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Malaria prevalence in district Okara, Punjab, Pakistan

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ABSTRACT

The present study was conducted to find out the prevalence of malaria in the human population of selected four villages of district Okara, province Punjab, Pakistan. Malaria is endemic from the last few years in this area. Blood samples were collected by passive case detection method from four private clinics and a major Basic Health Unit (BHU). Prevalence was determined using slide positivity rate (SPR) across various variables such as village residence, season, age and gender. Blood samples analysis indicated 26% malarial prevalence, out of which 18% cases were male positive and 8% were female positive. Children were more susceptible (43%) as compared to adults (22%) as revealed by passive case detection (PCD) screening for malaria. In general prevalence of malaria was greater in August (41%) as compared to June (22%) and July (23%). Infection with *Plasmodium vivax* was more predominant (98%) as compared to *P. falciparum* (2%). Current study indicated that malaria prevalence in selected villages of district Okara was 13 times more as compared to data from various regions of province Punjab.

**Key words:** Prevalence, Malaria, Okara, Pakistan, Slide positivity rate

INTRODUCTION

Malaria is one of the most prevalent diseases throughout the tropical and most of the sub-tropical areas of the world. WHO estimates that it is endemic in 104 countries (WHO, 2012) where more than three billion population living under threat of malaria. More than 200 million cases are reported each year throughout the world with almost one million deaths mostly among children (WHO, 2011). Gradual escalation of death rate from malaria involves different factors. However, lack of vigilance and adequate resources for its control are among major factors in many countries including Pakistan. The disease is widespread in rural as well as urban areas of Pakistan (Reisen & Milby, 1986). Global malaria situation is steadily rising and its economic cost is also enormous causing heavy annual economic losses (Gallup & Sachs, 2001; Goodman et al., 1999) for developing countries particularly (Brinkmann & Brinkmann, 1991).

Emerging evidences suggest that malaria is the foremost health problem for the population of South-East Asian (SEA) countries. Only Africa accounts for 90% of entire global malaria deaths and from remaining 10%, seventy percent deaths are reported in SEA region. Pakistan is considered to have medium level of malaria occurrence as about 50,000 deaths out of approximately 500,000 reported annual malaria cases. Recent data from Pakistan indicated that *P. vivax* has a higher prevalence. Falciparum malaria is responsible for about one million deaths annually (WHO, 2012).

Longevity of *P. vivax* in human host is more than that of *P. falciparum*, consequently disturbing health slowly (Kreier, 1980).

Many factors have significant effect on transmission of malarial parasite, however, heavy rainfall was found to initiate epidemics of malaria (Himeidan et al., 2007). Different climatic changes show different patterns of disease development in terms of incidence and prevalence. Malaria was found throughout the year however, it was clearly seasonal with epidemic outbreaks in the wet season from June to August (Lee et al., 2003). In Pakistan there is an increase in incidence of falciparum malaria during the last decade which is probably due to the role of climatic changes (Bouma et al., 1996). Temperature affects developmental stages of *Plasmodium* parasites and mosquito vectors. The extrinsic incubation period (EIP) is reliant on environmental temperature and species of *Plasmodium* involve in malaria (Pampanga, 1969). EIP usually varies from 9 to 10 days however, sometimes can be as brief as 5 days with increase in temperature (Bradley, 1987). *P. falciparum* and *P. vivax* have the shortest duration of intrinsic incubation period and therefore, outnumber *P. ovale* and *P. malariae* in prevalence (Oaks et al., 1991).

The prevalence of malarial parasites in the human population of urban areas of district Quetta, Pakistan was reported by Kakarsulemakhyel & Yasinzai (2004). *P. falciparum* had a higher incidence (16.31%) among the 21 years and above age groups in this report. In province Khyber Pakhtoon Khaw 88.75% patients were suffering from *P. vivax* and 7.5% from *P. falciparum* during...
1999-2004. Male were found more effected than females (Jalal Ud et al., 2006).

In a study of four and half years from Karachi, *P. vivax* was most prevalent (51.8%) as compared to *P. falciparum* (46.5%) while *P. malariae* was found least common (0.4%) (Beg et al., 2008). In city of Multan, province Punjab, prevalence of *P. vivax* was also more (3.17%) than *P. falciparum* (1.19%), with aged group 1-5 years having highest prevalence in males (5.55%) than females (3.17%) (Tasawar et al., 2003). A study of malaria prevalence in different age groups of Afghan refugees of Karachi and adjoining native population revealed that malaria was also prevalent in later age groups of Afghan refugees, while disease was restricted to younger age groups of native population (Suleman, 1988).

Malaria is one of the most important vector-borne diseases in Pakistan having major annual burden and still threatening millions of people due to the lack of satisfactory control measures (Pervez & Shah, 1989). Malaria is endemic in many rural areas with moderate prevalence and incidence in tehsil Depalpur, district Okara. An outbreak of falciparum malaria occurred in 2002 and it has become cause of many deaths in Havelikha of this district (unpublished data). The objective of this study was to find out the prevalence of malaria and involvement of *Plasmodium spp.* by passive case detection (PCD) in peak transmission season from selected villages of tehsil Depalpur.

**MATERIALS AND METHODS**

**Study area**

Depalpur belongs to district Okara (31°N and 74°E) in province Punjab, Pakistan with a total land area of 4,377 Km² and a population of more than 15 millions. Climatic conditions of the district are seasonal. The summer season ranges from April to October. May to July are the hottest months. The winter season lasts from November to March. December to February are the coldest months. The annual rainfall of the district is about 11 inches and July to September is rainy season. The region under study was a flat, irrigated, cultivated land, with rice and wheat as the leading crops, during peak malaria transmission months (July to November). It is estimated that rice crop is sown in 278,000 acres area and tube-well and canal system is used for irrigation purposes. The small water bodies developed during rainy season and flooded rice-fields provide breeding sites for Anopheline mosquitoes.

Four selected villages (Bahripur, Balesingh, Amlimoti, Godara) of tehsil Depalpur are located on Pakpattan road, 152 km south west of Lahore. Total population of these villages is 13584 (Bahripur 3220, Balesingh 3611, Amlimoti 4851, Godara 1902). House courtyards were built by mud and bricks mostly. Villagers usually provide different kinds of hosts and resting sites in their houses by keeping cattle and other domestic animals in courtyards. In this area malaria is endemic with seasonal fluctuations and moderate prevalence but severely lacks authentic record. In 2002, an outbreak of falciparum malaria following deaths occurred in tehsil Depalpur. The study will be useful to detect changes in trends or distribution of malaria and will also be helpful for measuring the effectiveness of anti-malaria program.

**Preparation of blood smears for SPR**

The Blood samples were collected twice a month from June to August 2007 (peak transmission season) by PCD from four private clinics and a major Basic Health Unit (BHU) located near village Amlimoti related to the selected villages. Blood smears were formed at the time of sampling. The second or third finger of the left hand of patient (suspected for malaria) was held and cleaned with swab, dipped in spirit and allowed to dry. By pricking the tip of a finger with a sterile disposable needle, two (large and small) drops of blood were placed on the same slide about 10 mm away from each other. The larger drop (3.0 to 4.0 µl) for thick smear was spread with the corner of another slide to form an approximate circle while the smaller drop (1.0 to 1.5 µl) for thin smear was spread by a second slide (the spreader) having a very smooth edge (Rickman et al., 1966). Slide numbers were marked and handled with care, only held by their edges. The thin smears were fixed by immersing slide in methanol for 15-30 seconds and stained by 10% Giemsa. Then slides were flushed with tap water, botted dried and examined under oil immersion (100x).

**Data analysis**

Malaria prevalence was expressed as percentage of SPR in all villages. Occurrence of this disease was estimated across different variables such as village residence, season, age and gender. Percent SPR was calculated as number of blood smears found positive for malarial parasite/number of blood smears examined×100. Monthly Blood smears Examination Rate (MBER) was calculated as number of blood smears collected during the month/population covered under surveillance×100 (WHO, 2013).
RESULTS

In current study total 116 blood samples were collected (June- August 2007) by PCD method from private clinics and a BHU related to four selected villages of district Depalpur. Out of which 30 were positive by SPR showing an average of 26% malaria prevalence in all four villages. Prevalence was greater in Amlimoti (36%) followed by Balesingh (24%), Bahirpur (20%) and Godara (19%). The results in the last three villages (except Amlimoti) were not significantly different P>0.05 (Figure 1A). Monthly data recorded as percent prevalence was higher in August (41%) than July (23%) and June (22%). Similarly monthly blood examination rate (MBER) was high in August (0.09%) as compared to July (0.07%) and June (0.06%) (Figure 1B).

Differences in Plasmodium seen in slides are shown in Figure 2. Prevalence with respect to gender distribution indicated 18% positive cases in males and 8% positive in females, indicating that males were more susceptible for malaria as compared to females. In children (1-10 years), prevalence of malaria was significantly higher (43%) than adults (22%) (10-70 years) (Figure 3).

DISCUSSION

The malaria indicators to track progress towards achieving target of halt spread of malaria by 2015 and goal to combat malaria and other different diseases of Millennium Development Goals (MDGs) include prevalence and mortality ratio related with malaria. Demographic survey in 2001 indicated that the malaria accounts for 0.5% deaths in Pakistan while data on prevalence is negligible in most of the areas of Pakistan. No study to date addresses the prevalence of malaria in Depalpur. Our study indicated an average of 26% prevalence of malaria with respect to SPR from June to August 2007 in all four villages. Total 98% malaria cases in selected area were due to P. vivax while 2% were recorded by P. falciparum.

In Pakistan as per SPR detection, malaria was reduced from 15% to <0.01% from 1961 to 1967. Then the disease reappeared in 1967 in the country and outbreak arose from 1972 to 1973 having 20% SPR each year in the Punjab province (De Zulueta et al., 1980). Munir et al. (1994) indicated long-term decline in SPR from 14.1% to 3.9% (1973 to 1994) in a report of national malaria statistics. Rowland et al. (2002) documented that malaria prevalence in Punjab province remained almost same with 2% SPR in 1980s and 1990s estimated by annual SPR at district level overtime. Current study indicated that malaria prevalence in selected villages was 13 times more as compared to data from all the Punjab. This could be due to the evaluation of prevalence by only PCD method.

Hozhabri et al. (2000) reported that prevalence of malaria by SPR was 5.9% in children of Jhangara town, Sindh, Pakistan, among which 65% was due to P. falciparum and 35% P. vivax. In current study prevalence was 43% in children and 22% in adults, indicating higher susceptibility in children than adults. In a case study of 160 children from a private clinic in Mansehra, 154 cases of malaria were confirmed positive during 1999-2004, which also indicated higher susceptibility of malaria in children (96%). The author also reported P. vivax was more prevalent (92%) as compared to P. falciparum (8%). In addition positive cases were more in male children (71%) as compared to female (29%) (Jalal Ud et al., 2006). Similar findings were observed in present study with respect to gender, 18% were male positive and 7.75% were female positive. Therefore, current findings are comparable with the study reported in Mansehra, Pakistan. More susceptibility of males over females may be due to more exposure to mosquito bites in males. In addition there could be some other physiological factors to attract the mosquitoes.

In conclusion 26% malaria prevalence was found in all selected villages. The prevalence was 43% in children (1-10 years) and 22% in adults (10-70 years) indicating that children were 2x more susceptible than adults. Moreover, susceptibility of males was recorded more (18%) as compared to females (7.75%).

Acknowledgements

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Fig., 1: Percent prevalence of malaria using Slide Positivity Rate (SPR) by Passive Case Detection (PCD).

(A) Prevalence in selected villages (B) Prevalence and MBER in selected months. Bars representing standard error of the mean (SEM).

* showing that values are not significantly different at p > 0.05.

Fig., 2: Different stages of *P. vivax* seen in slides. (A) S-Schizont (B) G-Gametocyte

Fig., 3: Distribution of malaria cases expressed as percentage among gender and age groups. Bars representing standard error of the mean (SEM).
Accumulation of Cadmium in Soil and Plants in Vicinity of Koh-E-Noor Textile Mills Rawalpindi, Pakistan

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ABSTRACT

Cadmium (Cd) is potentially toxic heavy metal that enters the food. Cadmium (Cd) pollution around the world is a serious issue demanding acceptable solutions, one of which is phytoremediation that is both cost-effective and eco-friendly. This study evaluated the potential of 23 plant species growing on contaminated sites. Phytoremediation has been used to remediate metal-contaminated site at Koh-e-Noor Textile Mills, Rawalpindi. Plant root, shoot and the soil samples were collected and analyzed for selected metal concentration values. To evaluate the potential of plant species for phytoremediation: Bioconcentration Factor (BCF), Biological Accumulation Coefficient (BAC) and Biological Transfer Coefficient (BTC) were calculated. The concentration of Cd in soils ranges from 1.5 to 2.7 mg/kg. The concentration of Cd in shoots from 0.2 to 6.2 mg/kg. The concentration of Cd in roots from 0.1-9.0 mg/kg. Among the plants, Achyranthus asper was the most efficient in accumulating Cd in its shoots (BTC = 37.0). Considering the BAC values 17 species for Cd possessed the characteristics of hyperaccumulator. None of the plant species was found as hyperaccumulator; however plants with high BCF (metal concentration ratio of plant root to soil) and low BTC (metal concentration ratio of plants shoots to roots) have the potential for phytostabilization and phytoextraction. The results of this study can be used for management and decontamination of soils with Cadmium (Cd) using plant species having with phytoremediation potential/characteristics.

Key Words: Hyperaccumulators of Heavy Metals, Phytoremediation, Phytoextraction.

INTRODUCTION

Phytoremediation is newly evolving field of science and technology to clean up polluted soil, water or air (Meagher, 2000). It may be defined as the using of green plants to remove, destroy or sequester hazardous substances from environment. Phytoremediation can provide a cost-effective, long lasting aesthetic solution for remediation of contaminated sites (Ma et al., 2001). One of the strategies of phytoremediation of metal contaminated soil is phytoextraction, i. e. through uptake and accumulation of metals into plant shoots, which can then be harvested and removed from the site. Another application of phytoremediation is phytoextraction where plants are used to minimize metal mobility in contaminated soils. Plant metal uptake is influenced by soil factors including pH, organic matter and cation exchange capacity as well as plant species, cultivar and age. The mobility and availability of heavy metals in soil are generally low, especially when soil is high in pH, clay and organic matter (Jung & Thornton, 1996; Roosselli et al., 2003).

There has been a continuing interest in searching for native plants that are tolerant to heavy metals; however few studies have evaluated the phytoremediation potential of native plants under field conditions (Shu et al., 2002; McGrath & Zhao, 2003). In recent years, phytoremediation has been widely considered as a cost effective approach to remediate metal or metalloid-contaminated soils (Chaney et al., 1997). In general, phytoremediation is a slow process, which reduces its applicability.

Cadmium is found in many domestic products like tobacco products, phosphate fertilizers, polyvinyl chloride (PVC) products, photocells, petrol, oils etc. Acute exposure to cadmium fumes may cause flu like symptoms including chills, fever and muscle ache sometime refer to as “the cadmium blues”. Cadmium is also believed to cause pulmonary emphysema and bone diseases. Hyperaccumulators which are often found growing in polluted areas can naturally accumulate higher quantities of heavy metals in their shoots than roots. In view of the fact metal removal from soil can be greatly enhanced by the judicious selection of plant species, the knowledge about the ability of various plant species or tissues to absorb and transport metals, will thus provide an insight into choosing appropriate plants for phytoremediation (Deng et al., 2004; Zhou & Song,
2004). Furthermore, the identification of hyperaccumulators is an imperative and important task for the successful implementation of phytoremediation (Zhou, 2002; Zhou & Song, 2004).

The hyperaccumulator was characterized first in members of the *Brassicaceae* and *Fabaceae* families (Salt *et al*., 1998). Presently at least 45 families are known to contain metal accumulating species. To date, more than 400 plant species of metal hyperaccumulator plants have been reported in the literature (Salt *et al*., 1998). Hyperaccumulation of metals have been found in temperate as well as in tropical regions throughout the plant kingdom, but is generally restricted to endemic plant species growing on mineralized soil and related rock types (Baker *et al*., 1989).

Cadmium has been identified as a major toxic heavy metal entering the food chain, directly through crop uptake and indirectly through animal transfers (Adriano, 2001). Health authorities in many parts of the world are becoming increasingly concerned about the effects of Cd on environmental and human health and its potential implications to international trade. The bioaccumulation of Cd in wheat and rice crops may have serious implications to animal and human health and to local and international cereal marketing (Nogawa & Kido, 1996). The objective of this study was to identify plant species which have the potential for phytoremediation in and around Koh-E-Noor Textile Mills Rawalpindi, Pakistan.

### MATERIALS AND METHODS

The plants and soil samples used in this study were collected from known metal contaminated sites at Koh-e-Noor Textile Mill, Rawalpindi that lies between 33° – 28' and 33° – 48' north latitude and 72° – 48' and 73° – 22' east longitude. The land is at the height of 490–500 m (a.s.l.).

Sampling was carried out in September & October, 2006. Forty-three plant samples of 23 species belonging to 12 families were collected (Table 1) along the drain discharging from Koh-e-Noor Textile Mills, Rawalpindi. The plants were carefully dug from the substrate and the majority of bulk soil was manually removed from the roots. A total of 6-10 plant samples including roots, stems and leaves of each species were collected to form a composite sample. Plant samples were placed loosely in labeled bag, and were transported to the Environmental Biology laboratory for further analysis. Soil samples (3-5 replicates), at 0-20 cm depth from rhizosphere of each plant were taken from each site from where plant sample was rooted.

Prior to the analysis of plant material, shoots and roots of plants were separated and carefully washed with tap and deionized water in order to remove soil or dust deposits. Plant samples were dried at room temperature for two weeks, pulverized and passed through 2 mm steel sieve.

For metal analysis, 0.5g of shoots along with leaves and roots sample were taken. 5 ml of nitric acid (65%) and 1 ml of perchloric acid (70-72%) was added (Wang *et al*., 2003). The digestion was allowed to proceed in microwave digester (CEM 2000 MARS XPRESS) at 80°C for 15 minutes. It was cooled and transferred to 50 ml calibrated flask. The volume was raised up to the mark with distilled water (Price, 1979), digestion followed by the measurement of total concentrations of Cd using Atomic Absorption Spectrophotometer (VARIAN, AA240FS).

The composite soil samples, after collecting from the field were brought to the laboratory in polythene bags. The samples were spread on the paper sheets, air dried, crushed and pulverized to pass through 2 mm sieve. Soil pH, EC and TDS were measured in a solution of 1:9 soils: water ratio with Milwaukee SM/802 smart combined meter. For measuring pH, it was calibrated with buffer solutions of pH 4, 7 and 9 while for measuring EC; it was calibrated with 1413 μS/cm at 25 °C/77 °F calibrating solution.

Soil texture was determined by Bouyoucos hydrometer method. On the basis of percentage of sand, silt and clay, textural class of each sample was determined with the help of standard textural class triangular (Brady, 1990).

For selected heavy metal in soil samples, 0.5 g of air dried, ground and sieved soil, was digested with 5:1 v/v of nitric acid (65%) and perchloric acid (70-72%) (Wang *et al*., 2003). The digestion was preceded in microwave digester (CEM 2000 MARS XPRESS) at 220°C for 30 minutes. Digested samples were cooled and volume was raised up to the 50 ml mark with distilled water, these extracts were used for total concentration of Cadmium (Cd). Total concentration of Cadmium in soil and plan samples was analyzed by using flame Atomic Absorption Spectrophotometer (VARIAN, AA240FS). All glass wares before use were washed with distilled water, soaked in nitric acid (30%) overnight, rinsed in deionized water and air dried.

