence were considered having damaged DNA. The percentage of spermatozoa with single-stranded DNA was calculated from the ratio of sperm with red, orange, or yellow fluorescence to all sperm counted per sample.

2.5. Statistical Analysis.

Statistical analysis was performed with STATISTICA for Windows software version 6.0 (Stat Soft Inc., USA). Student's t-test and ANOVA (One-way ANOVA) were used for the comparison of quantitative parameters. Correlation matrices were used for calculation of correlation coefficient. Data are stated as means ± Standard Error of the Mean (S.E.M). Statistical differences were considered significant at $p < 0.05$.

3. Results

3.1. Seminal Fluid Analysis

Seminal fluid analysis (SFA) for eighty four men attending the Infertility Unit at Farah Hospital (Amman, Jordan) was performed. According to WHO criteria, 1992, forty men were considered fertile as they had normal semen, and the other 44 men were considered infertile based on their SFA (Table 1). The mean age for fertile and infertile men was 39.5 ± 1.2 and 38.3 ± 1.5 years, respectively. The cause of infertility was due to male factor in all of the 44 infertile couples. Nineteen of the infertile couples had primary infertility while 25 couples had secondary infertility with a duration of 6.3 ± 1.2 years.

The fertile and infertile groups differed significantly in the volume of the semen ($p<0.01$), sperm concentration ($p<0.001$) and in the percentage of motile sperm ($p<0.001$), and normal sperm forms ($p<0.0001$) (Table 1).

Table 1. Seminal Fluid Analysis. Values are expressed as mean ± SEM.

Parameter	Fertile men (n= 40)	Infertile men (n=44)	p-value
Volume (ml)	3.2 ± 0.2	4.6 ± 0.4	<0.01
pH	8	8	-
Sperm count (106/ml)	55.9 ± 4.4	34.8 ± 4.1	P<0.001
Progressive motile sperm (%)	60.5 ± 1.2	50.9 ± 2.2	P<0.001
Normal sperm forms (%)	19.2 ± 0.8	10.1 ± 2.0	P<0.0001

4. Evaluation of Chromatin Integrity of Sperm DNA by Acridine Orange Staining.

Sperm DNA fragmentation was detected with the acridine orange staining method, in which sperm with single-strand DNA was identified by the presence of evident yellow to red fluorescence. No significant difference in the percentage of sperm with fragmented DNA was found between the fertile (21.9 ± 4.2%, 0.0-93.0%) and the infertile groups (26.7 ± 5.0%, 0.0-91.5%) (p>0.05).

4.1. ICSI Outcomes

No significant difference in the mean of the fertilization rate was found between the fertile (78.8 ± 2.6%) and infertile groups (69.4 ± 4.7%) (p>0.05). On the other hand, a significant difference was found in the developmental rate between the fertile (99.1 ± 0.6%) and the infertile groups (89.4 ± 4.8%) (p<0.05). Biochemical pregnancy was confirmed by the serum beta-HCG determination 15 days after ET. In both the fertile and infertile groups, the positive pregnancy rate was almost similar (39.4% and 38.1%, respectively, p>0.05) (Table 2).

Table 2. Comparison between ICSI outcomes in fertile and infertile groups. Values are expressed as mean □ SEM.

Parameter	Fertile group n=40	Infertile group n=44	P-value
DNA fragmentation rate (%)	21.9 ± 4.2	26.7 ± 5.0	P>0.05
Fertilization rate (%)	78.8 ± 2.6	69.4 ± 64.7	P>0.05
Development rate (%)	99.1 ± 0.6	89.4 ± 4.8	P<0.05
Pregnancy rate (%)	39.4 ± 1.9	38.1 ± 2.2	P>0.05

4.2. The effect of sperm DNA fragmentation on fertilizationrate

No correlation was observed between DNA fragmentation and fertilization rate in the fertile group (r= -0.02, p<0.00001) and the infertile group (r= -0.02, p<0.00001).

4.3. The effect of sperm DNA fragmentation on developmental rate

A small significant negative correlation was observed between DNA fragmentation and developmental rate in the fertile group (r= -0.20, p<0.00001). A moderate significant negative correlation was observed in the infertile group (r= -0.51, p<0.00001).

4.4. The effect of sperm DNA fragmentation on embryo quality

To elucidate the effect of DNA fragmentation on embryo quality, we divided the infertile group (data regarding the types of embryos were not available for the fertile group) according to DNA fragmentation rate into two groups, group I (sperm DNA fragmentation < 38%; n=32) and group II (sperm DNA fragmentation ≥38%; n=12) (Table 2). No significant difference was found between the two groups in terms of no. of embryos of type I and II (p>0.05, and p>0.05, respectively). However, there was a significant difference between the two groups in the no. of type III and IV embryos (p<0.05 and p<0.001, respectively) (Table 3).

4.5. The effect of sperm DNA fragmentation on pregnancy rate

DNA fragmentation had no effect on whether a pregnancy occurred or not. For the fertile group, DNA fragmentation rate was 19.4%± 6.3 for those couples who achieved pregnancy, and 25.3%±7.5 for those who did not, p>0.05. In the infertile group, DNA fragmentation rate was 26.8%±7.5 and 17.1%±4.6 %, respectively, p>0.05 (Figure 1).

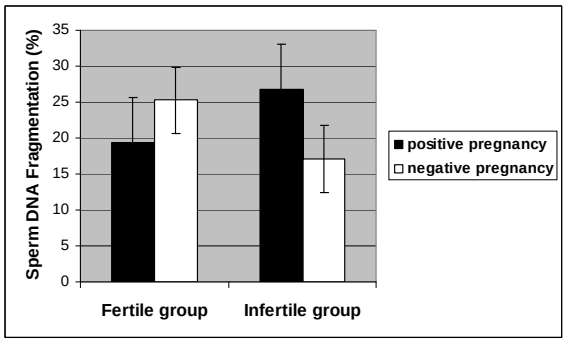

Figure 1. The relation between sperm DNA fragmentation and pregnancy result. Values are expressed as mean ± SEM. P >0.05.

4.6. Primary and secondary infertility and sperm DNA fragmentation and ICSI outcomes

To compare primary with secondary infertility according to DNA fragmentation, fertilization rate and developmental rate, the infertile couples were divided into two subgroups: primary infertility (n=19) and secondary infertility (n=25). No significant difference in DNA fragmentation rate (p>0.05), fertilization rate (p>0.05) and the developmental rate (p>0.05) was found between infertile couples suffering from primary or secondary infertility (Table 4). Also, no significant difference was found in the pregnancy rate between primary and secondary infertile males (32% vs. 43%, p>0.05) (Table 4).

5. Discussion

Acridine orange (AO) staining is an established cytochemical method for determining sperm DNA integrity, allowing the differentiation between normal, double-stranded and abnormal, single-stranded sperm

DNA, using the metachromatic properties of the dye (Virant-Klun *et al*., 2002; Martins *et al.,* 2007). The AO staining can be used to evaluate the integrity of the sperm nucleus, disorders of which can cause unexplained infertility or lower fertilization potential that may go undetected by routine analysis (Gopalkrishnan *et al*., 1999).

There has been a tremendous amount of controversy in literature in terms of the degree of sperm DNA fragmentation in fertile versus infertile men. This could be attributed mainly to two reasons; the method of testing for chromatin integrity, and/or the way researchers define "fertile" and "infertile" male. Many techniques have been developed to test chromatin fragmentation. Three of these

Table 3. Quality of embryos in the fertile group according to the percentage of sperm with DNA fragmentation

Infertile group	Excellent embryos Type I	Good embryos Type II	Fair embryos Type III	Bad embryos Type IV
Group I (n=32) (Fragmentation rate≤ 38%) No. of embryos= 79	46.2 ± 1.1%	27.9 ± 0.8%	24.6 ± 0.5%	1.3 ± 0.1%
Group II (n=12) (Fragmentation rate≥ 38%) No. of embryos= 112	36.9 ± 0.9%	18.7 ± 0.3%	38.2 ± 1.2%	6.2 ± 0.3%
P-value	P >0.05	P >0.05	P <0.05	P <0.001

Table 4. Comparison between DNA fragmentation and ICSI outcomes in primary and secondary infertile men subgroups. Values are expressed as mean ± SEM.