Biological Accumulation Coefficient was defined as the concentration of heavy metals in plant shoots divided by the heavy metal concentration in soil (Zu *et al*., 2005) and is given in equation 1.

\[
BAC = \frac{[\text{Metal} \text{ shoot}]}{[\text{Metal} \text{ soil}]} \quad \text{Eq. 1.}
\]
Biological Transfer Coefficient was described as the ratio of heavy metal concentration in plant shoot to that in plant root (Zu et al., 2005) and is given in equation 2.

\[
BTC = \frac{\text{[Metal] shoot}}{\text{[Metal] root}} \quad \text{Eq. 2}
\]

Bioconcentration Factor was calculated as ratio of concentration of heavy metal in plant roots to that of soil, (Yoon et al., 2006) and is given in equation 3.

\[
BCF = \frac{\text{[Metal] root}}{\text{[Metal] soil}} \quad \text{Eq. 3}
\]

## RESULTS AND DISCUSSION

The soil and plant samples collected from 15 sites were analyzed for their Cadmium contents. It appeared that cadmium concentration in different samples varies to a great extent from sample to sample. The values for Cd in soil samples varied between 1.5 mg kg\(^{-1}\) to 3.4 mg kg\(^{-1}\). The concentration of Cd varied in shoots from 6.2 mg kg\(^{-1}\) in \textit{P. oleracea} to 0.2 mg kg\(^{-1}\) in \textit{A. viridis}. The concentration in roots varied from 0.1 mg kg\(^{-1}\) in \textit{A. asper} to 9.0 mg kg\(^{-1}\) in \textit{R. communis} as well as \textit{P. oleracea} (Table 2). Higher concentration was also found in the roots of \textit{X. strumarium} (8 mg kg\(^{-1}\)). The BAC values for all plant species are given in Table 4. Among 43 plant species screened, BAC values for Cd for most species were greater than 1, maximum was 2.95 in \textit{P. oleracea}, minimum was 0.33 in \textit{C. rotendus}. Among 23 plant samples, maximum BTC for Cd (37) was reported in \textit{A. asper} while minimum was 0.40 in \textit{C. rotendus}. (Table 4). The highest BCF for \textit{P. hysterophorus} had the highest BCF for Cd (1.47) while \textit{A. asper} had the lowest (0.05) (Table 4).

The present study was planned to assess the status of cadmium contamination in soil and plants near Koh-e-Noor Textile Mills. The overall situation of cadmium pollution indicated that water channels running through these sites are used for disposal of effluents from Mill. It also showed elevated concentrations of Cd. The maximum concentration of Cd in soil (3.4 mg kg\(^{-1}\)) was recorded at site 12.

The results indicated that plants species differ greatly in their capacity of heavy metal accumulation in roots and shoots. None of the plant species accumulated Cd above 1000 mg kg\(^{-1}\) in the shoots, the criteria for a hyperaccumulator as given by Baker & Brooks (1989).

Cd is also a toxic element to plants. The mean concentration in the shoots is 1 mg kg\(^{-1}\) (Zu et al., 2004). Our results showed that the concentrations in the shoots of maximum species were higher than the mean concentrations in a normal plant (Table 2), which showed that the investigated species had a strong ability to tolerate this heavy metal.

The standard for hyperaccumulator has not been defined scientifically still. In the present study, the standard is described as four rules, i. e. the concentration of heavy metals in plant shoots reach hyperaccumulating level (Cd > 100 mg/kg) (Baker et al., 1994; Brown et al., 1994; Wei et al., 2002 & Nazir et al., 2010), the concentration of heavy metal in its above ground part is 10-500 times more than in plants from non polluted environments (Cd 1 mg/kg) (Shen & Liu, 1998), the metal concentrations in shoots are invariably greater than that in roots and enrichment coefficient > 1, showing a special ability of the plant to absorb from soils and transport metals and store them in their above-ground part (Baker et al., 1989; Baker et al., 1994; Brown et al., 1994; Wei et al., 2002). It is difficult to judge whether a plant species is hyperaccumulator or not, if the plant species do not accord with above four rules simultaneously. So define and use of a scientific standard for hyperaccumulator will be very necessary for hyperaccumulator choice and phytoremediation of soil polluted by heavy metal.

In this study, none of the plant species showed metal concentrations>1000 mg/kg in shoots. None of them are hyperaccumulators (Baker & Brooks, 1989). However, the ability of these plants to tolerate and accumulate heavy metals may be useful for phytostabilization. BAC, BTC and BCF can be used to estimate a potential for phytoremediation purposes. Considering BAC, out of 23 plant species, seventeen species such as \textit{A. viridis}, \textit{A. pungens}, \textit{A. asper}, \textit{C. pennisetiformis}, \textit{C. sativa}, \textit{C. dactylon}, \textit{C. album}, \textit{D. aegypticum}, \textit{E. alba}, \textit{E. conyzanthus}, \textit{I. hederacea}, \textit{P. hysterophorus}, \textit{P. oleracea}, \textit{P. barbatum}, \textit{R. communis}, \textit{S. halepense} and \textit{S. nigrum} for Cd may possess the characteristic of hyperaccumulator. Although the species having BAC lower than 1, could be primarily considered as potential hyperaccumulators. Biological accumulation occurs when a contaminant taken up by plant is not degraded rapidly, resulting in the accumulation in the plant. The process of phytorextraction generally requires the translocation of heavy metals to easily harvestable plant parts, i. e. shoots.


The BCF of 8 species such as \textit{A. viridis}, \textit{B. raptans}, \textit{C. sativa}, \textit{E. alba}, \textit{E. conyzanthus}, \textit{I.
hederacea, P. hysterophorus, S. halepense, and X. strumarium have greater than 1. Plants exhibiting BTC particularly BCF value less than one are unsuitable for phytoextraction (Fitz & Wenzel, 2002). A few species growing at the sites were capable of accumulating heavy metals in the roots and shoots, but most of them had low BAC, BTC and BCF values, which means limited ability of heavy metal accumulation and translocation by the plants.

This study was conducted to screen plants growing on contaminated areas of Koh-e-Noor Textile Mill to determine their potential for metal accumulation. Only species with BCFs, BACs and BTCs greater than one have the potential for remediation processes. Among forty-three plant samples, 23 plant species screened. None of them were identified as metal hyperaccumulator. However, several plants had BCFs, BACs or BTCs greater than 1. These plant species were considered suitable for growing in industrially polluted regions, as they accumulate considerable quantities of heavy metals from the soil with their root system and can be used as potential plant species for cleaning heavy metals. Phytoremediation potential of these plant species especially needs to be investigated. In order to reduce the present trend of soil contamination, it is suggested that the industries should follow the environmental regulations particularly waste treatment prior to discharge in the environment.

Table 1: Plant species identified and used in the study

<table>
<thead>
<tr>
<th>No</th>
<th>Family</th>
<th>Name of species</th>
<th>Life form</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amaranthaceae</td>
<td>Amaranthus viridis L.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>2</td>
<td>Amaranthaceae</td>
<td>Alternanthera pungens Kunth.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>3</td>
<td>Amaranthaceae</td>
<td>Alyphanthus asper L.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>4</td>
<td>Poaceae</td>
<td>Brachiaria raptans (L.)</td>
<td>Annual grass</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gardiner &amp; Hubbard</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Poaceae</td>
<td>Cenchrus pennisetiformis</td>
<td>Annual grass</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hochst. and Steud.ex Steud</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Canabinaceae</td>
<td>Canabis sativa L.</td>
<td>Perennial herb</td>
</tr>
<tr>
<td>7</td>
<td>Poaceae</td>
<td>Cydonon dactylon (L.) Pers.</td>
<td>Perennial grass</td>
</tr>
<tr>
<td>8</td>
<td>Chenopodiaceae</td>
<td>Chenopodium album L.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>9</td>
<td>Cyperaceae</td>
<td>Cyperus rotundus L.</td>
<td>Annual grass</td>
</tr>
<tr>
<td>10</td>
<td>Poaceae</td>
<td>Dactyloctenium aegypticum L.</td>
<td>Annual grass</td>
</tr>
<tr>
<td>11</td>
<td>Asteraceae</td>
<td>Eclipta alba (L.) Hasskl.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>12</td>
<td>Poaceae</td>
<td>Eleusine indica (L.) Gaertn.</td>
<td>Annual grass</td>
</tr>
<tr>
<td>13</td>
<td>Asteraceae</td>
<td>Erigeron conyzanthus L.</td>
<td>Annual forb</td>
</tr>
<tr>
<td>14</td>
<td>Convolvolaceae</td>
<td>Ipomoea hederacea Jacq.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>15</td>
<td>Malvaceae</td>
<td>Malvestrum coromandalinum (Linn.)Garcke</td>
<td>Perennial herb</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Asteraceae</td>
<td>Parthenium hysterophorus L.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>17</td>
<td>Portulaceae</td>
<td>Portulaca oleracea L.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>18</td>
<td>Polygonaceae</td>
<td>Polygonum barbratum L.</td>
<td>Annual forb</td>
</tr>
<tr>
<td>19</td>
<td>Polygonaceae</td>
<td>Rumex nepalensis Sprengle</td>
<td>Annual herb</td>
</tr>
<tr>
<td>20</td>
<td>Euphorbiaceae</td>
<td>Ricinus communis L.</td>
<td>Perennial shrub</td>
</tr>
<tr>
<td>21</td>
<td>Poaceae</td>
<td>Sorghum halepense (L.) Pers.</td>
<td>Annual grass</td>
</tr>
<tr>
<td>22</td>
<td>Solanaceae</td>
<td>Solanum nigrum L.</td>
<td>Annual herb</td>
</tr>
<tr>
<td>23</td>
<td>Asteraceae</td>
<td>Xanthium strumarium L.</td>
<td>Annual shrub</td>
</tr>
</tbody>
</table>
Table 2: Cadmium concentration (mg kg⁻¹) in soil, shoots and roots of plant samples.

<table>
<thead>
<tr>
<th>Scientific Name</th>
<th>Site #</th>
<th>Roots</th>
<th>Shoots</th>
<th>Soil</th>
</tr>
</thead>
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<tr>
<td>Achyranthes asper L.</td>
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<td>2</td>
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<td>Alternanthera pungens Kunth.</td>
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<td>1.4</td>
<td>3.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Amaranthus viridis L.</td>
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<td>2.8</td>
<td>3.7</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.5</td>
<td>0.5</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>1.8</td>
<td>4.1</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.9</td>
<td>0.2</td>
<td>2.2</td>
</tr>
<tr>
<td><em>Brachiaria raptans</em> (L.) Gardner &amp; Hubbard</td>
<td>10</td>
<td>2.8</td>
<td>1.3</td>
<td>2.2</td>
</tr>
<tr>
<td><em>Canabis sativa</em> L.</td>
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<td>2.7</td>
<td>3.9</td>
<td>2.3</td>
</tr>
<tr>
<td><em>Cenchrus pennisetiformis</em></td>
<td>1</td>
<td>1.1</td>
<td>5.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Hochst. &amp; Steud.ex Steud</td>
<td>2</td>
<td>1.5</td>
<td>2.8</td>
<td>2.1</td>
</tr>
<tr>
<td><em>Chenopodium album</em> L.</td>
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<td>1.7</td>
<td>2.9</td>
<td>2.1</td>
</tr>
<tr>
<td><em>Cynodon dactylon</em> (L.) Pers.</td>
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<td>0.4</td>
<td>3.3</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.0</td>
<td>3.3</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Cyperus rotundus</em> L.</td>
<td>14</td>
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<td>0.9</td>
<td>2.7</td>
</tr>
<tr>
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<td>2.0</td>
<td>3.9</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
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<td>1.2</td>
<td>2.4</td>
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<td><em>Eclipta alba</em> (L.) Hasskl.</td>
<td>11</td>
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<td>2.2</td>
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<tr>
<td><em>Erigeron conyzanthus</em> L.</td>
<td>15</td>
<td>2.4</td>
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<tr>
<td><em>Ipomea hederacea</em> Jacq.</td>
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<tr>
<td><em>Malvestrum coromandilianum</em> (L.) Garcke</td>
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<td>1.3</td>
<td>2.3</td>
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<tr>
<td></td>
<td>2</td>
<td>2.4</td>
<td>3.0</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.6</td>
<td>4.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Parthenium hysterophorus L.</td>
<td>1</td>
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<td>3.3</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.9</td>
<td>2.4</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.3</td>
<td>0.7</td>
<td>2.5</td>
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<td></td>
<td>9</td>
<td>3.2</td>
<td>5.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Polygonum barbatum L.</td>
<td>8</td>
<td>0.6</td>
<td>3.8</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.5</td>
<td>3.2</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.6</td>
<td>1.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Portulaca oleracea L.</td>
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<td>0.5</td>
<td>6.2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.2</td>
<td>2.5</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>9.0</td>
<td>1.5</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.0</td>
<td>3.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Ricinus communis L.</td>
<td>2</td>
<td>0.7</td>
<td>2.4</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.3</td>
<td>1.2</td>
<td>2.5</td>
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<tr>
<td></td>
<td>12</td>
<td>9.0</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.5</td>
<td>4.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Rumex nepalensis Sprenge</td>
<td>15</td>
<td>1.5</td>
<td>1.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Solanum nigrum L.</td>
<td>3</td>
<td>0.2</td>
<td>3.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Sorghum halepense (L.) Pers.</td>
<td>5</td>
<td>3.4</td>
<td>2.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Xanthium strumarium L.</td>
<td>3</td>
<td>2.5</td>
<td>1.7</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>8.0</td>
<td>2.1</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Table 3: Selected properties of soil samples collected from the contaminated sites.

<table>
<thead>
<tr>
<th>Site #</th>
<th>Soil pH</th>
<th>Soil Texture</th>
<th>Total Cd (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4</td>
<td>Sandy Loam</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>8.5</td>
<td>Loamy Sand</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>8.6</td>
<td>Sandy Clay Loam</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>8.3</td>
<td>Sandy Clay Loam</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>8.3</td>
<td>Sandy Loam</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>8.9</td>
<td>Sandy Loam</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>8.2</td>
<td>Sandy Clay Loam</td>
<td>2.2</td>
</tr>
<tr>
<td>8</td>
<td>8.1</td>
<td>Loamy Sand</td>
<td>2.4</td>
</tr>
<tr>
<td>9</td>
<td>7.5</td>
<td>Loamy Sand</td>
<td>2.2</td>
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<tr>
<td>10</td>
<td>8.3</td>
<td>Loamy Sand</td>
<td>2.2</td>
</tr>
<tr>
<td>11</td>
<td>8.0</td>
<td>Sandy Loam</td>
<td>2.2</td>
</tr>
<tr>
<td>12</td>
<td>7.7</td>
<td>Sand</td>
<td>3.4</td>
</tr>
<tr>
<td>13</td>
<td>8.2</td>
<td>Sand</td>
<td>2.6</td>
</tr>
<tr>
<td>14</td>
<td>7.8</td>
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<td>2.7</td>
</tr>
<tr>
<td>15</td>
<td>8.6</td>
<td>Loamy Sand</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Table 4: The Biological Accumulating Coefficient (BAC), Biological Transfer Coefficient (BTC) and Bioconcentration Factor (BCF) in selected plants.

<table>
<thead>
<tr>
<th>Species</th>
<th>BAC</th>
<th>BTC</th>
<th>BCF</th>
</tr>
</thead>
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<tr>
<td>Achyranthes asper</td>
<td>1.85</td>
<td>37.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Alternanthera pungens</td>
<td>2.2</td>
<td>2.35</td>
<td>0.93</td>
</tr>
<tr>
<td>Amaranthus viridis</td>
<td>1.60</td>
<td>1.32</td>
<td>1.21</td>
</tr>
<tr>
<td>Brachiaria raptans</td>
<td>0.59</td>
<td>0.64</td>
<td>1.27</td>
</tr>
<tr>
<td>Canabis sativa</td>
<td>1.69</td>
<td>1.44</td>
<td>1.17</td>
</tr>
<tr>
<td>Cenchrus pennisetiformis</td>
<td>2.21</td>
<td>1.86</td>
<td>0.47</td>
</tr>
<tr>
<td>Chenopodium album</td>
<td>1.38</td>
<td>1.70</td>
<td>0.80</td>
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<tr>
<td>Cynodon dactylon</td>
<td>1.57</td>
<td>0.47</td>
<td>0.19</td>
</tr>
<tr>
<td>Cyporus rotundus</td>
<td>0.33</td>
<td>0.40</td>
<td>0.81</td>
</tr>
<tr>
<td>Dactyloctenium aegypticum</td>
<td>1.69</td>
<td>1.95</td>
<td>0.66</td>
</tr>
<tr>
<td>Eclipta alba</td>
<td>1.09</td>
<td>0.82</td>
<td>1.31</td>
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<tr>
<td>Elusine indica</td>
<td>0.77</td>
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<td>Ipomoea hederacea</td>
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<td>0.56</td>
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<td>Parthenium hysterophorus</td>
<td>1.43</td>
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<td>Partulaca oleracea</td>
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<td>1.58</td>
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<td>0.68</td>
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<td>1.69</td>
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<td>0.85</td>
<td>2.26</td>
</tr>
<tr>
<td>Xanthium stromarium</td>
<td>0.73</td>
<td>0.68</td>
<td>1.08</td>
</tr>
</tbody>
</table>
REFERENCES


Phytochemical analysis and anthelmintic activity of extracts of aerial parts of *Solanum nigrum* L.

*ZEB SADDIQE, ALYA MAIMOONA & SABA KHALID*

Department of Botany, Lahore College for Women University, Lahore, Pakistan

**ABSTRACT**

In the present study crude methanol extract and subsequent solvent fractions of *Solanum nigrum* (Solanaceae) were evaluated for anthelmintic activity against sheep intestinal worms *Haemonchus contortus*. The extracts were also evaluated for total phenolic and total flavonoid contents using colorimetric methods. The ethylacetate extract showed significant anthelmintic effect with high death rate of worms at hourly interval at a concentration of 0.05 mg/ml. Total phenolic content in the crude methanolic extract was 342 ± 2.84 mg TAE/g dE. After fractionation the maximum concentration of phenols was measured in ethylacetate fraction (426 ± 3.87 mg TAE/g dE). Total flavonoid content in the crude methanol extract was 128 ± 2.34 mg QE/g dE. After fractionation highest concentration of flavonoids was measured in ethylacetate fraction (180 ± 2.51 mg QE/g dE). So the results indicated that the polar fractions of *S. nigrum* containing high concentration of phenolics and flavonoids possess high anthelmintic activity.

**Key words:** *Solanum nigrum*, helminthiasis, anthelmintic activity, phenolics, flavonoids

_______________________________________________________________________________________

**INTRODUCTION**

Parasitic diseases cause ruthless morbidity affecting principally population in endemic areas (Tagbota & Towson, 2001). Helminthiasis is a widespread parasitic infection among humans and animals caused by helminths. The disease is highly prevalent particularly in developing countries due to inadequate sanitary conditions and poor management practices (Dhar et al., 1982). Helminths are generally restricted to tropical regions and cause enormous hazard to health and contribute to the prevalence of undernourishment, pneumonia, eosinophilia and anemia (Bundy, 1994). Anthelmintics are drugs used to expel parasitic worms from the body by paralyzing or killing them. It has importance in humans and veterinary medicines (Holden-Dye & Walker, 2007). The gastrointestinal helminthes have become resistant to currently available anthelmintic drugs causing problem in treatment of helminthes diseases (Sondhi & Shahu, 1994). Hence there is an increasing demand towards natural anthelmintics.

Plants are a rich source of botanical anthelmintics (Satyavati et al., 1976; Lewis & Elvin, 1977). A large number of medicinal plants have been used for the treatment of helminthiasis in humans and animals (Chopra et al., 1956, 1958; Akhtar, 2000). Solanaceae is a family of flowering plants that includes a number of important agricultural crops, although many species are toxic plants. Many plants of the family are used by humans, and are important sources of food, spices and medicine.

Medicinally, as well as in terms of poisoning and psychotropic effects, members of Solanaceae have been valued for their alkaloid content and used throughout history (NHM 2008). The plants of family Solanaceae also have the properties of anti-malarial activities (Ramazani et al., 2010) and also used for the treatment of cold, eye diseases and heart pains.

The purpose of the present study was to evaluate the crude methanolic extract and subsequent solvent fractions of *Solanum nigrum* for anthelmintic activity. The extracts were also evaluated for total phenolic and flavonoid contents. The study can be of significant importance in developing cheaper and easily available anthelmintics with lesser side effects.

**MATERIALS AND METHODS**

Chemicals

*n*-Hexane, dichloromethane, ethyl acetate, acetone and methanol were all of analytical grade, purchased from Fischer Scientific. For purity measures, the chemicals were used after re-distillation. Aluminium chloride (AlCl₃), potassium hydroxide (KOH), ferric chloride (FeCl₃), sulphuric acid (H₂SO₄), Dragendorff reagent, sodium nitrite (NaNO₂), sodium hydroxide (NaOH), sodium carbonate (Na₂CO₃), Folin-Ciocalteu reagent, quercetin and tannic acid were purchased from Sigma Aldrich. Sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH₂PO₄) and dipotassium hydrogen phosphate (K₂HPO₄).
(K$_2$HPO$_4$) were purchased from RDH Laborchemikalien.

**Equipments**
Plant extracts were concentrated with rotary evaporator R-210 (BUCHI) Switzerland. All UV-Vis spectral analyses were carried out in methanol with UV-Vis double beam spectrophotometer (Hitachi, U2800). The sterilization of phosphate buffer solution was done in autoclave.