Parameter	Primary infertility n=19	Secondary infertility n= 25	P-value
DNA fragmentation rate (%)	16.9 ± 5.5	25.8 ± 6.6	P>0.05
Fertilization rate (%)	76.4 ± 4.7	80.7 ± 3.1	P>0.05
Development rate (%)	89.3 ± 0.7	89.0 ± 1.1	P>0.05
Pregnancy rate (%)	32.6 ± 2.3	43.9 ± 1.4	P>0.05

are Sperm Chromatin Structure Assay (SCSA) (Evenson and Wixon, 2006), In Situ DNA Nick End Labeling Assay (TUNEL) (Seli *et al.,* 2004, Huang *et al.,* 2005; Sergerie *et al.,* 2005), and Acridine Orange Staining (AO) (Tejada *et al.,* 1984; Virant-Klun *et al.,* 2002). Many researchers consider AO technique not to be sufficiently reliable for clinical use (Evenson and Wixon, 2005). The primary reason is that the metachromatic property of acridine orange requires a very strict equilibrium concentration of the dye and perfectly flat glass slides and cover slips (Kapuscinski *et al.,* 1983).

The second reason for the controversy arises from the fact that some researchers choose their "fertile, control" group as men with normal SFA (Seli *et al.,* 2004; Huang *et al.,* 2005), while others choose them as men of proven fertility (fathering a child or achieving pregnancy) (Sergerie *et al*., 2005). In the current work, our control, fertile men were chosen based on their normal seminal fluid analyses (SFA) (Table 1).

Our data show that sperm DNA fragmentation rate was not significantly different between fertile (21.9%) and infertile groups (26.7%) (p>0.05), which agrees with the results reported by Chohan and colleagues, 2004, and it might support the hypothesis that conventional SFA is not of a paramount value in choosing a sperm for ICSI. Indeed, some investigators have used sperm DNA fragmentation rate as a marker to differentiate fertile from infertile men (Lopes *et al.,* 1998).

In the current work, there was no significant difference between fertile and infertile men in terms of fertilization rate (78.8% and 69.4%, respectively) (p>0.05), and pregnancy rate (39.4% and 38.1%, respectively) (p=0.05). A significant difference was found in the developmental rate (99.1% and 89.5%, respectively) (p<0.5) (Table 2). It is possible that if the sperm DNA fragmentation rate is low, the oocyte might be able to repair the condition and the fertilization is successful (Huang *et al.,* 2005). On the other hand, the first steps of the development are subjected to maternal control, and the expression of paternal genes begins at the 4–8 cell stage. It is therefore at this stage that the consequences of paternal DNA integrity may become evident and thus impairing embryo development (Borini *et al*., 2006). Most studies found that when the level of spermatozoa with single-stranded DNA was increased, there was a significantly lower fertilization rate after ICSI (Lopes *et al*., 1998; Virant-Klun *et al*., 2002; Huang *et al*., 2005).

A significant negative correlation was observed between DNA fragmentation and developmental rate in the fertile (r= - 0.20) and in the infertile groups (r= -0.51). This is in agreement with the extensive literature which showed that sperm DNA fragmentation might affect the outcome of assisted reproduction through its effect on embryo development (Virant-Klun *et al*., 2002; Saleh *et al*., 2003). Sperm DNA fragmentation probably prevents the formation of blastocysts and embryo development (Borini *et al.,* 2006).

In terms of embryo quality, infertile men had more embryos of type III (fair embryos) and type IV (bad embryos). The number of type III and type IV embryos increased when DNA fragmentation was ≥ 38% (Table 3). This is in agreement with other reports. For example, a significant negative association between the percentage of sperm with DNA fragmentation and embryo quality was reported by Virant- Klun *et al*. (2002) and Huang *et al*., (2005). However, few studies indicated that pregnancy rate might be affected by sperm DNA fragmentation (Benchaib *et al*., 2003), while others showed that no correlation was found between DNA fragmentation and pregnancy rate (Virant-Klun *et al*., 2002; Gandini *et al*., 2004). Our data showed that DNA fragmentation had no effect on whether a pregnancy occurred or not (Figure 1). Usually, embryos of bad quality (type III and IV) are not transferred to the uterus, and if it happens it might then affect the pregnancy rate. Also, embryonic quality is not the only factor affecting implantation rate; implantation is a complex process involving several maternal factors (Virant-Klun *et al*., 2002).

Data presented in Table 4 show a comparison between DNA fragmentation and ICSI outcomes in primary and secondary infertile males' subgroups. Primary infertility is the term used to describe a couple that has never been able to conceive a pregnancy, after at least 1 year of unprotected intercourse. On the other hand, secondary infertility describes couples who have previously been pregnant at least once, but have not been able to achieve another pregnancy (WHO, 1999). No significant difference was found in sperm DNA fragmentation, fertilization rate, developmental rate and pregnancy rate if the patient suffered from primary or secondary infertility (Table 4).