**Collection of Plant Material**
Aerial parts of *S. nigrum* were collected from Lahore College for Women University, Lahore. Plants were identified and authenticated by Dr. Tahira Mughal, Associate Professor, Department of Botany, Lahore College for Women University (LCWU), Lahore. Voucher specimen was deposited in Prem Madam Herbarium, Department of Botany, LCWU, Lahore (voucher no. PM0138).

**Extraction**
The fresh plant material (360 g) was washed and dried in shade. The dried and powdered material (70 g) was extracted in redistilled methanol for seven days at room temperature away from sunlight, with occasional stirring. The soaking mixture was filtered to isolate the methanol extract. The process was repeated thrice and the combined extracts were concentrated under reduced pressure in the rotary evaporator to give crude methanol extract. For liquid-liquid partition the crude methanol extract was dissolved and suspended in double distilled water and filtered through filter paper. The water extract was partitioned between $n$-hexane, dichloromethane, ethyl acetate and acetone. The process afforded non-polar fractions of $n$-hexane and dichloromethane and polar fractions of ethyl acetate, acetone and water. The water fraction was freeze-dried to give water extracts. All the extracts were weighed (Table 1) and stored in tightly sealed dark glass containers at 4ºC for further analysis.

**Qualitative Phytochemical Analysis**
For qualitative phytochemical analysis standard chemical methods were performed (Harborne, 1973).

**Glycosides:** For glycosides 1 mL of freshly prepared 10% KOH was added to 1 mL of extract. The presence of glycosides was confirmed by the formation of brick red precipitates.

**Saponins:** For saponins, frothing test was performed in which 2 mL of the extract was vigorously shaken in the test tube for 2 minutes. Presence of frothing indicated saponins.

**Steroids:** Steroids were identified by adding 5 drops of concentrated H$_2$SO$_4$ to 1 mL of the extract in a test tube. Red coloration indicated the presence of steroids.

**Triterpenes:** For triterpenes, 5 drops of concentrated H$_2$SO$_4$ were added to 1 mL of extract. Appearance of blue green colour indicated the presence of triterpenes.

**Flavonoids:** Presence of flavonoids was tested by adding 1 mL of freshly prepared 5% AlCl$_3$ solution to 1 mL of extract. Yellow coloration indicated the presence of flavonoids.

**Phenolics:** For phenolics, two drops of 5% FeCl$_3$ were added to 1 mL of the extract in a test tube. Presence of greenish precipitate indicated the presence of phenolics.

**Alkaloids:** To detect the presence of alkaloids 0.2 gm of plant extract was warmed with 2% sulphuric acid in a test tube for 2 minutes. The mixture was filtered in a separate test tube and few drops of Dragendorff reagent were added and observed for the presence of orange red precipitates for the presence of alkaloids.

**Quantitative Phytochemical Analysis**
The crude extract and solvent fractions of *S. nigrum* were analyzed quantitatively for total phenolic and total flavonoid content using standard methods.

**Determination of total phenolics**
Total phenolic contents were determined by using Folin-Ciocalteu (FC) reagent (Cliffe *et al.*, 1994). For analysis 20 μL of plant extract was mixed with 1.58 mL of deionized water and 100 μL of FC reagent and incubated for 10 min at room temperature. To the reaction mixture 300 μL of 25% Na$_2$CO$_3$ solution (w/v) was added and again incubated at 40ºC. After cooling for 0.5 h, absorbance was measured at 765 nm against the blank (containing 20 μL of extracting solvent instead of plant sample). TPC of the sample was determined with a linear equation based on the standard calibration curve prepared under the same conditions using tannic acid as standard (Figure 1). The results were expressed as mg tannic acid equivalent (TAE)/g dry extract (dE).

\[ Y = 2.807x + 0.026; \quad r^2 = 0.914 \]

(where $Y$ is the absorbance and $x$ is the concentration of tannic acid mg/ml).

**Determination of total flavonoids**
Total flavonoid content (TFC) was determined by using aluminium chloride colorimetric method (Dewanto *et al.*, 2002). For analysis 250 μL of the extract was diluted with 500 μL of deionized water and 90 μL of 5 % (w/v) NaNO$_2$ solution was added and left to stand for 6 min. Then, 180 μL of 10% (w/v) AlCl$_3$ solution was added to the above
mixture and allowed to stand for another 5 min followed by the addition of 600 μL of 1 M NaOH solution. The final volume was made up to 3 mL with deionized water. Absorbance was measured at 510 nm against blank (250 μL of plant extract was replaced by 250 μL of extracting solvent). TFC was calculated from linear equation based the calibration curve of quercetin, used as standard, obtained under same experimental conditions as described above (Figure 2). The results were expressed as mg quercetin equivalent (QE)/g dE.

\[ Y = 0.131 \times +0.016; r^2 = 0.918 \]

(where \( Y \) is the absorbance and \( x \) is the concentration of quercetin in mg/ml).

Anthelmintic Activity

Preparation of Test Solution

Test solution was prepared by dissolving 0.5 mg of dried plant extract in 0.1 ml of DMSO and 9.9 ml of Phosphate Buffer Solution (PBS) to make the final volume 10 mL (0.05 mg/ml).

Anthelmintic activity of the plant extracts was examined by using the method of Ajaiyeoba et al. (2001). The assay was carried out on intestinal parasite of sheep *Haemonchus contortus* which resembles with intestinal worms of human beings. The worms were obtained from intestine (abomasi) of freshly slaughtered sheep. Intestine (abomasi) of sheep were collected from the local slaughter house and washed with normal saline solution to remove all the faecal matter. The intestines were then dissected and worms were collected and kept in normal saline solution. The average size of these worms was 1-2 cm.

The abomasi of the freshly slaughtered sheep were dissected and worms collected in a dish. The worms were washed and suspended in the PBS at room temperature. Ten worms per petri dish were used to study the effect of plant extracts. The experiments were performed in triplicates. The motility was recorded with hourly interval for 6 h. Finally the treated worms were kept for 30 minutes in the lukewarm fresh PBS to observe the revival of motility.

Control treatment

The control set-up was run by using PBS+DMSO in the petri dish to determine the effect of DMSO on the test organism.

Reference drug

The comparison was made with the standard anthelmintic medicine Levamisole (0.5 mg/ml).

Statistical application

The experiments were carried out in triplicate. All the results are reported as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Qualitative Phytochemical Analysis

The preliminary phytochemical analysis of different plant extracts evidenced the presence of multiple components in the extracts. The results revealed the presence of flavonoids, glycosides, tannins, steroids, saponins, terpenes, and phenolic compounds (Table 2).

Total phenolic content

The results of analysis of TPC are summarized in Figure 3. TPC in the crude methanolic extract was 342 ± 2.84 mg TAE/ g dE. After fractionation the highest concentration of phenols was measured in ethyl acetate fraction (426 ± 3.87 mg TAE/g dE) followed by dichloromethane fraction (416 ± 3.52 mg TAE/ g dE). The minimum amount of phenolics was determined in n-hexane fraction (228 ± 2.64 mg TAE/g dE).

Total flavonoid content

The results of analysis of TFC are summarized in Figure 3. TFC in the crude methanolic extract was 128 ± 2.34 mg QE/g dE. After fractionation highest concentration of flavonoids was measured in ethyl acetate fraction (180 ± 2.51 mg QE/g dE) followed by dichloromethane fraction (140 ± 2.30 mg QE/g dE). The minimum amount of flavonoids was determined in n-hexane fraction (44 ± 1.00 mg QE/g dE). High solubility of flavonoids in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction.

Anthelmintic activity

The results of anthelmintic activity of all the plant samples are summarized in Table 3 and Figure 4. All the extracts showed different anthelmintic activity in terms of mortality rate at the same concentration during the six hours time period. This difference in activity is attributed to difference in type and quantity of different phytochemicals present in each plant extract.

Several plants of family Solanaceae and particularly from the genus *Solanum* have been shown to possess anthelmintic activity (Gunasekile et al., 2010; Mathri et al., 2011; Yadav & Tangpu, 2012). In all these studies mostly the crude methanol or ethanol extracts and aqueous extracts were tested for anthelmintic activity. In the present
study the crude methanol extract was further partitioned using solvents of various polarity and the fractions were tested for anthelmintic activity. Among the extracts the ethyl acetate, acetone and aqueous fractions showed significant anthelmintic effect with high death rate in the given time interval at the tested concentration (0.05 mg/ml). Levamisole used as positive control showed 100% mortality rate after 3 h. The results can be considered significant since the extracts are crude samples with a number of compounds and can be a source of phytochemicals with anthelmintic activity comparable to standard drugs used. Although the rate of death of worms after each hour was different for each fraction, at the end of six hour time period the rate of death of worms was same. The effect of extracts on the death of the worms, according to the result may be indicated as ethyl acetate > acetone > aqueous > dichloromethane > crude methanolic > n-hexane extracts. In particular ethyl acetate extract exhibited an increased death of worms at hourly interval.

A number of studies are available for anthelmintic activity of tannins, alkaloids and flavonoids (Anthnasiadou et al., 2001; da Silva et al., 2008; Wang et al., 2010). The presence of these phytochemicals may be responsible for the observed anthelmintic activity of plant extracts in present study. Tannins have been shown to interfere with coupled oxidative phosphorylation thus blocking ATP synthesis in these parasites (Martin, 1997). Tannins may also bind to the cuticle of the helminth’s body surface making it immobile causing the parasite to become paralysed leading to its death (Thompson & Geary, 1995). Presence of tannins in ethyl acetate, acetone and aqueous fractions may be responsible for high anthelmintic activity of these extracts.

The difference in activity of different plant extracts may also be due the difference in total phenolic and flavonoid contents in these extracts. Since the polar fractions contained a high phenolic and flavonoid content than the non-polar fractions this may explain the observed difference in the anthelmintic activity of these extracts.

CONCLUSION

In the present study the crude extracts and fractions of S. nigrum were evaluated for anthelmintic activity against sheep intestinal worms H. contortus and significant activity was observed for the polar fractions. The extracts were also tested for qualitative and quantitative analysis of selected plant metabolites. The extracts were found to be rich both in quality and quantity of metabolites especially phenolics and flavonoids. The study thus concludes that the polar extracts of S. nigrum can be used for the treatment of parasitic diseases such as helminthiasis and are a cheap source of phenolics such as alkaloids, tannins and flavonoid with biological activities including antioxidant, antibacterial and antifungal.

REFERENCES


### Table 1: Amount (g) and % yield of different solvent fractions of *S. nigrum*

<table>
<thead>
<tr>
<th>MeOH</th>
<th>n-Hexane</th>
<th>CH₂Cl₂</th>
<th>EtOAc</th>
<th>Acetone</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt.</td>
<td>%</td>
<td>wt.</td>
<td>%</td>
<td>wt.</td>
<td>%</td>
</tr>
<tr>
<td>(g)</td>
<td>age</td>
<td>(g)</td>
<td>age</td>
<td>(g)</td>
<td>age</td>
</tr>
<tr>
<td>11.4</td>
<td>16.28</td>
<td>2.2</td>
<td>19.3</td>
<td>0.3</td>
<td>2.63</td>
</tr>
<tr>
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</tr>
</tbody>
</table>
### Table 2: Qualitative phytochemical analysis of extracts

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Flavonoids</th>
<th>Glycosides</th>
<th>Tannins</th>
<th>Steroids</th>
<th>Saponins</th>
<th>Terpenes</th>
<th>Phenolics</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>–</td>
<td>+</td>
<td>–</td>
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<td>–</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>EtOAc</td>
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<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Acetone</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Aqueous</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

(+) = indicates presence, (−) = indicates absence

### Table 3: Comparison of *in vitro* anthelmintic activity of different extracts of *S. nigrum*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number of worms surviving at hourly interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>MeOH</td>
<td>10.00 ± 0.00</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>10.00 ± 0.00</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>10.00 ± 0.00</td>
</tr>
<tr>
<td>EtOAc</td>
<td>10.00 ± 0.00</td>
</tr>
<tr>
<td>Acetone</td>
<td>10.00 ± 0.00</td>
</tr>
<tr>
<td>Aqueous</td>
<td>10.00 ± 0.00</td>
</tr>
<tr>
<td>Levamisole</td>
<td>10.00 ± 0.00</td>
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</table>
**Fig., 1:** Calibration curve for tannic acid

\[ y = 2.807x + 0.026 \]
\[ R^2 = 0.9188 \]

**Fig., 2:** Calibration curve for quercetin

\[ y = 0.1313x + 0.0168 \]
\[ R^2 = 0.9188 \]

**Fig., 3:** Total phenolic and flavonoid content in crude extract and fractions of *S. nigrum* extracts

**Fig., 4:** Anthelmintic activity of crude extract and fractions of *S. nigrum*
The Effect of Neem (*Azadirachta indica*) Leaves Extract on the Ecdysis and Mortality of Immature Stages of Common House Mosquito *Culex pipiens fatigans*

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**ABSTRACT**

Larvicidal effect of *Azadirachta indica* on mosquitoes has been studied in the present work. The water extracts of air dried leaves and fresh leaves separately have been used for the study. Different concentrations were made of all these extracts and twenty second instar larvae of *Culex pipiens fatigans* were placed in each concentration separately. Control was set up for each experiment. Mortality and ecdysis inhibition effect were used as effective parameters. Observations on mortality were carried out after each 24 hours. The 25% stock solution of dry leaves extract showed greatest larvicidal activity i.e. 100% mortality within first 24 hours as compared to the mortality rate of fresh leaves and simple dry leaves extracts. The LC$_{50}$ of 25% stock solution extract, fresh leaves and dry leaves extracts was found to be 19%, 33%, 40% respectively. Percentage mortality per day, percentage pupation and percentage emergence from pupae are calculated. In lower concentrations 1%, 5%, 10%, pupation was delayed by two or three days. In 40%, 50% and 60% concentration of all these extracts, pupation did not occur. All the larvae died at larval stage but the larval period was extended to 12-14 days. Conclusively it can be said that simple aqueous extract of neem leaves have some biologically active components which show insecticidal activity. So they can be applied easily for the biological control of mosquitoes.

**Key words:** Neem, Leaf extract, *Culex pipiens fatigans*

**INTRODUCTION**

*Culex pipiens fatigans* (Diptera: Culicidae) is a common house mosquito. It is a major vector of filariasis, Eastern equine encephalitis and St. Louis encephalitis. It may also be involved in the transmission of bird malaria, heart worm of dogs and fowl pox (William & Maurice, 1961). It is prevalent in urban areas and is one of the most important biting nuisance mosquitoes, having high densities in nearly all residential areas of the cities (Youdeowei & Service, 1983; Curtis, 1994; Collins & Paskewitz, 1995). It breeds in highly polluted, stagnant waters. Different insecticides are used for the chemical control of mosquitoes. But these insecticides and larvicides are very expensive. They also cause pollution and toxicity to man, crop, plants, domestic animals, wild life and also kill the other desirable fauna by introducing the toxicant in food chain. The mosquitoes are becoming resistant to a wide range of pesticides (Rathore et al., 1986). This makes room to consider some other larvicides or bio insecticides which must be cheap and appropriate and could safely be used for vector control.

Every part of *Azadirachta indica* (neem) has been advocated to possess medicinal properties. Pruthi (1937) first proved scientifically the insecticidal effect of neem. “Azadirachtin a microcrystalline compound isolated from neem kernel extract is a promising larvicide against *Culex pipiens*. Naturally occurring bio pesticides could be an alternative to chemical pesticides” (Abdelouaheb et al., 2009). It has been reported that it possesses many substances which interfere with insect molting, food uptake, reproduction and provides a nontoxic insect controlling agent for use in agriculture. These cause growth inhibition, abnormal development, elongation of larval period and no pupation (Ascher, 1984; Isman, 1993; Ladd, 1984; Mari, 1989; Naqvi et al., 1991; Naqvi et al., 1994).

Aqueous neem kernel extracts were used for warding off insect attack on crops. Neem leaf juice is used for expelling worm and curing jaundice and skin diseases. Oil from nuts and leaves is a stimulant insecticide and antiseptic. It inhibits feeding in a variety of insects and also inhibits ecdysis at much lower concentrations (Mari & Watanabe, 1989). This prevents the insect larvae from developing into mature insects which could further multiply and produce new generations. It blocks receptor of ecdysteroids which are needed for larval development (Govindachari, 1992). Azadirachtin also increased residence time in the feeding and nonfeeding immature stages, larva treated with 1.6µg of azadirachtin for example, had
significant longer larval periods than did untreated larvae; length of prepupal and pupal stages was extended (Ladd, 1984). Lin & Liu (2006) studied properties and efficacy of pesticides from neem tree, and found them effective antifeedants for pest control. Azadirachtin were growth inhibitors. They interfere with neuroendocrine regulation of juvenile and molting hormone titers (Rembold, 1988).

Toxicity and abnormalities caused by neem fractions, RBU-9, RB-b and Margosan-OTM were determined against fourth instar larvae of Aedes aegypti; partially emerged adults were found with crumpled and entangled legs in puparium (Naqvi et al., 1994).

Simple formulations of neem derivatives, such as leaf or kernel powder or extracts are safe to non-target organisms including humans (Saxena, 1988). Recent studies encouraged the investigation of insecticidal properties of plant-derived extracts; and concluded that they are environmentally safe, degradable, and target specific (Senthil et al., 2006).

Neem is a natural insecticide and is nonhazardous to man and other mammals (Oudegans, 1991). Therefore simple nonhazardous and inexpensive methods of extraction should be developed to enable practical use of neem (Feuerhake, 1984).

The aims and objectives of the present work is to develop a simple inexpensive and nontoxic method for the control of mosquito larvae by A. indica (neem), which can be applied easily by the ordinary man without use of costly spraying equipment.

MATERIALS AND METHODS

Culex pipiens fatigans larvae were chosen as experimental insect for this study, because it is the major vector for filariasis and is one of the most important biting nuisance mosquitoes, having high densities in nearly all residential areas of the cities. Larvae of Culex pipiens fatigans were reared in the insectary and second star larvae were selected for experimental work.

Extracts were prepared from leaves of neem in water and no organic solvent was used. The extracts of air dried and fresh leaves were prepared separately. For fresh leaves extract, 1000 gms of fresh leaves were crushed in 1000 ml of water and kept for 24 hours. This mixture was then filtered with the help of muslin cloth and the filtrate was used for the experiment.

For dry leaves extract, 1000 gms of fresh leaves were dried in shade for 15 days and then powdered. 500 gms of dry powder of leaves was mixed in 1000 ml of water and kept for 48 hours. This mixture was then filtered with the help of muslin cloth and the filtrate was used for the experiment.

Another method was adopted for the preparation of neem leaves extract. For this, 500 gms powder of dry leaves was mixed in 1500 ml of water and kept for 48 hrs. This mixture was then filtered with muslin cloth and the filtrate was heated on water bath, till all the water was evaporated, 25 gm. dry neem extract was dissolved in 75ml of water to make the total volume 100ml. so that 25% stock solution was prepared. This 25% stock solution was used for the preparation of further dilutions.

All the above extracts were used for the preparations of different concentrations i.e. 1%, 5%, 10%, 20%, 30%, 40%, 50% and 60%. Second instar larvae of about same age were treated with such concentrations according to WHO method. Three replicates were taken for each concentration. In control only 250 ml water was added. Then 20 second instar larvae were placed in each beaker. A pinch of liver powder was sprinkled on each beaker, which serves as larvae’s food. The beakers were covered with nets to avoid egg laying of mosquitoes. The room temperature and beaker’s temperature were noted. The survival and mortality of these were checked after each 24 hours and the results were recorded in tabular form.

Statistical analysis

The data was analyzed statistically. % mean mortality per day was calculated. Regression lines were plotted on graph papers to determine the LC50 values. And the time taken by the larvae to pupate and for emergence was also noted. %age pupation, %age emergence were calculated.

RESULTS

Effects of neem leaf extracts on the larval mortality of C. pipiens: Larval mortality was noted after every 24 hrs. for 8 days. In the first set of experiments using 25% stock solution of dry leaves extracts, the 100% mortality was observed in 30%, 40%, 50% and 60% concentrations (Table 1). In the second set of experiments using dry leaves extracts, the 100% mortality was observed in 50% and 60% concentrations (Table 2). In the third set of experiments using fresh leaves extracts, the 100% mortality was observed in 40%, 50% and 60% concentrations (Table 3). Average percentage mortalities of eight concentrations were calculated and regression line was plotted on graphs papers (fig 1, 2, 3). The LC50 value of 25% stock solution, dry and fresh leaves were 19% (fig 1), 40% (fig 2) and 33% (fig 3)
### TABLE 1: Effect of 25% stock solution of dry leaves extract of *Azadirachta indica* on larvae/pupae of mosquito

<table>
<thead>
<tr>
<th>conc.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>% Pupation</th>
<th>% Emergence from pupae</th>
<th>Pupation Period in days</th>
<th>Emergence Period in days</th>
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<tr>
<td>1%</td>
<td>8</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>15</td>
<td>20</td>
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<td>5%</td>
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<td>61</td>
<td>73</td>
<td>76</td>
<td>80</td>
<td>81</td>
<td>90</td>
<td>_</td>
<td>_</td>
<td>0</td>
<td>0</td>
<td>No pupation</td>
<td>_</td>
</tr>
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<td>30%</td>
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### Table 2: Effect of dry leaves extract of *Azadirachta indica* on larvae/pupae of mosquito

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<th>6</th>
<th>7</th>
<th>8</th>
<th>% Pupation</th>
<th>% Emergence from pupae</th>
<th>Pupation Period in days</th>
<th>Emergence Period in days</th>
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<tr>
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<td>13</td>
<td>16</td>
<td>18</td>
<td>20</td>
<td>25</td>
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<td>63</td>
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<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>95</td>
<td>100</td>
<td>3.5</td>
<td>5.6</td>
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</tbody>
</table>
Table 3: Effect of fresh leaves extract of *Azadirachta indica* on larvae/pupae of mosquito

<table>
<thead>
<tr>
<th>Conc.</th>
<th>1%</th>
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<th>3%</th>
<th>4%</th>
<th>5%</th>
<th>6%</th>
<th>7%</th>
<th>8%</th>
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</thead>
<tbody>
<tr>
<td>% Pupation</td>
<td>90</td>
<td>87</td>
<td>–</td>
<td>–</td>
<td>75</td>
<td>80</td>
<td>3.5</td>
<td>5.7</td>
</tr>
<tr>
<td>% Emergence from pupae</td>
<td>50</td>
<td>60</td>
<td>–</td>
<td>–</td>
<td>37</td>
<td>4.7</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Pupation Period in days</td>
<td>40</td>
<td>3.6</td>
<td>5.7</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Emergence Period in days</td>
<td>93</td>
<td>75</td>
<td>0</td>
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</tbody>
</table>

Effect of neem leaf extracts on the ecdysis of *C. pipiens*:
The %age pupation, %age emergence and % mortality were drawn in the form of graphs, which showed the difference in different types of extracts (Fig. 4, 5, 6). In lower concentrations 1%, 5%, 10%, pupation was delayed by two or three days. In 40%, 50% and 60% concentration of all these extracts, pupation was nil. All the larvae died at larval stage but the larval period was extended to 12-14 days on the average and the ecdysis of larvae was inhibited. In fresh leaves extracts, only 40% and 18% larvae could pupate in 20% and 30% (Fig., 6).

Fig. 1: Toxicity curve showing % mortality and LC \(_{50}\) value of *Culex pipiens fatigans* larvae in 25% stock solution extracts.

Fig. 2: Toxicity curve showing % mortality and LC \(_{50}\) value of *Culex pipiens fatigans* larvae in dry leaves extracts.

Fig. 3: Toxicity curve showing % mortality and LC \(_{50}\) value of *Culex pipiens fatigans* larvae in fresh leaves extracts.
DISCUSSION

Simple aqueous extract method was used, so that an ordinary man could prepare it at home, without using expensive organic solvent and scientific equipment. The 25% stock solution was most effective as compared to fresh leaves and simple dried leaves extract. The molting period of larvae was delayed. Most of the larvae could not pupate and remained alive in larval stage till 12 days. Similar results was also achieved by Abdelouaheb et al. (2009) that Azadiractin treatment prolonged the duration of the larval stage of Culex pipiens. And the results of study indicate that plant-based compounds such as Azadiractin may be an effective alternative to conventional synthetic insecticides for the control of Culex pipiens.

Larvicidal activity of Azadirachta indica against various species of mosquitoes has been observed by various researchers (Wandscheer, 2004; Chavan, 1984; Virendra et al., 2009; Aliero et al., 2003; Vatandoost & Vaziri, 2004; Abdelouaheb et al., 2009; Senthil et al., 2006). The extracts produced some abnormalities in larvae. Larval pupal intermediates were observed. Partially emerged adults showed crumpled legs and entangled in pupation. All these abnormalities were also reported by Naqvi (1987).

The development of insects’ growth regulators (IGR) has gained considerable attention for selective control of insect of medical and veterinary importance and has produced mortality due to their high neurotoxic effects (Wandscheer et al., 2004; Senthil et al., 2006). Lucantoni et al., (2006) results indicated that the neem, revealed a delay in oocyte development in the vitellogenesis of female mosquito, Anopheles stephensi. This disruption of reproductive capability could lead to significant population decline over time.

In addition to azadirachtin, a number of other active ingredients have also been isolated and identified from different parts of the neem tree, such as salannin, meliantriol and nimbin (Mulla & Su, 1999; Ruskin, 1992). Two new triterpenoids (22, 23-dihydronimocinol and des-furano-6-alpha-hydroxyazadiradione) were isolated from a methanolic extract of the fresh leaves of Azadiracta indica along with a known meliacin, 7-alpha-senecioy-(7-deacetyl)-23-O-methylnimocinolide (Siddique, 2002). Neem components show multiple effects against different insects such as mosquitoes, flies, triatomine bugs, cock-roaches, fleas, lice and ticks (Mulla & Su, 1999; Ruskin, 1992). Neem leaf and seed extracts also showed efficacy against stored grain pests (Sharif et al., 2007). Neemarin, at the recommended concentrations in field studies of
1 and 2 L/hectare, significantly reduces the frequency of larvae and the estimated residual effect was 7 days (Vatandoost & Vaziri, 2004).

The extracts of neem leaves in different solvents (petroleum ether, ether and EtoH) were evaluated for mosquito (Culex p. fatigans) larvicidal activity according to W.H.O. method. The 1% petroleum ether extract showed 100% mosquito larval activity, it also had good residual activity (for 144hr) at 0.2% (Chavan, 1984).

Conclusively it can be said that neem has some biologically active components which show insecticidal activity. This conclusion is supported by the previous investigations of various workers. So neem products may be used as mosquito population controlling agent, which is a vector of many diseases. They are cheaper and biodegradable and can be used easily by an ordinary man without hazardous effects. Moreover resistance does not develop in insects against them, due to their multiple mode of action on insects (Vollinger, 1987).

REFERENCES


Ethnopharmacological Studies on Phytochemicals obtained from Skimmia laureola (DC.) Zucc. Ex Walp. of Pakistan.

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¹Department of Botany, GC University, Lahore, Pakistan. ²FNRC Department, PCSIR Laboratories, Ferozepur Road, Lahore, Pakistan. ³ACRC Department, PCSIR Laboratories, Ferozepur Road, Lahore, Pakistan. ⁴University of Management and Science, Township Lahore. Pakistan.

ABSTRACT

A detailed study regarding total antioxidant capacity (TAC), radical scavenging and antimicrobial effects of the essential oils from Skimmia laureola leaves (SL), stem (SS) and roots (SR) were investigated. Essential oils obtained by steam distillation process were subjected to various assays to determine antioxidant capacity. TPC values were found to be 9.07, 20.90 and 71.95 mg/L gallic acid equivalent and 4.08, 22.96 and 329 mg/L quercetin equivalent for SL, SR and SS, respectively. A significant correlation between the percent inhibition of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation and the amount of essential oils viz., R² = 0.9971, 0.9851 and 0.996 for SS, SL and SR respectively. The percent superoxide anion radical scavenging activity was found to be 51.75, 45.37 and 56.5 percent for SL, SR and SS samples, respectively. The reducing power in terms of ferric reducing antioxidant power (FRAP) values were found to be 0.114, 14.33 and 1.2 mM FeSO₄ for SL, SR and SS respectively. All samples exhibited good metal chelating activities. The percent inhibition of the complex formation was found to be 80.37, 44.35 and 25.96 for SL, SR and SS respectively. The oils showed antimicrobial activities comparable to chloramphenicol. The data obtained from oils demonstrate the powerful antioxidative, radical scavenging and antimicrobial properties of the plant.

Keywords: Antioxidant Potential, Radical Scavenging, TAC, Skimmia laureola.

INTRODUCTION

Skimmia laureola is a shrub found throughout the temperate Himalayas from Murree to Mishmi and Khasia mountains. It is an extremely aromatic, gregarious, evergreen shrub. The smoke of burning leaves is supposed to purify the air (Nandkarni, 1982). Chromones and coumarins, including the new chromone, skimminin were isolated and characterized from Skimmia laureola (Waight et al., 1987). Skimmia oil obtained from leaves is used in high grade perfume and as incense (Niad, 1997). Oil is antiseptic, effective against Staphylococcus, Streptococcus and leaves used to treat small pox (Skane, 2010), used in flavouring curries (Johans, 2005). Skimmia laureola leaf paste mixed with cow's urine and the paste applied twice a day for 4 to 28 days for the treatment of psoriasis, leucoderma. Plant contains essential oil containing terpenes, l-linalool, l-linalyl acetate, azulene and bergaptenene. It also contains alkaloid skimmianin, furoucomarin, isopimpinellin, umbelliferone, laureoline (Ranaa et al., 2010). Similarly the extracts of Phyllanthus urinaria, Thevetia nerifolia, Jatropha gossypifolia Saraca asoca, Tamarindus indica, Aegle marmelos, Acacia nilotica, Chlorophyllum borivilianum, Mangifera indica, Woodfordia fruticosa and Phyllanthus emblica showed antimicrobial activity in a range of 75-1200 µg/ml.

The diverse antidisease activities claimed for Skimmia laureola extracts/oils and the increasing demand for antioxidant compounds from natural sources encouraged us to undertake a comprehensive study of the antioxidant and radical scavenging activities of the plant. The main objective of this study is to investigate and compare the total antioxidative capacity (TAC) and radical scavenging activities of extracts from root, leaves and stem of Skimmia laureola. TAC and radical scavenging activities were determined in terms of ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996), ABTS, DPPH and superoxide anion radicals scavenging activities, total phenolic and total flavonoid contents and metal chelating activity.

MATERIALS AND METHODS

The plant material (leaves, roots and stem) of S. laureola was collected from Abbotabad, Pakistan. Water-cleaned and shade-dried plant material was subjected to steam distillation. The samples obtained were used either neat or in diluted...
form in various antioxidant and radical scavenging assays.

Antioxidant assays
Total soluble phenolic compounds were determined following the method of Singleton & Rossi (1965) using gallic acid as a standard phenolic. FRAP (Ferric reducing antioxidant power) values were determined following the method of Benzie & Strain (1996). Final results were expressed as FRAP values (mM FeSO\(_4\) \(\cdot\) 7H\(_2\)O). 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging activity was determined by following the method of Re et al., (1999) with minor changes. (2,2'-Diphenyl-1-picrylhydrazyl Radical Scavenging Activity) DPPH free radical scavenging activity of the plant oils was measured by using the method of Shimada et al., (1992). Total antioxidant activity of the essential oils was determined according to the method employed by Mitsuda et al., (1992). Superoxide anion radical scavenging activity was determined using the method of Nikishimi et al., (1972). Metal (Ferrous ion) chelation by plant samples was estimated according to the method employed by Dinis et al., 1994.

RESULTS AND DISCUSSION

Percent yields and total phenolic content.
The percent yields and total phenolic contents of the sample oils were calculated and shown in Table 1. In comparison with SR and SL about 15 times greater percent recovery of oil was found for SR sample. SS oil of the plant was found richer in phenolic contents. It is quite obvious that in spite of low percent recoveries, the TPC values were quite good, showing richness of the oil samples with phenolic components. High TPC contents indicated high antioxidant and radical scavenging capacities for all the samples.

ABTS radical scavenging capacity and relationship between TEAC and TPC
ABTS radical cation produced as a result of reaction between ABTS and potassium persulfate in aqueous solution at physiological pH has considerable stability and sensitivity towards crude and specific antioxidants (Re et al., 1999). The reduction potential of ABTS radical cation is very similar to that of hydroxyl radical cation. So in test environment it may be taken as equivalent to hydroxyl radical produced in vivo during certain disorders and metabolic reactions. ABTS radical scavenging ability of the test samples was evaluated using ABTS radical cation decolorization assay.

### Table 1: Percent recovery, total phenolic content (TPC) of essential oils from leaves, roots and stem of *Skimmia laureola*.

<table>
<thead>
<tr>
<th>Property</th>
<th>Leaves(SL)</th>
<th>Roots(SR)</th>
<th>Stem(SS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of specimen (Kg)</td>
<td>2.200</td>
<td>1.765</td>
<td>1.560</td>
</tr>
<tr>
<td>Weight of oil (g)</td>
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<td>0.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Percent recovery</td>
<td>0.020</td>
<td>0.028</td>
<td>1.000</td>
</tr>
<tr>
<td>Color of oil</td>
<td>Clear yellowish</td>
<td>very light yellow</td>
<td>Clear light pale</td>
</tr>
<tr>
<td>TPC(mg/L GAE)</td>
<td>9.07</td>
<td>20.90</td>
<td>71.95</td>
</tr>
</tbody>
</table>

Figure 1 shows the dose dependent percent inhibition of ABTS radical cation by essential oils from leaves, roots and stem of *S. laureola*.
Small berries have been reported to be rich sources of phenolic compounds such as phenolic acids as well as anthocyanins, proanthocyanidins, and other flavonoids, which display potential health promoting effects (Fukumoto & Mazza 2000; Hakkinen et al., 1999; Wang et al., 1996; Block et al., 1992; Bomser et al., 1996; Feldman 2001 and Saito et al., 1998). Total phenolic contents in terms of gallic acid equivalents of all the extracts were determined using Folin-Ciocalteu's method. The extracts showed high GAE values. The amount of total phenolics for SS, SL and SR samples were found to be 71.95, 9.07 and 20.90 mg/L gallic acid equivalent respectively. High values of TPC obtained for all the samples demonstrated presence of various phenolic acids and flavonoid components in these samples. It is also evident from the data that ABTS radical cation decolorization assay is more linearly related to TPC. Attempts have been made to derive a relationship between the phenolic contents and antioxidant activity. Controversial results have been obtained regarding a linear relationship between TPC and antioxidant activity (Faure et al., 1990).

The present study showed a relatively good relationship between TPC and antioxidant activity determined through ABTS radical cation decolorization assay and FRAP Assay. Non-acquisition of absolutely linear relationship between TPC and the two assays may be due to different response of different phenolics in Folin-Ciocalteau Reagent (Gazzani et al., 1998) difference in the pH of the medium of assays and the reduction potential of the oxidized species. Furthermore the antioxidant activity strongly depends upon the chemical structure of phenolic compounds. Therefore no definite quantitative relationship could be obtained for general application to all the plant extracts.

**DPPH, Lipid Peroxyl and Superoxide Anion Radicals Scavenging Activities**

DPPH and lipid peroxide free radicals have been used to evaluate reducing properties and to assess free radicals chain breaking abilities of phyto-chemicals. Figure 2 demonstrates the kinetics of DPPH radicals scavenging by SS, SL and SR. All EOs showed time dependant quenching of DPPH radicals. SL sample was found to be a better quencher of DPPH radicals than other EOs. The absorbance continued to decrease with almost a uniform gradient throughout the time span of 30 minutes showing the presence of a good amount of slow reacting antioxidant components in both the mixtures.
through Iron (III) complex with thiocyanate, spectrophotometrically. The antioxidative components in proportionate to their amount halt this conversion by trapping peroxyl radicals. Figure 3 shows that all samples had considerable resistance to lipid peroxidation which is quite comparable with that of trolox (10 µM).

Superoxide (SO) anion radical is one of the important ROS which is produced first after oxygen is taken inside the body. The subsequent dismutation of SO leads to the formation of other injurious ROS. So the capacity of samples to scavenge ROS can play a very crucial role in determining the overall strength of antioxidant activity. The percent superoxide anion radical scavenging activity was found to be 51.75, 45.37 and 56.5 percent for SL, SR and SS sample, respectively.

Reducing and Metal Chelating Activities
FRAP assay was employed to estimate the ferric reducing activity of the samples using FeSO₄ as the standard reducing agent. At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to intense blue colored ferrous form can be examined by measuring the change in absorption at 593nm. Being a non specific reaction any half reaction that has lower redox potential, under the test conditions, than that of Fe(III)/Fe(II) reaction will convert Fe(III) to Fe(II). The change in absorbance reflects cumulative reducing power of the electron donating antioxidants present in the reaction mixture. The FRAP values were found to be 0.114, 14.33 and 1.2. mM FeSO₄ for SL, SR and SS respectively. The metal chelating activity was found by determining the chelating activity of the sample with ferrous ion in the presence of ferrozine (a ferrous chelating agent). In the presence of chelating components of the sample the formation of Fe(II)-ferrozine complex, which may be monitored at 562 nm spectrophotometrically is halted. The percent inhibition of the complex formation was found to be 80.37, 44.35 and 25.96 for SL, SR and SS respectively.

Antibacterial and antifungal activity
Agar well diffusion method was performed to calculate zones of inhibition for the samples. The results obtained (Table-2) indicated that all the samples showed varied affectivity against all the organisms tested except Salmonella typhimurium and Rhodutula minuta

<table>
<thead>
<tr>
<th>Strains / Treatments</th>
<th>Zone of Inhibition (cm)</th>
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<tbody>
<tr>
<td></td>
<td>SL*</td>
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<tr>
<td>Bacillus cereus</td>
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<tr>
<td>Staphylococcus aureus</td>
<td>1.2</td>
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<tr>
<td>Salmonella typhimurium</td>
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</tr>
<tr>
<td>Escherichia coli</td>
<td>2.4</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.8</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>1.2</td>
</tr>
<tr>
<td>Streptococcus equi</td>
<td>1.1</td>
</tr>
<tr>
<td>Micrococcus roseus</td>
<td>2.0</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>2.2</td>
</tr>
<tr>
<td>Penicillium chrysogenum</td>
<td>2.6</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>2.0</td>
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<tr>
<td>Rhizopus oligosporus</td>
<td>1.8</td>
</tr>
<tr>
<td>Rhodutula minuta</td>
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</tr>
</tbody>
</table>

*100 microlitres of each oil and the standard was used in the experiment

CONCLUSION

The data presented here shows that S. laureola extracts have great antioxidant and radical scavenging activity and thus may be used as a good source of natural antioxidants. The in vivo efficacy of S. laureola oils against diabetes mellitus or other degenerative diseases may be partially attributed to radical scavenging and antioxidant activity of the plant. As the oils also showed significant activity against tested organisms, they could be potential sources of new antimicrobial agents.

REFERENCES

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Incidence of White Spot Disease in Freshwater Ornamental Fishes imported to Pakistan

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ABSTRACT

The aim of this study was to investigate incidence of white spot disease in four types of tropical freshwater ornamental fishes imported to Pakistan. One hundred and twenty ornamental fishes (goldfish, comet, shubunkin, oranda, n=30 each) were examined for Ichthyophthirius multifiliis infection. Goldfish showed highest prevalence (26.6%) and mean intensity 5.25. The prevalence was 20% in shubunkin but it had highest mean intensity (9.16). Whereas, comet and oranda showed low prevalence (13.13% and 10%) but mean intensity was 4.0 and 6.66 respectively. The maximum white spots in a fish were seen in goldfish (16) and minimum (7) in comet. Only gills were infected in goldfish and shubunkin. The fins were the only infected sites in oranda. But, in comet 50% infection was on gills and other 50% infection was on fins of the host. Gills seemed to be more infected as compared to fins. Both immature and mature trophonts were observed on fins, which indicate either infection is a continuous process or different growth rate of parasite in the host. There was little inflammatory response by the fins tissue around the parasite. However, affected gill filament appeared degenerated. The variable infection rate and mean intensity in these four fish types indicates that, there is probably more than one strain of I. multifiliis which differs in its pathogenicity and virulence to different varieties of goldfish.

Key Words: Ichthyophthirius multifiliis, white spot disease, ornamental fishes, goldfish

INTRODUCTION

White Spot disease (Ichthyophthiriasis) is commonly called as “itch”, and is caused by a ciliate parasite Ichthyophthirius multifiliis Fouquet, 1876. Ichthyophthirius multifiliis is a three stage parasite with direct life cycle (Ewing and Kocan, 1992). When the adult parasite leaves the infected fish, it is called tomont. Tomont is attached to a suitable substrate in water and form a thin walled cyst. Within the cyst tomont divide many times and form a thin walled cyst. The tomites when expelled from the cyst is elongated in shape (20-60mm) and become theront. The theront swim to fish host and penetrate into the epidermis using a penetrating gland and the strong swimming action of their cilia (Durborow et al., 1998). After penetration into the fish skin they are called trophont, which grow inside fish epithelium and become 1mm in diameter (Lom & Dykova, 1992). The body form a somatic cyst around the trophont (Price & Bone, 1985) and cysts appear as white spot, which are easily visible and countable. All life stages are extremely temperature dependents which ranges from 18-25°C (Osman et al, 2009).