In summary, many factors affect the outcomes of ICSI. One of these factors is the integrity of sperm chromatin. Therefore, it becomes a priority to look for an assay which tests the chromatin integrity of the sperm used in ICSI technique. Such an assay must not be invasive or fatal for that sperm.

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Antihyperglycemic and antihyperlipidaemic activities of root extracts of *Calotropis procera* (Ait.) R.Br on streptozotocin induced diabetic rats.

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Abstract

The root extracts of *Calotropis procera* were investigated for its anti-hyperglycemic effect in Male Wister Albino rats. Diabetes was induced by administration of single dose of streptozotocin (STZ, 50 mg/kg, I.P) Pet. ether, methanol and aqueous extracts of roots of *C. procera* at dose of 250 mg/kg, b. wt were administered as a single dose, per day to diabetes rats for the period of 15 days, respectively. After extracts treatment, the blood glucose levels were decreased from 238.6±2.30 to 198.8±1.8 and 236.0±1.58 to 154.0±1.58, 236.0±1.58 to 142.6±2.07 and 238.6±2-64 to 164.4±2.70 mg/dL respectively. The effect of *C. procera* on blood glucose level was measured in the diabetic rats. Serum lipid profile like total cholesterol (TC), triglycerides (TG), phospholipids (PL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and high density lipoprotein (HDL) were measured in the diabetic rats. The activities were also compared to that effect produced by a standard anti-diabetic agent, glibenclamide 500µg/kg. The present investigation established pharmacological evidence to support the folklore claim that it is an anti-diabetic agent.

Keywords: *Calotropis procera*, Glibenclamide, Hyperglycemia, Streptozotocin.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder affecting approximately 10% of the global population. Besides hyperglycemia, several other factors including dislipidemia or hyperlipidemia are involved in the development of micro and macro vascular complications of diabetes which are the major causes of morbidity and death (Bennet, 1998). Plants have played a major role in the introduction of new therapeutic agents. A medicinal plant, *Galega officinalis*, led to the discovery and synthesis of metformin (Aiman, 1970). Despite considerable progress in the treatment of diabetes by oral hypoglycemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations. In recent times, there has been a renewed interest in the plant remedies (Dinesh Puri, 1997; Ratnakar, 1996).

C. procera (Aselepiadaceae) is a small plant and found in tropical and subtropical regions of India. The wood is used as cheap fuel and latex is used in tanning industry, and pharmacologically latex is used as a wound healing agent by traditional healers and as an abortificient in folk medicines. The plants were considered sacred and the leaves were used in Vedic times in sun worship. Polysaccharides, four cardenolids were isolated from leaves (Qudrat-I-Khuda, 1969) triterpenes from root bark of *C. procera* (Ansari, 1999).

The plant selected for this present work is locally available in the Salem district and has been used for long a time in local folklore medicine for the treatment of diabetics.

2. Materials and Methods

2.1. Materials and Methods

The plant materials were collected in the forest area of Ghaziabad (Uttar Pradesh). The plant was authenticated and identified at raw material herbarium and museum, National Institute of Science Communication and Information Resources, Delhi. The plant material was shade dried at room temperature for 10 d, coarsely powdered with the help of a hand-grinding mill, and the powder was passed through sieve No.60 and used for extraction.

2.2. Preparation of the extract

The powdered *C. procera* root material (1000g) was extracted separately using pet. Ether, methanol by Soxhlet and aqueous extract by cold maceration. The extracts were dried under reduced pressure.

2.3. *Animals*

Male albino rats, 9-12 weeks old with an average weight of 150-175 g were used for the study. They were housed in polypropylene cages and fed with a standard chow diet and water ad libitum. The animals were exposed

to an alternating 12 h and light cycle. Before each experiment, the animals were fasted for at least 18 h. The experimental protocols were approved by Institutional Animal Ethical Committee.
NISCAIR/RHMD/CONSULT/2008-09/996/27,

2.4. Toxicity evaluation in mice

Male albino mice, 6-8 weeks old with an average weight of 25-30 g were used for the study. The methanol and aqueous extracts were tested for their toxicity in mice by administrating, a single oral pet. ether, methanol, and aqueous extracts of *C. procera* in different dose to different groups of mice (6 mice were used for each group). The control group received Tween. Mortality and general behavior of the animals were observed periodically for 48 h. The animals were observed continuously for the initial 4 h followed by 6 h, 24 h and 48 h after drug administration. The parameters observed were grooming, hyperactivity, sedation, respiratory rate and convulsion.