Ichthyophthirius multifiliis is one of the most important protozoan pathogen of almost all freshwater fishes worldwide (El-Dien & Abdel-Gaber, 2009) and is distributed in tropical, subtropical, temperate regions and even extends up to Arctic Circle (Matthews, 1994, Ventura & Paperna, 1985). White Spot disease is considered as the most pathogenic diseases of fish, which cause significant economic losses of affected cultured fish. The parasite is capable of killing large number of fish in short period of time (Durborrow, et al, 1998). This parasite can cause serious epizootic in different species of fish in aquarium, ponds and hatcheries and even in wild fish populations (Ezz El-Dien et al, 1998; Kim et al 2002, Thilkaratne et al, 2003). White spot disease is more common in intensive fish farming system due to the confinement of fish under stressful conditions. The aim of this study was to investigate incidence of white spot disease in some tropical freshwater ornamental fishes imported to Pakistan.

MATERIALS AND METHODS

One hundred and twenty freshwater ornamental; goldfish (n=30) and three of its varieties (shubunkin, oranda and comet, 30 each) were examined for Ichthyophthirius multifiliis infection from Feb. to Oct. 2012. The fishes were obtained from local pet shops in Lahore and brought to laboratory in sterilized polyethylene bags containing aerated water and maintained in glass aquarium separately. Total length and body weight of each specimen was recorded. The fish skin and fins were
examined for the ulcer and lesions and presence of white spots and symptoms of the disease. Gills were removed and placed into petri dish containing distilled water and examined for the presence of *I. multifiliis*. Parasites quantification was performed directly on wet mount of fins and gills under microscope. The identification of parasites was made following Kabata (1985), Durborow *et al.*, (1998); Elsayed *et al.*, (2006) and Osman *et al.*, (2009). Mean intensity and prevalence of parasites were determined according to Margolis, *et al.*, (1981) and compared in all four types of fishes. The photographs were taken with Olympus camera fitted on microscope.

**RESULTS**

The morphometric data total length, standard length, body width and body weight of the experimental fishes is given in Table 1.

Table 1: Morphometric observations of four types of experimental fish

<table>
<thead>
<tr>
<th>S. No</th>
<th>Fish</th>
<th>No. of Fish exam</th>
<th>Total length range (cm)</th>
<th>Standard length range (cm)</th>
<th>Body width range (cm)</th>
<th>Body weight range (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Goldfish</td>
<td>30</td>
<td>5.6-9.5</td>
<td>3.5-5.6</td>
<td>1.9-3.9</td>
<td>4.3-7.9</td>
</tr>
<tr>
<td>2</td>
<td>Comet</td>
<td>30</td>
<td>6.5-11.4</td>
<td>4.1-6.9</td>
<td>1.7-3.0</td>
<td>5.3-11.3</td>
</tr>
<tr>
<td>3</td>
<td>Shubunkin*</td>
<td>30</td>
<td>3.3-11.6</td>
<td>2.4-3.7</td>
<td>1.1-3.7</td>
<td>1.2-11.8</td>
</tr>
<tr>
<td>4</td>
<td>Oranda</td>
<td>30</td>
<td>5.8-6.6</td>
<td>3.9-4.2</td>
<td>1.9-3.6</td>
<td>4.3-11.3</td>
</tr>
</tbody>
</table>

(*From Iqbal & Hussain 2013*)

**Infection of fish with *Ichthyophthirius multifiliis***

The gills and fins were two sites of infection in these fishes. In goldfish, shubunkin and oranda, 100% infection was on one sites either gills or fins. The prevalence in these fishes was 26.6%, 20.0% and 10.0%; mean intensity was 5.25, 9.16 and 6.6, respectively (Table 2).

Table 2: Occurrence of *Ichthyophthirius multifiliis* in four types of ornamental fishes

<table>
<thead>
<tr>
<th>S. No</th>
<th>Fish</th>
<th>No. of Fish exam</th>
<th>Fish infect</th>
<th>Prevalence (%)</th>
<th>Maxl. Parasites</th>
<th>Total Parasites</th>
<th>Mean Intensity</th>
<th>Parasites No and Site of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Goldfish</td>
<td>30</td>
<td>8</td>
<td>26.66</td>
<td>16</td>
<td>42</td>
<td>5.25</td>
<td>42 and 16</td>
</tr>
<tr>
<td>2</td>
<td>Shubunkin*</td>
<td>30</td>
<td>6</td>
<td>20.0</td>
<td>13</td>
<td>55</td>
<td>9.16</td>
<td>55 and 23</td>
</tr>
<tr>
<td>3</td>
<td>Comet</td>
<td>30</td>
<td>4</td>
<td>13.33</td>
<td>07</td>
<td>32</td>
<td>4.0</td>
<td>09 and 23</td>
</tr>
<tr>
<td>4</td>
<td>Oranda</td>
<td>30</td>
<td>3</td>
<td>10.0</td>
<td>09</td>
<td>20</td>
<td>6.66</td>
<td>- and 20</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>120</td>
<td>21</td>
<td>17.50</td>
<td>16</td>
<td>149</td>
<td>7.09</td>
<td>106 and 43</td>
</tr>
</tbody>
</table>

(*From Iqbal & Hussain 2013*)

In comet both gills and caudal fins were infected in the same host at the same time in all infected fishes. The prevalence was 13.33%. However, mean intensity of infection on gills was 2.25 and that of fins was 5.75. The detail of gills infection by *I. multifiliis* and *Dactylogyrus* sp. in shubunkin is discussed elsewhere (Iqbal & Hussain, 2013). Infected fish showed typical but mild clinical signs and symptoms. Mucus secretion from gills and skin was clear which was sloughing off. Shubunkin showed aggressive flashing behavior in the aquarium. The other three fishes showed less aggressive behavior than shubunkin. The white spots on the fins were very clear. Both mature and immature stages of parasite, (the trophont) were located under mucus and layers of cells (epithelium) on gills and between fin rays. The wet mount preparation of the parasites from the gills and fins showed, oval to round shape parasite (trophont) with characteristic C-shaped or horseshoe shaped macronucleus (Fig.2). In the immature stages of parasites, the macronucleus was not prominent (Fig. 1). Not much pathological changes due to parasitic infection on fins were observed. The tissue surrounding the trophont did not show any damage. There seems to be little inflammatory response by the fin tissue to the parasite. *Ichthyophthirius multifiliis* were more dominant on gills than on fins. The infected gill filament looked degenerated and desquamated. The gill surface became thick and
showed edema at the point of attachment of the parasite (Fig. 3)

Fig. 1: Variable sizes of trophonts of *I. multifiliis* on the caudal fin of comet

Fig. 2: A trophont of *I. multifiliis* with C-shaped macronucleus in the middle of caudal fin of comet

Fig. 3: A *I. multifiliis* trophont and a *Dactylogyrus* sp. on gill filament of shubunkin.

DISCUSSION

The prevalence and mean intensity in these fishes varied from 10.0% to 26.6% and 4.0 to 9.16 respectively. Thilakaratne et al., (2003) reported low prevalence of *I. multifiliis* from five ornamental fishes. Pizza et al., (2006) found variable infection of *I. multifiliis* from platy, *Xiphophorus maculates*, sword tail *Xiphophorus helleri* and molly *Poecilia sphenops*. Tavares-Dias et al., (2010) also reported variable mean intensity of *I. multifiliis* from six ornamental fishes, *Carnegiella strigata*, *Carnegiella martae*, *Hyphessobrycon copelandi*, *Nannostomus eques*, *Nannostomus unifasciatus*, and *Pterophyllum scalare* in Brazil.

Goldfish shows high susceptibility to *I. multifiliis* as exhibited by natural repeated outbreak of Itch (Ezz Eldin et al., 1998). The moderate prevalence and mean intensity and maximum number of cysts in one goldfish in present study support this point that goldfish is more susceptible to Itch, than other three fishes, shubunkin, oranda and comet. Shubunkin with highest mean intensity and maximum parasites (13) in a fish seems having fair susceptibility for Itch. Comet and oranda with low prevalence and mean intensity can still can be placed as less susceptible to Itch compared to goldfish. The variations in infection level and mean intensity among four varieties of goldfish probably point the variable susceptibility of these fishes to *I. multifiliis* attack. This explanation may be compared with care to Hines et al., (1974) who indicated that different fish species show significant difference in their ability to resist diseases.

There are reports indicating that different fish species have significant difference in their resistance to Itch. These differences in susceptibility have been associated primarily to environmental factors or genetic make up of the host (Hines et al., 1974; Clayton & Price, 1992, 1994; Price & Clayton, 1999, Gleeson et al., 2000). Butcher (1947) reported that rainbow trout *Oncorhynchus mykiss* were more susceptible to infection by *I. multifiliis* than brown trout *Salmo trutta*. Clayton & Price, (1992) stated that susceptibility to Itch varies between different strains of platy. Our results show slightly higher infection in goldfish and shubunkin compared to other two varieties. Variation in infection of these four types of fishes with *I. multifiliis* may be associated with one assumption that there is probably more than one strain of *I. multifiliis*. This view is strengthened by the fact that the source of ornamental fish was different in each sample. This point has already been highlighted by Leff et al., (1994). The mild infection in comet and oranda may be explained by already reported fact that maternal
antibodies pass from mothers to their offspring directly via egg (Mor & Avtalion, 1988, Sin et al., 1994). The presence of atypical signs such as poor inflammatory response on fins in our fishes such as comet and oranda is probably an indication of the success of the infection process, but at the same time points to non-specificity of the parasite strain for the host rather than host resistance. Molnar et al., (2012) stated that the protozoan parasites of fish, such as; Eimeria anguillae, E. daviesae, E. praecae, E. Variabilis, E. rutii and E. nemethi are related but distinct from those that infect terrestrial vertebrates, such as Eimeria bovis, E. zuernii, E. cylinderica, E. subsphera reported from cattle (Qamar et al., 2011).

Probably goldfish and shubunkin show similar interaction with I. multifiliis as both fishes had infection on gills. Comet showed different pattern by having infection on gills and fins at the same time and oranda showed entirely different pattern of infection by having infection just on fins. Eissa (2004) reported that sublethal infection provides the fish with a protection against re-infection. Mild infection observed in present study may also be explained by one point that infection with monogenean gill parasites such as Dactylogyrus sp. seems to protect the fish partly against Itch. This is just one explanation of low I. multifiliis infection, as observed in case of shubunkin, where Dactylogyrus was attached to gill along with I. multifiliis. But in contrast to this Buchmann et al., (1999) found that the activation of the response against other parasites did not induce any protection against Itch. The four types of fishes investigated in this study basically belonged to one species Carassius auratus. Hence, the understanding of their susceptibility to Itch, host response, success of establishment of parasite in host and finally its maturity and completion of life cycle needs a very detailed in vitro investigation.

ACKNOWLEDGEMENT

We are grateful to the University of Punjab Lahore, for funding this study under faculty development program 2011-12.

REFERENCES


punctatus (Rafinesque), against two immobilization serotypes of *Ichthyophthirius multifiliis* (Fouquet). *J. Fish Dis.*, **17**: 49-432.


New remains of *Giraffa priscilla* from Parrhewala Chinji Formation, Northern Pakistan

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**ABSTRACT**

New giraffid remains comprising upper dentition recovered from the Parrhewala outcrops (Upper Chinji Formation) of the Lower Siwaliks, northern Pakistan. The remains are assigned to *Giraffa priscilla*. *Giraffa priscilla* is endemic to the Lower Siwaliks and is unknown from outside of this region. *G. priscilla* is found in the Middle Miocene (14.2-11.2 Ma) of the Siwaliks. All the described specimens have broad crown, strong styles and median ribs.

**Keywords:** Mammalia, Giraffidae, Giraffinae, Middle Miocene, Siwaliks.

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**INTRODUCTION**

The foothills of Himalayas, Potwar, Suleman Range (Punjab), Kirthar (Sind), Waziristan (NWFP) and many scattered patches in Baluchistan are known as Siwalik Group. The Siwalik formations are also located in India, Nepal and Bhutan ranging 6-90 km in width (Acharyya, 1994). Siwalik Hills are famous for its fossil fauna. Almost all major vertebrate and invertebrate phyla have been recorded from these formations.

The Siwalik Hills are relatively low, having an altitude from 300 to 1200 meter above sea level (Ahmed, 1995). The Hills actually comprise a series of parallel ridges, forming a belt some 13 km in width (Colbert, 1935). Fossil record of Pakistan Potwar Plateau is best for the interval from 18 to 6 million years ago (Lihoreau *et al.*, 2004).

The Suborder Ruminantia (Order Artiodactyla) is well represented in the Siwaliks from the small tragulid (*Darcotherium minus*) to the large giraffid (*Bramatherium*) (Barry & Flynn, 1989). Since then a number of genera and species of Family Giraffidae have been recovered from various formations of the Siwaliks by different workers such as Matthew (1929), Colbert (1935), Sarwar (1990).

Matthew (1929) described *Giraffa priscilla* from the Lower Siwaliks. However, the fossils of this species are limited. Pilgrim (1911) found upper left second and third molars of *Giraffa priscilla*, but post-cranial skeleton is not known. Basu (2004) identified *Giraffa priscilla* from lower Siwaliks of Ramnager (India). According to him, fossils of *Giraffa priscilla* are Middle Miocene in age. Pakistan Lower Siwaliks i.e. Chinji Formation represent the same fauna.

**Geography and geology:**

The Chinji Formation lies in the Potwar Plateau of Pakistan. It is composed of bright red clays and sub-ordinate brown grey sandstones. The age of Chinji Formation is Middle Miocene from 14.2-11.2 Ma (Barry *et al.*, 2002; Nanda, 2002, 2008). New remains of *Giraffa priscilla* described here have been recorded from locality of Parrhewala which is 12.2-11.2 Ma in age.

**Parrhewala:**

Parrhewala (Late. 32° 41’ N; Long. 72° 16’ E) is a local farm located at about 1 km south east of the Chinji village (Fig. 1). Upper Chinjian rocks are well exposed bearing Lower Siwaliks fauna. Bright red clays with abundant of siliceous nodules forming pseudo conglomerates are the dominating feature (Sarwar, 1990).

**Abbreviations:**

Ma, Million years ago; P, Premolar; M, Molar; l, Left; r, Right; GCUPC, Government College University Paleontological Collection, Lahore, Pakistan; PUPC, Punjab University Paleontological Collection, Lahore, Pakistan; GSI, Geological Survey of India; AMNH, American Museum of Natural History, New York, USA; W/L, Width/Length ratio; mm, Millimeters.
MATERIALS AND METHODS

The studied material was collected from the Parrhewala (Upper Chinji Formation) of Lower Siwaliks Pakistan. The clay was removed with the help of needles and brushes. The unwanted sediments were washed in the paleontology laboratory of Zoology Department of GC University, Lahore with the help of phosphoric acid. Each specimen was catalogued e.g. 1164/13, the nominator shows the serial number of the collection and the denominator denotes the collection year.

Length and breadth measurements were taken at occlusal level by a digital vernier caliper. The material is compared with the specimens present in the Indian Museum Calcutta, American Museum of Natural History New York and Punjab University Paleontological Collection in Zoology Department, University of the Punjab, Lahore, Pakistan.


SYSTEMATIC PALEONTOLOGY

Order ARTIODACTYLA Owen, 1848
Suborder RUMINANTIA Scopoli, 1777
Infraorder PECORA Linnaeus, 1758
Superfamily GIRAFFOIDEA Gray, 1821
Family GIRAFFIDAE Gray, 1821
Subfamily GIRAFFINAE Zittel, 1893
Genus GIRAFFA Brisson, 1756

Type species. Giraffa giraffe Brisson, 1756

Generic diagnosis. Members of this genus are medium sized with much elongated neck and limbs. Basicranial and basipalatal inclined at a small angle. Both sexes have two short ossicones on parieto-frontal and a median naso-frontal protuberance. A pre-lachrymal vacuity is present (Colbert, 1935). Dentition is moderately brachyodont. Premolars are complex and molariform. Buccal enamel coarsely rugose (Harris et al., 2010). The enamel forms outgrowths into the central cavity from the crescents. Upper teeth are with strong external ribs. Breadth is in excess of length. Lower incisors and canines are robust. Lower molars not elongated, generally rudimentary tubercles but a large one always present in M1 and generally in M3 (Mathew, 1929; Colbert, 1935).

Geographic distribution. Giraffa is best known from Pakistan, India, Ethiopia, Kenya, Malawi, Tanzania, South Africa and Uganda (Pilgrim, 1911; Basu, 2004; Bhatti, 2005). Giraffa priscilla Matthew, 1929

Lectotype. GSI B511, a left M3

Type locality. Chinji, Lower siwaliks, Punjab, Pakistan (Matthew, 1929).

Stratigraphic range. Lower Siwaliks (Chinji Formation of Pakistan and India) (Matthew, 1929; Colbert, 1935; Basu, 2004; Bhatti, 2005; Khan et al., 2012)

Specific diagnosis. Teeth are broad crowned and more brachyodont as compared to Giraffokeryx. Anterior rib and metastyle are very strong; in M3 the more oblique-set inner crescents, broad third lobe with strong accessory basal cusp in front of it, as well as shorter crown (Matthew, 1929).


DESCRIPTION

P2. The specimen (GCUPC 1164/13) is an isolated lower right second premolar (Fig. 2(1)). The tooth is excellently preserved and moderately worn out. The dentine is exposed all over the crown surface. It is quadrangular in general contour. A thick layer of cingulum is present all over the crown surface. The enamel layer is thick, shiny and corrugated at the anterior, posterior and lingual sides. It is broad crowned tooth having four types of cones. The protocone and metaconule are low in vertical height than the labial cusps i.e. paracone and metacone.
The protocone is moderately worn out and its dentine is exposed. It is contiguous with the metaconule posteriorly. The metaconule and protocone are not differentiated due to wearing. The metaconule is connected with metacone by cingular ridges. The paracone is present anterolabially, preparacrista is shorter than postparacrista. The enamel lining of paracone is thick and shiny. It is pushed outward and backward to form protostyle. It is visibly distinct and free at anterior part of cusp.

The metacone is present posterior to paracone. It is inverted “V” shaped. Its enamel border is thick and corrugated. The metacone is pushed outward posteriorly to form pillar like structure called metastyle. The enamel folding of metacone extend anteriorly and paracone posteriorly and outwardly to form a very strong and thick mesostyle which is supported by cingular ridges. A large V-shaped central cavity is present between lingual and labial cusp. Longitudinal valleys are wavy and shallow while transverse valley is open lingually but closed labially in the middle of crown.

P*. GCUPC 1147/09 is rP4. The tooth is finely preserved and square in outline (Fig. 2(2)). The cingulum is not visible labially due to thick layer of cement, but quite thick at the lingual side of crown. The enamel layer is rugose and somewhat shiny; mostly it is rough perhaps due to weathering. All the four principal cusps i.e. protocone, metaconule, paracone and metacone are clearly visible.

The protocone is present at anterior lingual side of crown. It is moderately worn out and dentine is exposed. It is surrounded by thick layer of cement. The metaconule is present at posterior side of protocone. Its enamel border is thick and rugose. It is completely worn and filled by a thick layer of cement. The metaconule and protocone are almost worn out forming a continuous structure. Therefore its dental morphology cannot be obscured.

The paracone is inverted V-shaped, having a thick and rugose enamel border. It is largely worn out to form dentinal island with metaconule. The enamel surface of paracone is folded back anteriorly forming a thin pillar like parastyle. The metacone is present at posterior labial side of tooth. The metacone is somewhat spade shaped having a thick labial enamel lining.

The enamel lining of metaconule posteriorly folded back to form a very strong pillar like structure called metastyle running up at the base of crown surface. The enamel lining of metaconule extend anteriorly and paracone posteriorly and outwardly to form a very strong and thick mesostyle. The central cavity between lingual and labial cusps is not visible due to cement. The transverse valley is open at the lingual side but it is closed labially by the mesostyle. The longitudinal valleys are straight and open both anteriorly and posteriorly.

M*. GCUPC 1138/09 is IM2 (Fig. 2(3)) and PUPC 68/13 is rM2 (Fig. 2(4)). Both the teeth are broad crowned. A pressure mark is present antero-posteriorly, which indicates that they are second molars. The lingual cingulum is quite thick and it forms a shelf like structure which is highly corrugated and shiny while the labial cingulum present on the paracone and metacone are incipiently developed.