2.5. Preliminary phytochemical screening

The extracts were subjected to preliminary screening for various active phytochemical constituents (Kokate, 1994).

2.6. Drugs and chemicals

Alloxan monohydrate was purchased from S.D Fine chemicals Ltd., Boisar. Glibenclamide was procured from Aventis Pharma, Mumbai, India. All other chemicals were obtained from local sources and were of analytical grade.

2.7. Preparation of extract, reference and STZ

The extract was administered orally to rats, as a suspension in 1% Carboxy Methyl Cellulose (CMC). Glibenclamide (500 µg/kg) was suspended with 1% w/v CMC and administered orally (Standard drug). STZ was dissolved in 0.9% ice-cold saline immediately before use.

3. Experimental design: Long-term experiment

3.1. Streptozotocin-induced diabetic rats

Streptozotocin (STZ) was dissolved in 0.9% ice-cold saline immediately before use. Diabetes was induced in rats by intra peritoneal (i.p) injection of streptozotocin at a dose of 50 mg/kg, dissolved in saline (Pulok K Mukarjee, 2002). Forty eight hours after streptozotocin administration, blood samples were drawn from tail and glucose levels determined to confirm diabetes. The diabetic rats exhibiting blood glucose levels higher than 200 mg/dl were selected for the studies.

3.2. Experimental procedure

In experiment, a total of 30 rats were used (36 diabetic surviving rats, 6 control rats) for the execution of the experiment. The rats were divided as follows into six groups

- Group I : Control rats (Vehicle treated)
- Group II: Diabetic control (Received 0.5 ml of 5% Tween 80)
- Group III: Diabetic rats given Glibenclamide 500 µg/kg (Received 0.5 ml of 5% Tween 80) (Augusti, 1996)
- Group IV, V and VI : Diabetic rats given pet. ether, methanol and aqueous extracts of *C. procera* 250 mg/kg b. wt,

Blood samples were collected from the tail for glucose estimation just before drug administration on the first day and 1 h after drug administration on days 4, 7, 10 and 15. Blood samples were collected and centrifuged to separate serum for estimation of lipid profile and other biochemical parameters (Harold varley, 1983).

3.3. Anti-hyperlipidaemic activity

Total cholesterol, HDL- C, LDL-C, VLDL-C, and triglycerides were analyzed from serum. Total cholesterol was estimated according to Liebermann Burchard Reaction Method Richterich, 1981). LDL cholesterol was estimated indirectly by Friedwald's method (Friedwald, 1972). Triglycerides (TG) were determined using Hantzsch condensation method (MacDonald, 1970).

3.4. Statistical evaluation

All the data are presented as mean ±SEM, n= 6. The differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by the Dunnette multiple comparisons test. $P<0.01$ was considered to be significant.

4. Results

4.1. Preliminary phytochemical test

Phytochemical studies indicated that extracts of roots of *C. procera* contains alkaloids, flavanoids, glycosides, saponins and terpenes.

4.2. Acute toxicity studies

In performing preliminary test for pharmacological activity in rats, aqueous extract did not produce any significant changes in the behavioral or neurological responses up-to 2500 g/kg b. wt. acute toxicity studies revealed the non-toxic nature of the pet. ether, methanol and aqueous extracts of the roots of *C. procera.*

4.3. Antihyperglycemic activity

The effects of extracts on blood glucose levels in diabetic rats are reported in Table 1. Blood glucose levels of the STZ treated rats were significantly higher than those in normal rats. In STZ (50 mg/kg) induced rats, the blood glucose level significantly increased from 93.89±1.47 to 238.2±2.22 mg/dl. Pet. Ether, methanol and aqueous extracts (250 mg/kg) given up-to 15 days. After extracts treatment, the blood glucose levels were decreased from 238.6±2.30 to 198.8±1.8 and 236.0±1.58 to 154.0±1.58, 236.0±1.58 to 142.6±2.07 and 238.6±2-64 to 164.4±2.70 mg/dL respectively, Whereas in glibenclamide treated rats, blood glucose levels were decreased from 237.0±1.00 to 136.8±3.11mg/dL.