All the four cusps i.e. protocone, metaconule, paracone and metacone are mostly worn out; dentine is exposed forming isolated dentinal islands. The preprotocrista and postprotocrista are almost same in size. It is contiguous with paracone anteriorly by thick enamel vertical fold. It is bounded by thick layer of enamel and supported by cingular ridges and protostyle. The metaconule is present posterior to protocone. Its premetaconule crista is V-shaped while postmetaconule crista is crescent in shape. The metaconule is surrounded by thick layer of enamel. The enamel lining of the metaconule at the lingual side extends backward and connected with posterior border of protocone. The metaconule is supported by cingular ridges.

The paracone is present antero-labially. It is perfectly V-shaped and its enamel lining antero-labially directed forward and backward to form a very thick low in vertical height pillar like structure parastyle. The preparacrista and postparacrista are nearly equal in size. The paracone has a very strong and thick labial rib. The metacone is higher than paracone. The premetaconule is elongated in contrast to postmetaconule. The metastyle and mesostyle running at the base of teeth are quite broad and strong. Median ribs are quite thick and pillar like present in the middle of proto- and metacone.

The anterior and posterior fossettes are V-shaped and quite shallow, surrounded by thick layer of enamel border. Transverse valley is linear and shallow, open both lingually and labially while longitudinal valley is wavy and extend antero-posteriorly. Two root fangs are much clear at the base of the protocone and metaconule.
Fig., 2: *Giraffa priscilla*: 1. GCUPC 1164/13, rP2. 2. GCUPC 1147/09, rP4. 3. GCUPC 1138/09, lM2. 4. PUPC 68/13, rM2 (a, occlusal view; b, lingual view; c, labial view). Scale bar equals 10 mm.

COMPARISON AND DISCUSSION

The teeth are selenodont having rugose enamel sculpture so they can be attributed to the family Giraffidae (Pilgrim, 1911). In the Lower Siwaliks, only small genera of this family are present, so the specimens can be compared with the maxilla of *Giraffokeryx* or *Giraffa* (Colbert, 1935; Bhatti, 2005). However, these two differ greatly in their dental morphological characteristics. In *Giraffokeryx*, major cusps are in a straight line (Pilgrim, 1911; Bhatti, 2005). Styles are weakly developed (Pilgrim, 1911; Colbert, 1935; Bhatti, 2005; Bhatti *et al*., 2012). Median ribs are very faint (Matthew, 1929). Spur is present on anterior fossette (Bhatti, 2005). Crown is comparatively broad (Bhatti *et al*., 2012).

But in *Giraffa*, major cusps are not in a straight line (Pilgrim, 1911; Bhatti, 2005). Styles are strong and pillar like (Colbert 1935; Bhatti *et al*., 2012). Median ribs are prominent (Matthew 1929; Colbert, 1935). Spur is absent on anterior fossette (Bhatti, 2005). Crown is narrow as compared to *Giraffa* (Bhatti *et al*., 2012).

Regarding size and morphological dental features, the specimens are very close to genus *Giraffa* (Table 1, Fig. 3). Three species of this genus are present in the Siwaliks of Pakistan i.e. *Giraffa priscilla*, *Giraffa punjabiensis*, and *Giraffa sivalensis*. *Giraffa sivalensis* is a large species present in the Upper Siwaliks of Pakistan. The posterior half of tooth is much reduced as compared to other species of this genus. Metastyle is not prominent in this species (Colbert, 1935; Bhatti, 2005). *Giraffa punjabiensis* is recorded from Middle Siwaliks of Pakistan. It is distinguished from other species by having less reduced posterior half of tooth and weak metastyle. *Giraffa priscilla* is reported from Lower Siwaliks of Pakistan. It differs from other two species of *Giraffa* by having less reduced posterior half and strong pillar like metastyle.

The described dental material is collected from the Parrhewala (Upper Chinji Formation) of Lower Siwaliks Pakistan. All the specimens under study have broad crown. In all teeth, cusps are not in a straight line. Styles and median ribs are well developed especially metastyle is very strong. The posterior half of the tooth is also reduced as compared to *Giraffokeryx*. On the basis of these similarities i.e. morphological features, measurements and W/L index (Table 1, Fig. 3), all the premolar and molars refer to *Giraffa priscilla* and can be compared with the specimens discussed by Matthew (1929), Pilgrim (1911), Colbert (1935), Bhatti (2005) and Bhatti *et al*., (2012). This species was identified by Matthew (1929) from Lower Siwaliks and is known only from Middle Miocene Lower Siwaliks localities of Pakistan and India (Colbert, 1935; Basu, 2004; Bhatti, 2005).
Table 1: Comparative dental measurement of the cheek teeth of the Siwalik *Giraffa priscilla* in mm (millimeters). *the studied specimens. Referred data are taken from Matthew (1929), Bhatti (2005) and Bhatti *et al.* (2012).

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Number</th>
<th>Nature</th>
<th>Length</th>
<th>Width</th>
<th>W/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. Priscilla</em></td>
<td>GCUPC 1164/13*</td>
<td>P²</td>
<td>21.7</td>
<td>14.5</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>GCUPC 1147/09*</td>
<td>P⁴</td>
<td>20.3</td>
<td>22.5</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>GCUPC 1138/09*</td>
<td>M²</td>
<td>25.2</td>
<td>28.6</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>PUPC 68/13*</td>
<td>M²</td>
<td>25.0</td>
<td>27.5</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>PUPC 02/99</td>
<td>P⁴</td>
<td>19.5</td>
<td>21.0</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M²</td>
<td>25.0</td>
<td>28.0</td>
<td>1.12</td>
</tr>
<tr>
<td><em>G. punjabiensis</em></td>
<td>GSI K 13/349</td>
<td>P⁴</td>
<td>22.0</td>
<td>20.0</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M²</td>
<td>32.0</td>
<td>24.0</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>PUPC 95/23</td>
<td>P⁴</td>
<td>20.0</td>
<td>23.0</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M²</td>
<td>34.0</td>
<td>28.0</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>PUPC 86/84</td>
<td>M²</td>
<td>34.0</td>
<td>27.0</td>
<td>0.79</td>
</tr>
<tr>
<td><em>G. sivalensis</em></td>
<td>PUPC 69/123</td>
<td>P⁴</td>
<td>18.0</td>
<td>22.5</td>
<td>1.25</td>
</tr>
</tbody>
</table>

*Fig.* 3: Scatter diagram showing dental proportions of the Siwalik *Giraffa* species. Referred data are taken from Matthew (1929), Bhatti (2005) and Bhatti *et al.* (2012).
ACKNOWLEDGEMENTS

We thank Sayyed Ghyour Abbas Kazmi for his help during fieldworks and Adeeb Babar for his help in photography and preparing plates.

REFERENCES


Heavy Metals Toxicity in *Psidium guajava* irrigated by Polluted Water of Hudiara Drain in District Lahore, Punjab Pakistan

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**ABSTRACT**

The present study was aimed to investigate the accumulation of heavy metals in Guava (*Psidium guajava*) fruit from three sites (Lallo, Mohlanwall and Khurdpur in District Lahore) irrigated by Hudiara Drain water along its whole stretch (55km) in Punjab, Pakistan. Hudiara Drain is a case of trans-boundary pollution between Pakistan and India. Eleven heavy metals like Na, Mg, Al, K, Ca, Ti, Cr, Mn, Fe, Ni and Zn were detected by Proton Induce X-Rays emission (PIXE) technique. The mean concentrations of these heavy metals are Na (1448.33-32151ppm), Mg (1255.33-22996.67ppm), Al (927-16239.67ppm), K (33362.67-25817.67ppm), Ca (836-37820.67ppm), Ti (0-1210ppm), Cr (0-241.33ppm), Mn (0-240ppm), Fe (103.67-3636ppm), Ni (0-91.67ppm) and Zn (106-145.33ppm) in all samples of Guava respectively. On the basis of adequate intake and recommended dietary allowance it is evident that *P. guajava* is supplying highest amount of bio elements to the organisms of the Area. The concentrations of heavy metals in the study area were significantly high, when compared to permissible international standards. These results are signals of threat to the entire ecosystem including human population which can receive these pollutants directly. Still the consequences may be wide spread as the drain dumps its polluted water in Ravi River that irrigates lot of agricultural land in the province of Punjab.

**Key words;** Hudiara Drain, *Psidium guajava*, Metals toxicity

**INTRODUCTION**

Use of water from non-conventional resources like polluted water of industrial discharge is common practice in countries that are facing the problem of water shortage (Al-Ansari et al., 2013). This uncontrolled irrigation of crops with sewage water leads to the accumulation of some potentially toxic metals in soil and have very adverse effects on growth of the plants (Vasu et al., 1998). Pakistan has largest irrigation canal system in the world as it is an agricultural country. About 70% of the area is irrigated by water of Indus basin for crop production. But this water is not adequate to cope the water demand for crops and other agricultural practices due to uncertain environmental condition and rapid increase in population. Therefore, it is now common practice in many parts of the country to use municipal sewage water that contain both industrial effluents and domestic liquid waste for irrigation purpose (Iram et al., 2009; Mushtaq & Khan, 2010). This waste water contain few important plants growth nutrients viz, Potassium (K), Zinc (Zn), Phosphorous (P), Nitrogen (N) and organic solids (Gibs et al., 2006). Whereas, it is a prime source of potentially hazardous organic and inorganic toxic material. The organic pollutants have transient and slight effect on environment of the soil and metabolize into carbon dioxide and other simple substances. However heavy metals are present in higher concentration among the inorganic substances and chelated by organic matter in waste water (Singh et al., 2013). When agricultural fields are applied with such type of waste water, soil starts to accumulate these heavy metals and fixe in soil components products (Mushtaq & Khan, 2010). Thus continuous waste water application result in high enrichment of soil with heavy metals and have long lasting effects on soil environment as they remain persistent for indefinite period of time (Kabata-Pendias & Pendias, 2002). Heavy metals are one of the important groups of environmental pollutants and mainly absorbed by the plants roots in higher quantities from the contaminated soil and enter into food chain through consumption of these plants by animals and humans (Muhammad et al., 2013; Singh, 2013). In addition heavy metals are also one of the major food supply contaminants and consumers considered them as important problems so there is an ever increasing demand of healthier, safe and good quality food in the market (Grembecka & Szefer, 2013).

Human body depends upon 72 trace elements for optimum metabolic rate in cell, healthy immune system and reproduction. Some metals are required in greater amount and are called macro
elements, others are trace metals and some are among the possible essential trace elements (Bahemuka & Mubofu, 1999). Metals like lead and mercury have no beneficial role in the body and are known as xenobiotics which are harmful even in small quantity. Iron (Fe) plays important role in highly complex reactions that take place at molecular level and very essential for human life e.g., oxygen transport to the body (Dreosti, 1980), Manganese (Mn) is very essential for proper functioning of various body parts like nerves and brain (Steenland & Boffetta, 2000), Nickel (Ni) is highly important for many metabolic reactions in living organisms (Nadeem et al., 2010) and about 200 enzymes require Zinc (Zn) as a coenzyme. But essential metals have extremely serious effects on the body when in higher concentration (Khillare, et al., 2012; Muhammad, et al., 2013). Toxic metals enter into human body through consumption of contaminated fruits and vegetables. Fruits are considered as protective supplementary food because they provide minerals, vitamins, carbohydrates, dietary fibers and essential amino acids. They are mandatory for neutralization of acid which is formed during digestion in the stomach (Hashmi et al., 2007). Furthermore, fruits are very effective in treatment of many diseases due to presence of antioxidants and other biologically active ingredients (Tucker, 2009; Park, et al., 2011; Grembecka & Szefer, 2013). Word Health Organization (WHO), Food and Agricultural Organization (FAO), State Environmental Protection Administration China (SEPA), and other regulatory bodies of many countries have established the standards for maximum permissible concentration of heavy metals in food stuffs (Xue, et al., 2012).

Higher concentration of heavy metals in soil have very deleterious effects on plants, like inhibition of different metabolic processes, inhibition of root and shoot development and growth, roots tip damage, chlorosis, enzyme system damage and reduced uptake of nutrients and water (Mushtaq & Khan, 2010; Muhammad, et al., 2013). Hence, this study was aimed to determine the concentration of heavy metals in Guava (P. guajava) fruit irrigated by Hudiara Drain. Guava fruit is eaten directly by bite, but are preferred seeded and served sliced in salads or as desert.

MATERIALS AND METHODS

This study was designed to determine the concentration of heavy metals in P. guajava irrigated by polluted water of Hudiara Drain at three sites in District Lahore, Pakistan. Lallo village was selected as first sampling site (R-I) to determine the bioaccumulation of heavy metals in P. guajava because it is the first point where Drain enters into Pakistan boundary from India. Near Multan road, Mohlenwal village was selected as second sampling site (R-II) because Hudiara Drain receive one of the major polluted tributary Satu-Katla drain before this site. Khurdpur village was selected as a third sampling site (R-III). This site is located where Hudiara Drain joins River Ravi. Fresh samples of Guava Fruits were collected from the trees located in agricultural fields that are permanently irrigated by Drain water. At the place of collection twenty seven samples were washed with the clean water, kept in labeled polythene bags and transported to laboratory and again washed with the distilled water and stored in refrigerator at 0°C. Each sample was weighed and placed in oven at 65-75°C for 72 hours for drying. Each dried sample was grinded into fine powder mass by pestle and mortar. 5mg powder of each sample was taken from each sample and placed in Aluminum foil to avoid any contamination especially the moisture. 5mg of powder of each sample was fixed on the center of transparent triangular sheet with calculated volume of Yttrium salt (YaNo3). Each triangular sample attached sheet was attached with the target holder rod. All triangular sheets each with a particular sample were attached with the sample holder and were placed in PIXE (Proton Induced X-rays Emission) chamber for metals analysis.

Proton Induced X-rays Emission (PIXE)

PIXE analysis is more authentic and accurate as compared to other techniques. This is a time saving technique and can analyze 72 inorganic elements. It is non-destructive process (Carmona et al., 2010).

RESULTS

Hudiara Drain

The detailed description of Hudiara Drain layout is given elsewhere (Muhammad et al., 2013).

Heavy metals concentration in P. guajava

Eleven heavy metals (Na, Mg, Al, K, Ca, Ti, Cr, Mn, Fe, Ni and Zn) were detected in the P. Guajava. Na concentration (1051ppm) was lowest at R-I and highest (34595ppm) at R-III respectively. The lowest and the highest concentration of Mg (657ppm) and (23661ppm) were detected at R-I and at R-III. Lowest concentration of Al (0ppm) was observed at R-II and highest concentration of Al (17115ppm) was observed at R-III. The Lowest and the highest concentrations of K (7677-79841ppm) were detected at R-I. Ca was found in lowest concentration (540ppm) at R-I and highest concentration (53361ppm) was found at R-II. Ti was
detected in lowest and highest concentration (1101-1291ppm) at R-III only because it was not detected at R-I and R-II. Cr was also only detected at R-III with lowest and highest concentration (177-284ppm). It was not detected at R-I and R-II. Similarly lowest and highest concentration of Mn ranged from (208-266ppm) at R-III and it was not detected at R-I and R-II. Lowest concentration of Fe (57ppm) was studied at R-I and highest concentration (3758ppm) was studied at R-III. Ni was only found at R-II with lowest and highest concentration (68-106ppm). It was not detected at R-I and R-III. The lowest concentration (98ppm) of Zn was detected at R-I and highest concentration (154ppm) was detected at R-III table 1.

DISCUSSION

In the study area high concentration of eleven metals (Na, Mg, Al, K, Ca, Ti, Cr, Mn, Fe, Ni and Zn) were detected in *Psidium guajava* fruit because the orchards are irrigated by polluted water of Hudiara Drain. In vicinities of Lahore many industries are synthesizing chemicals and pesticides. Textile industry require considerable amount of water for dyeing and printing. The Hudiara Drain is receiving the waste water from these industries and is supplying water for irrigation purposes. The permanent irrigation of soil with this contaminated water causes the accumulation of metals in the soil. From the soil these metals are transported to plant body and start to accumulate in higher concentration in roots, shoots and fruits and ultimately reach the body of human beings. Heavy metals start to interact with essential and non-essential ions of the body and leads to toxicity (Iram et al., 2009). The water of Hudiara Drain is highly polluted by addition of untreated industrial and sewage contaminants and must not be used for irrigation Purpose (Ayub & Tabinda, 2000; Khan et al., 2003; Iram et al., 2009; Muhammad et al., 2013). The concentration of all metals except K and Ca increased from site R-I through R-II to R-III. Highest concentration of K was present at site R-I and concentration of Ca increased from site R-I to R-II but decreased at R-III as compared to site R-II. This increased concentration of heavy metals in fruits of *Psidium guajava* is due to increased number of industrial units along the banks of Drain as it travels towards its confluence point with River Ravi (Muhammad et al., 2013).

Highest concentration of K was detected in fruits of *Psidium guajava* followed by Ca, Na, Mg, Al, Fe, Ti, Cr, Mn, Zn and Ni. The mean concentration of Na in guava fruit was higher at site R-III and R-II but lower at site R-I than the previously recorded in leaves of *Brassica compestris* at the same sites. The mean concentration of Mg in the study area is also low at site R-I and R-II but almost equal at site R-III as compared to *B. compestris* at the same sites. But the mean concentrations of K and Ca are less in fruits of guava at all sites as compared to *B. compestris* at the same sites as reported by Muhammad et al. (2013). The mean concentration of Al at site R-I, R-II and R-III were 927, 1502.67 and 16239.67ppm respectively and significantly higher than the FAO/WHO (2011) permissible standards. For the children 2-6mg/day of Aluminium is suggested in their daily dietary intake and 6-14mg/day is suggested for the adults (Havas & Jaworski, 1986). The mean concentration of Al at all sites is lower in the guava fruit as compared to *B. compestris* (Muhammad, et al., 2013). The mean concentrations of Calcium in the study area at the R-I, R-II and R-III sites were 836, 21287 and 37820.7ppm and these were lower than the *B. compestris*. FNB (2010), set Tolerable Upper Intake Levels (ULs) of Calcium for children and young adults are 2500-3000mg/day and 2500mg/day respectively. The concentration of aluminium is only with in the safe limit at site R-I but at sites R-II and R-III its concentration greatly exceeded than the standards of FNB (2010).

Iron was detected at all sites R-I, R-II and R-III with mean concentration of 103.67, 264.33 and 3636ppm in guava fruit. Iron concentrations were found to be higher at all sites as compared to FAO/WHO standards (Ayub & Tabinda, 2000: Ahmad et al., 2012), but lower than results of Iram et al., (2009) and Muhammad et al., (2013) at Site R-I and R-II. Titanium was detected only at sit R-III with mean concentration 1210ppm and it was not detected at Sites R-I and R-II. Iram et al., (2009) detected Titanium in Hudiara Drain water with mean concentration 4.807mg/kg. The high concentration of Titanium like other metals in guava fruit is due to the fact that plants accumulate high concentration of metals in their body tissues as compared to soil and water (Khan et al., 2010). Ti is not harmful for human body even in higher concentration and it is eliminated in intact form. Chromium was only detected at Site R-III with mean concentration of 241.33ppm. It was not detected at site R-I and R-II. The concentration of Chromium was lower in the study area than the results of Iqbal, et al., (2011) but significantly higher than the permissible limit of Chromium for plants set by WHO (1996) and the results of Bukhari et al., (2013). Chromium (VI) is carcinogenic and allergic for human body even in minor quantity. Similarly, Manganese was also detected at only site R-III and its concentration in guava fruit with mean concentration of 240ppm was

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almost equal to as detected in *B. compestris* by Muhammad *et al.* (2013). The concentration of Manganese in the study area was significantly higher than the permissible standards set by FAO/WHO (1976), and Bukhari *et al.* (2013) but lower than the results of Iram *et al.*, (2009). Zinc was detected at all sites R-I, R-II and R-III with mean concentrations of 106, 120 and 145.33ppm respectively. Zinc concentrations in the study area were higher than the permissible limits set by FAO/WHO (2001), SEPA (2005), Mushtaq & Khan (2010), Al-Ansari *et al.* (2013), Grembecka & Szefer (2013) and Bukhari *et al.* (2013), but lower than Iram *et al.*, (2009) and Muhammad *et al.* (2013). Nickel was not detected at R-I and R-III probably Nickle pollution was added around R-II with mean concentration of 91.67ppm hence it was only detected at that site. The Nickel concentration was higher in the study area than the permissible standard of FAO/WHO (1976), SEPA (2005), Bukhari *et al.*, (2013), Grembecka & Szefer (2013) and Iqbal *et al.*, (2011), but lower than the results of Iram *et al.*, (2009). Nickel is very important trace element it is necessary for DNA, RNA and many enzymes of Plants and animals. But its higher concentration has allergic and carcinogenic effects on the body. The concentration of all heavy metals especially Al, Cr, Mn, Fe, Ni and Zn is higher than the permissible international standards set by FAO/WHO (1976, 2001, 2011) and SEPA (2005). The concentration of Zn was within the safe limit in only one sample of guava fruit at site R-I according to SEPA (2005) standards but higher than the FAO/WHO standards.

**CONCLUSION**

This study indicates that guava fruit was enriched with heavy metals. Therefore guava fruit has potential health risk for the local peoples and animals that consume these contaminated fruits. It is the responsibility of the Government of the Pakistan to formulate strict rules and regulation for industrial discharge and sewage waste according to International Standards. It is also responsibility of the Government to educate the local peoples about the health hazards of heavy metals in the study Area. It is further suggested that farmers should not use the polluted water of Hudiara Drain for irrigation purpose and they should be educated and trained. Furthermore the pollution level in Hudiara Drain should be decreased to its minimum level because Drain dumps its polluted water in the River Ravi. It is severely dangerous for the aquatic life of River Ravi and thousands of acres of agricultural land in the province of Punjab that is irrigated by the River water. The present study draws our attention to contamination of our natural food by heavy metals. Further study to determine heavy metals concentration in animals and human beings of the local area is strongly recommended.

**ACKNOWLEDGEMENT**

We are thankful to Pakistan Science Foundation, Islamabad to provide financial support to complete this study.

**REFERENCES**


HEAVY METALS TOXICITY IN PSIDIUM GUAVA

Standards Program, ALI-NORM 01/12A: PP:1-289.


**Table 1**: Metals Contents (ppm) in *P. guajava* Irrigated with Hudiara Drain at Three Sampling Sites.

<table>
<thead>
<tr>
<th>Metals</th>
<th>SITE 1 Range</th>
<th>Mean</th>
<th>S.D</th>
<th>SITE 2 Range</th>
<th>Mean</th>
<th>S.D</th>
<th>SITE 3 Range</th>
<th>Mean</th>
<th>S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>1051-1777</td>
<td>1448.33</td>
<td>±367.83</td>
<td>4295-4508</td>
<td>4413</td>
<td>±108.34</td>
<td>30541-34595</td>
<td>32151</td>
<td>±2151.83</td>
</tr>
<tr>
<td>Mg</td>
<td>657-1843</td>
<td>1255.33</td>
<td>±593.07</td>
<td>4861-5377</td>
<td>5050</td>
<td>±284.33</td>
<td>22514-23661</td>
<td>22996.7</td>
<td>±594.68</td>
</tr>
<tr>
<td>Al</td>
<td>564-1124</td>
<td>927</td>
<td>±314.75</td>
<td>0-2393</td>
<td>1502.7</td>
<td>±1308.6</td>
<td>15595-17115</td>
<td>16239.7</td>
<td>±785.81</td>
</tr>
<tr>
<td>K</td>
<td>7677-79841</td>
<td>33362.7</td>
<td>±40325.7</td>
<td>59999-65178</td>
<td>63124</td>
<td>±2751.77</td>
<td>23566-27522</td>
<td>25817.67</td>
<td>±2034</td>
</tr>
<tr>
<td>Ca</td>
<td>540-1170</td>
<td>836</td>
<td>±316.71</td>
<td>4992-53361</td>
<td>21287</td>
<td>±27778.09</td>
<td>36292-39111</td>
<td>37820.67</td>
<td>±1424.53</td>
</tr>
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<td>Ti</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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<td>1101-1291</td>
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<td>Cr</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>177-284</td>
<td>241.33</td>
<td>±56.69</td>
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<tr>
<td>Mn</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>208-266</td>
<td>240</td>
<td>±29.46</td>
</tr>
<tr>
<td>Fe</td>
<td>57-158</td>
<td>103.67</td>
<td>±50.93</td>
<td>246-279</td>
<td>264.33</td>
<td>±16.80</td>
<td>3498-3758</td>
<td>3636</td>
<td>±130.74</td>
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<tr>
<td>Ni</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>68-106</td>
<td>91.67</td>
<td>±20.64</td>
<td>0</td>
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<td>Zn</td>
<td>98-113</td>
<td>106</td>
<td>±7.55</td>
<td>116-123</td>
<td>120</td>
<td>±3.61</td>
<td>136-154</td>
<td>145.33</td>
<td>±9.02</td>
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</table>

**Table 2**: Comparison between the concentrations of heavy metals (ppm) in the study area with International Permissible Standards.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Al</th>
<th>Cr</th>
<th>Mn</th>
<th>Fe</th>
<th>Ni</th>
<th>Zn</th>
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<tr>
<td>Present</td>
<td>564-17115ppm</td>
<td>177-284ppm</td>
<td>208-266ppm</td>
<td>57-3758ppm</td>
<td>68-106ppm</td>
<td>98-154ppm</td>
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<td>study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAO/WHO</td>
<td>1.0mg/kg/day</td>
<td>1.30mg/kg</td>
<td>2.0-5.0mg/day</td>
<td>0.8mg/kg</td>
<td>10mg/kg</td>
<td>9.4mg/kg</td>
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<td>Standards</td>
<td></td>
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</table>
New remains of *Brachypotherium* (Mammalia, Rhinocerotidae) from Dhok Pathan Formation of Middle Siwaliks, Northern Pakistan

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ABSTRACT

New dental material – one left maxillary fragment with fourth premolar and complete series of molar (P4-M1-3) has been recovered form the Dhok Pathan type locality of the Dhok Pathan Formation in northern Pakistan. The material has been ascribed to the large Siwalik rhinocerotid *Brachypotherium* and the species *B. cf. perimense*. The Dhok Pathan assemblage is composed of the elements that are characteristic of the Late Miocene rhinocerotid fauna of the Siwaliks. The purpose of the paper is to provide more information about the large rhinocerotid fossil record in the Late Miocene deposits of the Siwaliks.

**Key words**: Vertebrate, Perissodactyla, Rhinocerotidae, Siwaliks, Late Miocene.

INTRODUCTION

Dhok Pathan Formation of Middle Siwaliks is widely exposed in Potwar Plateau of Pakistan (Shah, 2009; Khan M.A. *et al.*, 2009, 2010, 2011, 2012). The village Dhok Pathan (Lat. 33° 07’ N; Long. 72° 14’ E) situated in the Chakwal district, northern Pakistan, is designated the type locality of the formation. It is of Late Miocene-Early Pliocene in age and the thickness of this area is from 950-1200 m (Barry *et al.*, 2002). The Dhok Pathan Formation is composed of alternate sandstone, claystone/siltstone beds with occasional lenses of gravels. The sandstone is hard, well cemented to very soft and poorly cemented having thickness from few meters to more than 90 m. While claystones are hard dark grey, greenish grey or brown over consolidated silty clays, laminated at places containing some calcium carbonate. The clays are orange brown in colour (Shah, 2009).

The Dhok Pathan Formation of the Middle Siwaliks in the Potwar Plateau shows two contemporaneous, interfingering fluvial systems, namely the Blue-gray system and the Buff system. The Blue-gray system characterized by widespread sheet with low sand/mud ratio, was deposited by larger braided system while the Buff system characterized by shoe-string sand bodies was attributed to frequent avulsion in a 10-20 km wide floodplain. The difference in the Blue-gray system and the Buff system was explained on the basis of difference in source area analogous to mountain-fed rivers in fan areas and foothill-fed river systems in interfan areas (Barry *et al.*, 2002).

The Dhok Pathan Formation was deposited in semi and sub tropical climatic conditions and it contains plant fragments and vanadium rich minerals. In some areas the Formation contains considerable amount of humic acid pyrite, formed during diagenesis and these served as important reductants from uranium deposits. A few places, crevasse-splay sheets, around 30 cm thick, clast-supported conglomerates occur. The conglomerate beds often contain unidentified bone and tooth fragments. The sandstone beds gradually thicker as well as multistoried. These substantially thicker, vertically stacked and laterally extensive individual grey sandstone units from a fining-upward sequence with thinner dull red to brown siltstone on the top. Varicolored, mottled, highly bioturbated paleosol horizon form distinct and lateral extensive units within the siltstone or at the transition of the sandstone to siltstone facies (Barry *et al.*, 2002).

The specimen is collected from the Late Miocene Dhok Pathan type locality in northern Pakistan (Fig. 1). The description of the new specimen is the objective of the present study.

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MATERIAL AND METHODS

Surface collection is the primary method to collect the fossils of *B. perimense*. The material was washed, cleaned and prepared for the identification. The material was cleaned by needles, fine chisels, small brushes and light hammers. Broken parts were assembled by using various gums such as Peligon, Magic stone, Alphy and Araldite.

The specimen is catalogued in series i.e., yearly catalogue and serial catalogue, so number on the specimen represents the collection year and the serial number. The collection year was written in numerator and the serial number of that respective year was written in denominator (e.g., PUPC 69/680). PUPC – Punjab University Palaeontological Collection is an institutional abbreviation. A vernier caliper was used to take the measurements and the measurements expressed in millimeters (mm). Capital letters indicate the upper dentition. A Canon Digital Camera Power Shot S×30 IS was used for the photography of the specimen. The terminology and measurements method in this paper follows Heissig, 1972.

SYSTEMATIC PALAEONTOLOGY

Order Perissodactyla Owen, 1848
Family Rhinocerotidae Owen, 1845
Subfamily Rhinocerotiane Dollo, 1885
Tribe Rhinocerotini Gray, 1821
Subtribe Teloceratina Roger, 1902

Genus *Brachypotherium* Roger, 1904

*Brachypotherium perimense* (Falconer & Cautley, 1847)


**Fig. 2:** *Brachypotherium perimense*. PUPC 69/680, a fragment of left maxilla having P4-M1-3, collected from Dhok Pathan, the Chakwal district, northern Pakistan. (i) buccal view, (ii) occlusal view.

**Distribution:** The genus is well known from the Lower and Middle Siwaliks (Heissig, 1972; Khan A.M. et al., 2012) and Gaj Series in the Bughti Hills of Pakistan (Lydekker, 1884; Pilgrim, 1912; Heissig, 1972).

**Horizon:** Middle Siwaliks.
**Table 1: Comparative dental measurements of the cheek teeth of Brachypotherium perimense from the Dhok Pathan and the Chinji formations in mm. * the studied specimens. Referred data are taken from Colbert (1935).**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Formation</th>
<th>Number</th>
<th>Nature</th>
<th>L</th>
<th>W</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. perimense</td>
<td>Dhok Pathan</td>
<td>PUPC 69/680*</td>
<td>P4</td>
<td>83.5</td>
<td>64.0</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M1</td>
<td>84.0</td>
<td>59.0</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M2</td>
<td>81.0</td>
<td>65.0</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M3</td>
<td>87.5</td>
<td>78.5</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Chinji</td>
<td>AMNH 19470</td>
<td>M1</td>
<td>81.0</td>
<td>88</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M2</td>
<td>87.0</td>
<td>94</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Chinji</td>
<td>AMNH 19454</td>
<td>P1</td>
<td>37.0</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P3</td>
<td>46.0</td>
<td>62</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P4</td>
<td>51.0</td>
<td>74</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M1</td>
<td>60.0</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M2</td>
<td>69.0</td>
<td>78</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M3</td>
<td>63.0</td>
<td>63</td>
<td>-</td>
</tr>
</tbody>
</table>

**Locality:** Dhok Pathan, Chakwal district, the Punjab Province, Pakistan.

**New material:** PUPC 69/680, left maxillary fragment with P4 and M1-3.

**Description**

*P4:* Premolar is hypsodont and broad. The protocone is somewhat constricted. The parastyle is well developed and has a vertical paracone fold. The protoloph is well developed in the premolar. The metacone is bitheled. The paracone is very prominent and the vertical groove is present along its length. The metacone looks like a pillar but it is damaged at the apex. The enamel is rugose and has weathering cracks. The ectoloph has no median rib, rather flat in appearance. The premolar is hypsodont and molariform. It has lingual cingula at the entrance of the well developed median valleys.

The posterior cingulum is united to the metaconal cingulum closing a posterior fossette. A small crista is present, remaining just a smooth undulation with a wear. From the metaconal a very strong crochet projects into the median valley. The metaconal and protoconal are parallel, obliquely placed. The protoconal gradually increases in thickness from the apex to the cingular level. Internal pass of the median valley is very shallow. The hypcone is completely bound in with the metaconal.

*M1:* The cingulum is well developed along the proto-metaconal and it is absent on the ectoloph. The protocone is somewhat constricted and extends backwardly to form a strong crochet. The lingual margin of the protocone is flat. The parastyle and metacone are well developed. The metaconal is short. The tooth is narrow posteriorly. A well developed crochet is absent. The hypocone is not constricted and the molar is covered with thin irregular cement on its anterior and posterior sides.

*M2:* The tooth is extremely broad and hypsodont. The paracone, metacone and hypocone are slightly damaged at apices. The parastyle and metacone are well developed. The anterior and posterior faces show very strong pressure marks caused by the respective teeth. The cingulum is present anteriorly along the base of the crown and looks like a shelf. The antecrochet extends toward the median valley from the protoloph, and the strong crochet runs into the median valley, almost subdividing it. There is a rudimentary crista, which extends from the ectoloph. The mediocingulum and postcingulum possess a thick enamel investment. The protocone is well separated from the hypocone pillar due to the presence of deep vertical median valley. The protoloph and metaconal are roughly parallel.

*M3:* The molar is triangular in shape. The parastyle is marked forming an obtuse angle with the ectometaloph. The protoloph is continuous, sigmoid with strong anterior constriction and antecrochet at the base of the crown. The lingual side of the protocone is very long and flat without any groove. The median valley is widely open lingually. The ectometaloph is convex without any constriction. It is an unworn molar that presents two spur like enamel projections into the median valley and extends along its height, which corresponds to the double crochet. The enamel is moderate in thickness. The molar is in middle wear and presents a simple crochet.

**COMPARISON**

PUPC 69/680 can be recognized as the teeth of a large rhinocerotid: the teeth shows lophodonty, the development of antecrochet, crochet, crista and cristella and ectoloph with parastyle fold (Prothero et al., 1989; Heissig, 1989). The teeth are characterized by their large size, the slightly constricted protocone, the presence of antecrochet, the well developed crochet, and the absence of cingulum at the protoloph lingually. These characteristics closely match with the teleceratine rhinoceros *Brachypotherium*, documented from the Miocene of Eurasia and Africa (Hooijer, 1966; Heissig, 1972; Guérin, 1980).

The sample can be associated to *Brachypotherium perimense* from the Middle to Late
Miocene of the Indian Subcontinent and Myanmar in having a weakly constricted protocone, distinct crochet, crista and rudimentary cristella (Colbert, 1935; Heissig, 1972). However, these dental characteristics, such as a strongly constricted protocone, a large antecrochet, a cingulum and a crista, have appeared independently several times in different rhinocerotid groups, providing no reliable taxonomic assignment for most isolated teeth at the generic/species level. Nevertheless, morphometrically, the specimen is closely related to B. perimense.

**DISCUSSION**

*Brachypotherium* is widely distributed in MN 6 to MN 9 of Europe and the eastern Mediterranean region (Heissig, 1972, 1989), in the Middle to Late Miocene of Africa (Hooijer, 1966), in the Early to the Late Miocene Bugti and the Siwalik Hills of the Indian Subcontinent (Heissig, 1972) and in the Miocene of Myanmar (lower part of the Irrawaddy sediments). *Brachypotherium* is distinguished from the other Siwalik rhinoceroses by its great size. Lydekker (1884) in his detailed description of the species (AMNH 19470) in the American Museum collection is quite indicative of the large proportions of the skull that are characteristics of *Brachypotherium perimense*. Owing to the massiveness of the skull of *Brachypotherium perimense* it gives the impression of being extraordinarily great in size (Colbert, 1935).

The diversity of the Siwalik rhinoceroses is clearly dependent on climatic conditions. The change in climatic conditions has resulted in shrinkage and growing of dry and wet areas and their special vegetation (Heissig, 2003). The hypsodonty and brachydonty are also directly related to species environment. The hypsodont species could eat coarse grasses, so lived in open habitat where environmental conditions were intermediate (Lacombat, 2005). The high crowned general i.e. *Chilotherium* and *brachypotherium* were abundant in those areas of the Chinji, Nagri and Dhok Pathan formations where the climatic conditions were intermediate.

**CONCLUSIONS**

The present discovery suggests the extensive distribution of *Brachypotherium* in the Late Miocene of the Siwaliks. *Brachypotherium* is also recorded from the Middle Miocene of the Lower Siwaliks and it is absent in the Siwalik Late Pliocene. The last occurrence of *Brachypotherium* in Pakistan is at the start of Pliocene. At the end of Pliocene, *Brachypotherium* have been on decline probably due to changing climatic conditions. A number of species became extinct and rhino no longer survived in Pakistan.

**REFERENCES**


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Teratogenecity caused due to Oral Administration of Neomercazole to Albino Mice

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ABSTRACT

Neomercazole (carbimazole) is an antithyroid drug. Present study was designed to evaluate the teratogenic and embryotoxic effects of above mentioned drug given to mice during organogenesis. The dose groups used were 0.2, 0.4, 0.6 and 0.8 µg/gBW. The pregnant mice were exposed to these dose groups on days 8 and 12 of gestation. Fetuses were recovered on day 18 of gestation and were subjected to morphological, morphometric and skeletal studies. Morphological studies revealed anomalies like distorted axis, hydrocephaly, microphthalmia, open eye lid, agnathia, micromelia, syndactyly, subcutaneous hemorrhages and kinky tail. Moreover, there was overall decline in litter size and upsurge in percentage of fetal resorptions. Detailed study of skeletal parameter presented reduction in ossification in skull, ribs and limb region.

Keywords: Neomercazole, Carbimazole, embryotoxicity, teratogenicity.

INTRODUCTION

Ratio of occurrence of hyperthyroidism is 5:1 in women as compared to men and the chance of hyperthyroidism during pregnancy is 0.2% (Mestman, 1997). Glioner (1998) focused on two main causes of hyperthyroidism observed in pregnancy i.e., Graves’ disease and gestational transient thyrotoxicosis. The prevalence of hyperthyroidism may represent 3-4% of all pregnancy. But these patients need immediate therapy with antithyroids and regular monitoring for signs of fetal and maternal hyperthyroidism and hypothyroidism (Patil-Sisodia & Mestman, 2010). Autoimmune fetal hyperthyroidism can generally be treated by optimizing therapy of mother, such as by increasing the dose of antithyroid drugs (Polak, 2011).

Antithyroid drugs (thionamides) are most commonly used for the therapy of an overactive thyroid (hyperthyroid). Propylthiouracil, methimazole, carbimazole and radioactive iodine are being used as effective antithyroids (Gupta et al., 1992) with potential teratogenic risk also. Most commonly occurring defect due to these antithyroid drugs is aplasia cutis congenita in new borns (Perger et al., 2011). Aplasia cutis congenita (a circumscribed absence of skin that usually involves scalp) is one of the major anomaly associated with methimazole exposure during pregnancy (Rodriquez-Garcia et al., 2011). Among other anomalies related to prenatal methimazole exposure are choanal and esophageal atresia, minor facial anomalies, and psychomotor delay (Clementi et al., 1999). A case report reveals embryopathy caused by maternal thiamazole usage during first trimester of pregnancy. Embryopathy is mainly characterized by choanal atresia, esophageal atresia, minor dysmorphic facial features, growth retardation and delayed psychomotor development. The baby boy born to antithyroid treated mother at the age of 4 years showed delayed speech and language development (Ozgen et al., 2006).

Neomercazole (carbimazole) has been found to be very valuable against Graves' disease. Carbimazole is found to be effectual if given within the range of 20mg/day (minimum) and 40mg/day with minimum risk of iatrogenic hypothyroidism to treat hyperthyroidism (Page et al., 1996). Some patients are also given 30mg/day once methimazole (Mafauzy et al., 1993). It is also found that drug transfer across the placenta and into the breast milk is higher for lipid soluble methimazole (an ultimate metabolite of carbimazole) than any other antithyroid drug (Kampmann & hensen, 1981). Kriplani et al. (1994) described a cohort study of 32 pregnancies with thyrotoxicosis and majority of mothers were using carbimazole. Problems observed were preterm labour (25%), pregnancy induced hypertension and one maternal death also. While 13% IUGR and 6% unspecified congenital anomalies were found in infants.