4.4. Antihyperlipidaemic activity

The lipid profiles in control and experimental rats are depicted in Table 2 in STZ induced diabetic rats, there was a significant ($P<0.001$) increase of total cholesterol, triglycerides, phospholipids, and low density lipoproteins (LDL) and very low density lipoprotein (VLDL)

cholesterol and significant ($p<0.001$) decreases in high density lipoprotein (HDL) cholesterol in serum compared with normal control. The extracts-treated rats indicated significantly ($p<0.001$) decreased total cholesterol, triglycerides, phospholipids and LDL and VLDL cholesterol and significantly ($p<0.001$) increased HDL.

Table 1. Effect of extracts of *C. procer*a on blood glucose level on streptozotocin-induced diabetes in rats

Treatment mg/kg	Blood glucose level (mg/dl)			
	Day 1	Day 5	Day 10	Day 15
Normal control	123.89±1.47	124.2±2.26	123.4±2.46	123.2±0.22
Diabetic control	238.2±2.22	242.6±2.24	240.8±4.24	248.6±4.48
Glibenclamide 500 µg/kg	237.0±1.00	204.8±2.58	192.6±2.07	136.8±3.11
Pet. Ether extract (R) 250	235.6±2.30	219.8±2.74	215.2±1.92	203.2±1.59
Methanol extract (R) 250	234.8±1.48	208.8±1.58	197.6±1.58	144.2±2.22
Aqueous extract (R) 250	235.2±1.58	230.2±3.11	214.2±2.91	163.2±1.30

Values are mean ±SEM, n= 6 (One way ANOVA Followed by Dunnette multiple Comparisons test).

Super script *, **, denotes statistically significance of P<0.05, P<0.01, P<0.001, when compared with respective diabetic control

Table 2. Antihyperglycemic effects of extracts of leaves of *C. procera* on STZ induce diabetic rats

Treatment mg/kg	Changes in mg/dL level					
	Serum total cholesterol	Triglycerides	Serum HDL	Serum LDL	Serum VLDL	Serum Phospholipids
Normal control	81.5 ± 7.9	89.3 ± 7.5	23.8 ± 2.1	35.3 ± 3.3	13.3 ± 1.7	146.3 ± 7.7
Diabetic control	195.3 ± 11.6*	170.3 ± 5.1	13.3 ± 3.1*	71.7 ± 8.3*	30.3 ± 1.1*	255.8 ± 10.6*
Glibenclamide 500 µg/kg	133.7 ± 9.2**	114.7 ± 2.8**	19.2 ± 1.0**	40.2 ± 4.5**	17.8 ± 2.0**	178.8 ± 7.6**
Pet. Ether extract (R) 250	142.8± 0.2	130.0±0.22	19.6±4.8	45.3±3.2	26.8±4.6	188.0±2.2
Methanol extract (R) 250	138.8± 4.6	122.8±2.2	20.5± 2.8	44.8±4.2	18.6 ± 8.2	180.4±0.2
Aqueous extract (R) 250	144.0± 8.6	132.2±0.2	18.4±4.8	47.8±6.2	22.8±2.2	192.0±4.8

Values are mean ±SEM, n= 6 (One way ANOVA Followed by Dunnette multiple Comparisons test).

Super script *, **, denotes statistically significance of P<0.05, P<0.01, P<0.001, when compared with respective diabetic control.

5. **Discussion**

Diabetic mellitus is a metabolic disease associated with impaired glucose metabolism which in effect alters intermediary metabolism of lipids and proteins adversely. Most of the complications of the diabetic state are initiated by the generation of free radicals: for instance LDH oxidative modification, leading to atherosclerosis

(Felmeden 2003; Bhakdi, 2004) occurs only in the presence of free radicals.

The presence study was conducted to study the antihyperglycemic and antihyperlipidaemic activities of extracts of *C. procera* in rats as well as to provide on introductory approach for the evaluation of its traditional preparation in order to scientifically validate the therapeutic preparation of this plant in the control of diabetes.

It has been established that diabetes mellitus altered the normal metabolism of lipids in diabetic rats. It is seen that cholesterol and triglycerides are elevated in the diabetic condition; such an elevation represented the risk factor for coronary heart disease. There was a significant reduction in the cholesterol and triglycerides level of diabetic rats after *C. procera* treatment for 15 days.

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