In view of the above mentioned literature, the present study was designed and carried out in
mice embryos because of their possible extrapolation in human application.

**MATERIALS AND METHODS**

8-10 weeks old mice weighing 30gms each were used during this research. Four dose groups i.e., NI, NII, NIII and N IV with Control groups were designed having 10 mice each.

Neomercazole (Carbimazole) was used as an antithyroid. The drug is soluble in water. The dose was prepared by diluting a tablet of 5mg in distilled water in such a way that each 0.1 ml of the solution contained desired dose. Four doses used during this experiment were 0.2, 0.4, 0.6 and 0.8µg/gBW, keeping in view minimum and maximum dose per gm body weight during human treatment. These doses were administered orally on days 8 and 12 of gestation. A control group was also managed alongside, which was administered as 0.1 ml of distilled water.

On day 18 of gestation, the treated dams were weighed and anesthetized with anaesthetized Ether. The dams were given midline incision in the abdomen and uteri were exposed. The number of implantations and resorptions were recorded. Fetuses were removed from the uteri. The foetuses were placed in Bouins fixative for 48 hours after being weighed. After 48 hours, foetuses were transferred to 70% ethanol. Various morphological anomalies were studied in the regions of craniofacial, trunk, limbs, tail and axis. Finally these were microphotographed with the help of digital camera. The morphometric analysis included fetal weight, crown rump length, head circumference, eye circumference, lengths of fore limb, hind limb and tail. The head and eye circumference values (p=mm²) were calculated for each fetus with the help of computer based programme Ellipse circumference calculator (CSGN 2006). The morphometric data was subjected to AONVA by using SPSS software.

Fetuses were preserved in 95% ethanol by Richmond & Bannett (1938) method (skeletal preparation). These foetuses were eviscerated through small abdominal incision and all thoraco-abdominal organs were removed, then these were shifted to 2% KOH solution for the complete removal of flesh. On appearance of bones, foetuses were placed in Alizarin Red for 30 min. The deeply stained foetuses were then shifted to 1% KOH until the skeleton become clearly visible through surrounding tissue and finally cleared in 20% glycerinated 1% KOH. The stained specimens were preserved in 50% ethanolic glycerol for microscopic observations and macrophotography (Kawamura et al., 1990).

**RESULTS**

During present study, maternal toxicity showed a rise in maternal weight of vehicle control. While in treated groups slight rise in weight was observed as compared to the control then gradual decline in weight with increase in dose i.e., 4.1% increase in 0.2µg/g BW, 3.9% in 0.4 µg/g BW, 4.1% in 0.6 µg/g BW and 2.09% in 0.8µg/g BW. Detailed study of fetal toxicity described increase in malformations in dose treated groups as compared to the control. Similarly resorption rate was also found high in higher dose groups as compared to control (Table 1).

**Table 1: Effects of Neomercazole on maternal weight and fetal toxicity after maternal exposure on days 8 and 12 of gestation.**

<table>
<thead>
<tr>
<th>Dose Groups</th>
<th>Maternal wt %age rise</th>
<th>% of malformed fetuses</th>
<th>% of resorptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>1.12</td>
<td>0</td>
</tr>
<tr>
<td>0.2µg/g BW</td>
<td>4.1</td>
<td>8.9</td>
<td>5.1</td>
</tr>
<tr>
<td>0.4µg/g BW</td>
<td>3.9</td>
<td>12.5</td>
<td>5.5</td>
</tr>
<tr>
<td>0.6µg/g BW</td>
<td>3.6</td>
<td>15.7</td>
<td>5.7</td>
</tr>
<tr>
<td>0.8µg/g BW</td>
<td>2.09</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

In this exposure, pregnant mice were treated with Neomercazole on day 8 and 12 of gestation. Recovered fetuses of the dose group 0.2µg/gBW, showed morphological deformities such as kyphosis microcephaly, open eye lid and hemorrhagic spot (Fig., 2). Where as in 0.4µg/gBW anomalies observed were hydrocephaly, dysplasia of hind limb (Fig., 3) and kinky tail. Malformations expressed in dose group 0.6µg/gBW included kyphosis, open eyelid, anagathia, hind limb dysplasia (Fig., 5), malrotated limb (Fig., 4), hemorrhagic spots and degenerate tail. Dose group N-IV (d) (0.8µg/gBW) showed abnormalities like hydrocephaly, syndactyly, microphthalmia (Fig., 6), hind limb micromelia and hemorrhagic spots (Table 2).
Teratogenicity caused due to neomercazole

Fig. 1-6: Macrophotographs of 18 days old fetuses recovered from mothers followed by treatment with different doses of neomercazole on days 8 and 12 of gestation. Fig. 1: Vehicle control; Fig. 2: 0.2 µg/g BW; Fig. 3: 0.4 µg/g BW; Fig. 4 & 5: 0.6 µg/g BW; Fig. 6: 0.8 µg/g BW. MC, microcephaly; MO, microphthalmia; HG, hygroma; KY, kyphosis; K, kinky tail; MR, malrotated limb; HFL, hyperflexion of forelimb; MM, micromelia; Yellow Star, limb dysplasia, Arrow; haemorrhagic spots.

Skeletal preparations of foetuses recovered from 0.4 µg/g BW dose group indicated less ossification in hind limb region. While there was complete absence of ossification in skull, forelimb, hind limb and ribs of fetuses recovered from dose group 0.8 µg/g BW (Fig. 7).

Fig. 7: Fetal skeleton showing varying degree of ossification. A, Vehicle control skeleton showing well developed ossified skeleton; B, skeleton of 0.4 µg/g BW neomercazole treated fetus presenting reduced ossification in hind limb region; C, skeleton of 0.8 µg/g BW neomercazole treated fetus presenting complete absence of ossification in skull, forelimb, hindlimb and ribs.
Table 2: Morphological anomalies induced in 18 days old foetuses recovered from pregnant mice treated with different doses of Neomercazole on days 8 and 12 of gestation.

<table>
<thead>
<tr>
<th>Dose groups</th>
<th>Axis %</th>
<th>Brain%</th>
<th>Eye%</th>
<th>Snout%</th>
<th>Limbs %</th>
<th>Claws%</th>
<th>Skin %</th>
<th>Tail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td></td>
<td></td>
<td></td>
<td>Degenerat (1.12)</td>
</tr>
<tr>
<td>0.2µg/g BW</td>
<td>Kyphosis (1.16)</td>
<td>Microcephaly (3.8)</td>
<td>Open eyelid (2.5)</td>
<td>------</td>
<td>---</td>
<td></td>
<td></td>
<td>Hind limb dysplasia (2.22)</td>
</tr>
<tr>
<td>0.4µg/g BW</td>
<td>Hydromegaly (6.9)</td>
<td>------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6µg/g BW</td>
<td>Kyphosis (4.2)</td>
<td>------</td>
<td>Open eyelid (7.1)</td>
<td>Agnathia (1.4)</td>
<td>Hind limb dysplasia (1.7)</td>
<td>Malrotated limb (1%)</td>
<td>------</td>
<td>Kinky tail (2.7)</td>
</tr>
<tr>
<td>0.8µg/g BW</td>
<td>------</td>
<td>Microcephaly (8.3)</td>
<td>Microphalymia (11.6)</td>
<td>-------</td>
<td>-------</td>
<td>Micromelia (10)</td>
<td>Syndactyly (3.3)</td>
<td>Hemorrhagic spots (6.6)</td>
</tr>
</tbody>
</table>

Morphometric observations during this research showed a (p<0.001) significant reduction in weight, crown rump length brain and eye circumference and fore and hind limb lengths and tail lengths as compared to the control group foetuses (Table 3).

Table 3: Effects of Neomercazole on different parameters of foetuses after maternal exposure on days 8 and 12 of gestation.

<table>
<thead>
<tr>
<th>Dose Groups Δ</th>
<th>Fetal weight</th>
<th>Fetal CRL</th>
<th>Fetal Brain circumference</th>
<th>Fetal eye circumference</th>
<th>Fetal fore limb</th>
<th>Fetal hind limb</th>
<th>Fetal tail</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>1663 ± 9.39</td>
<td>22.51 ± 0.68</td>
<td>22.90 ± 0.033</td>
<td>7.07 ± 0.056</td>
<td>6.92 ± 0.017</td>
<td>7.77 ± 0.00512</td>
<td>10.892 ± 0.0104</td>
</tr>
<tr>
<td>N-I</td>
<td>892.1 ± 9.55</td>
<td>16.15 ± 0.035</td>
<td>22.16 ± 0.357</td>
<td>6.77 ± 0.018</td>
<td>5.6110 ± 0.01531</td>
<td>6.0990 ± 0.0198</td>
<td>9.918 ± 0.0227</td>
</tr>
<tr>
<td>N-II</td>
<td>801.8 ± 0.55</td>
<td>13.31 ± 0.055</td>
<td>16.21 ± 0.044</td>
<td>4.43 ± 0.032</td>
<td>5.00 ± 0.00277</td>
<td>5.7910 ± 0.021</td>
<td>7.0080 ± 0.01104</td>
</tr>
<tr>
<td>N-III</td>
<td>635.62 ± 29.70</td>
<td>10.99 ± 0.342</td>
<td>14.41 ± 0.048</td>
<td>3.66 ± 0.011</td>
<td>4.2060 ± 0.3256</td>
<td>4.5260 ± 0.03256</td>
<td>6.4520 ± 0.01143</td>
</tr>
<tr>
<td>N-IV</td>
<td>369.87 ± 38.66</td>
<td>8.73 ± 0.661</td>
<td>10.16 ± 0.025</td>
<td>3.08 ± 0.039</td>
<td>3.026 ± 0.1861</td>
<td>3.426 ± 0.235</td>
<td>4.5278 ± 0.388</td>
</tr>
</tbody>
</table>

ANOVA (Inter group Comparison) *** *** *** *** *** *** ***

*** Significant difference (p<0.00
DISCUSSION

Present study showed that Carbimazole induced overall reduction in growth rate. It included significant reduction (p<0.001) in different parameters like weight, crown rump length, brain size, eye size, limb size and tail size. Growth rate displayed retardation with increase in dose concentration exposure time. As neomercazole lowers the thyroid secretion and rapidly crosses placenta and ultimately cause fetal hypothyroidism. Clinical hypothyroidism is associated with high incidence of fetal loss, reduction in fetal weight and congenital system malformation. This condition is complemented with preterm delivery, poor vision development and neurodevelopmental delay (Su et al., 2011). El- Bakry et al., (2010) reported that in an animal study where methimazole was orally administered to albino rats in drinking water from gestation day 1 to lactation day 21. New borns showed severe growth retardation along with different deformities.

In the recent course of study various morphological defects were studied including head /brain anomalies like microcephaly, hydrocephaly, exencephaly, open eye and microphthalmia, hygroma; kyphosis, kinky tail, malrotated limb, hyperflexion of forelimb, micromelia, limb dysplasia(Fig 1-6). These results are in agreement with the case history in which maternal treatment with methimazole (an ultimate metabolite of carbimazole) caused neonatal hypothyroidism. As a consequence of which abnormalities of central nervous system like incomplete maturation of neuronal and glial cells, reduction in synaptic densities and myelin deficiency (Wong & Leung, 2001). In another animal study where methimazole induced fetal mental retardation as it produced deleterious effects on neural growth and development like reduced synaptic activity, delayed myelination and alteration in neurotransmitter level (Kormilas et al., 2010). El- Bakry et al. (2010) reported some anomalies and developmental deformities in the cerebral cortex and cerebellar regions of brain due to methimazole (ultimate metabolite of carbimazole). These degenerations became more obvious and widely spread at the 3rd postnatal week. Consequently due to these deformations reduced growth in neurons of these regions was observed. In an experimental work, female rats were fed with the same drug from day 1 till 21st day of lactation in order to induce hypothyroidism. This condition resulted severe growth retardation in neurons of cerebral and cerebellar region. Basically these factors produce mal-development of neuron and dendrites in different brain regions of fetuses (Ahmed et al., 2012). Komoike et al. (2013) worked on fertilized egg of zebra fish. Eggs were grown on culture plates containing methimazole. The embryos showed iridic and retinal coloboma, loss of pigmentation, hypoplastic hind brain, turbid tissue in fore brain, swelling of notochord and curly trunk. Histological sections also showed delayed development and hypoplasia of whole brain and spinal cord along with severe disruption of retina.

Another major defect of the recent study was skin haemorrhagic spots, which is supported by a review of case history of 29 females given carbimazole during their pregnancy. Outcomes of the study revealed various anomalies like skin defects (62%), oronasal anomalies (48%), facial dysmorphism (38%), gastrointestinal anomalies (33%) and abdominal wall defect (19%). Out of 27 babies studied in this review, 3 had aplasia cutis (a major skin defect) (Ting et al., 2013). In another study where maternal therapy with methimazole induced a skin defect in the fetus called aplasia cutis congenital (absence of skin fold) (Lollgen et al., 2011). Six cases of embryopathy were reported due to use of carbimazole in the first trimester. The anomalies included facial dysmophia, patent omphalomesentric duct, aplasia cutis congenital, choanal atresia with aorta coarctation (Koenig et al., 2010).

Research work also showed skeletal defects which clearly indicated that ossification and mineralization of skeleton was reduced with higher doses and more exposures (Fig 7). These results are being justified by the findings of Gripp et al. (2011) who analysed a number of anomalies after carbimazole administration to pregnant albino rats from 10th day of gestation till parturition. This treatment proved to be embryotoxic as different fetal anomalies were observed like clinodactyly of the fifth finger. Growth retardation was observed particularly in long bones (tibia and ulna). In another study experimental rats were orally given carbimazole throughout the pregnancy. Results showed reduction in crown rump length of fetus as well as reduction in thickness of epiphyseal growth plate in ulna and tibia of treated fetuses (Shaikh et al., 2013). Amara et al., (2012) described an experimental study on rats, who were administered methimazole. Fetuses ultimately showed reduced femur length. Reduction in body weight was also observed. Calcium and phosphorus level also declined in bones of the treated fetuses.
CONCLUSION

Purpose of this study is to raise awareness about potential teratogenicity of carbimazole. Above study indicates that carbimazole exerts potentially adverse effects on development as it is reducing the size of different body organs as well as inducing skeletal defects. Therefore, it is suggested that this drug should be prescribed with extreme care and there must be better reporting of congenital anomalies in children of women with Graves disease with or without in utero exposure to antithyroid drug.

REFERENCES


Optimization Studies of Lipase Production from locally isolated *Bacillus* spp.

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**ABSTRACT**

The objective of this study was to determine the lipase production, from locally isolated *Bacillus* spp., of soil, under different cultural conditions and optimize them. Mustard oil cake was found as an ultimate source of carbon for the production of lipase (1.8 U/ml). Among different nitrogen sources yeast extract was found to be the ideal one for maximum lipase production (1.9 U/ml). Lipase production was reduced when inorganic nitrogen source (ammonium sulfate) was used. 0.5% of salt concentration was considered to be the optimum concentration for the lipase production (1.8 U/ml). Lipase production was found maximum at the 48 hours of growth (1.8 U/ml). Olive oil was able to prompt more lipase than the other natural oils (1.9 U/ml). Similarly pH value of 8 and temperature of 37°C were observed to be ideal for maximum lipase production (1.9 U/ml). Lipase was also partially characterized and estimation of protein was done for the maximum lipase units. It was observed that specific activity of lipase was maximum at pH 8.5 and at temperature 55°C (66.6 U/mg).

**Keywords:** Lipase, Optimization, *Bacillus* spp., Bacterial

**INTRODUCTION**

Enzymes have occupied the space of nature’s catalysts and are also termed as the biocatalysts. The use of enzyme-dependent processes can be found in ancient civilizations. Because of its versatile application, lipase is considered as one of the most important enzyme following amylase and protease (Cavalcanti et al., 1997; Saxena et al., 1999). Out of 4000 enzymes about 200 are used at commercial level and majority of the industrial enzymes that have been extracted until now are of microbial origin. The total sales of enzymes until 1960 was not much but with the advancement in the fermentation processes, understanding of biochemistry and also improved extraction and recovery of enzymes have made an immense effect on the enzymes production and increased their market tremendously. There are 12 major producers while 400 minor producers, the majority of the minor producers are from Europe (Godfrey & West, 1996; Wilke, 1999).

Microbial enzymes are more potent than the enzymes extracted from animals and plants sources (Saxena et al., 1999; Sharma et al., 2001). Similarly, microbial lipases are more conveniently produced than that of their alternative sources.

Lipase (triacyl glycerol acylhydrolases, EC 3.1.1.3) belongs to the group of hydrolases that act on the triacylglycerols in the aqeous conditions to synthesize the free fatty acids and also glycerol (Lutz, 2004; Kempka et al., 2008).

Triacylglycerols contain high energy carboxyl ester bonds. Lipases can be produced by different species of fungi, bacteria and antinomycetes. Microorganisms that are mostly used for the lipase production are, *Bacillus* spp., *Aspergillus niger*, *Rhizopus* species, *Rhizomucor mehei* and *Penicillium* species etc.; (Chahinian et al., 2000 and Herrgard et al., 2000). Gopinath et al. (2005) reported about 34 wild fungal species associated with edible oil mill wastes. Fungi and bacteria are present on the wide range of the substrates and help in the production of extracellular and intracellular lipase. Because of the presence of sulfide bonds extracellular enzymes are more stable than that of the intracellular enzymes.

Lipases can be produced by both solid state fermentation and submerged fermentation. Lipase production is more convenient in submerged fermentation because of the better availability of nutrients for the microorganisms.

In recent times, lipases have appeared as key enzymes in expeditiously growing biotechnology, owing to their multidimensional properties, which find usage in a wide array of industrial applications, such as technology of detergent, food, chemical industry and biomedical sciences. Lipases are considered to be the third largest group following proteases and amylases, based on total sales volume. Because of its extensive range of applications lipase production is a billion dollar business (Jaeger & Reetz., 1998).

In the present work we report the optimization studies for increased production of lipase from locally isolated *Bacillus* species.

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MATERIALS AND METHODS

Sample collection and processing
Soil samples were taken in the plastic bags at 4-5 cm depth with the help of spatula from different places like dump areas, waste product containing soil (sewage) and soil from roadside in the vicinity of G. C. University, Lahore. The samples were brought to the laboratory after collection and then 1 g of soil samples were suspended into 100 ml of distilled water and after mixing the stock solution was kept for further microbial isolation.

Screening of microorganism
0.5 ml from different stock solutions were poured on nutrient agar plates for primary isolation and the plates were incubated at 37°C for 24 hours. Single cell generated colonies were produced with the help of streaking. With help of guidelines of Bergey’s Manual; it was confirmed that our isolates belong to Bacillus genus.

Selection of strain with more lipase production
Eight different isolated colonies were screened for the production of extracellular lipases through shake flask fermentation method. The screening was performed in 250 ml Erlenmeyer flasks. In 50 ml of fermentation medium loop-full of microbial cells were poured from different sources and were screened before and after 24 hours incubation at 37°C. Then after incubation 5ml of the inoculum was transferred to the medium containing solid substrate and incubated them for 48 hours at 37°C. The colony that showed maximum activity was picked for further studies.

Fermentation techniques

Substrate selection
Different substrates were used earlier for lipase production. Mustard oil cake, coconut oil cake, wheat bran and nutrient broth from the local market of Lahore were used as substrates for lipase production (Bora et al., 2007).

Inoculum preparation
In order to prepare inoculum minimal media was used that contained 6.0 g Na₂HPO₄, 5.0 g NaCl, 3.0 g KH₂PO₄, 0.1 g MgSO₄, and 2.0 g NH₄Cl in 1 L of distilled water. 50 ml of the minimal media was poured in 250 ml flask and transferred a loop-full of microbial cells into the media and incubated at 37°C in a shaking incubator at 180 rpm for 24 h.

Media preparation
90 ml of the minimal media was transferred in each 250 ml flask and 10 g of the desired substrate was weighed and suspended in them separately. The flasks were then autoclaved for sterilization for 20 minutes.

Solid state fermentation
After autoclaving, 5 ml of inoculum was aseptically transferred to the flasks containing autoclaved medium. The flasks were then incubated at 37°C for 48 hours and 200 rpm in shaking incubator. After the specific time of incubation the contents of the flasks were used for the estimation of enzymes activity.

Enzyme extraction
Simple extraction methods were used for the extraction of the enzyme from fermentation broth. 90 ml of 0.1 M of Sorenson phosphate buffer (pH 8.0) was mixed with the fermented material and incubated again at the same shaking fermenter for 1 hour for thorough mixing at 37°C and 200 rpm. The fermented material was then centrifuged at 5000 rpm and the supernatant was used for the extracellular lipase activity (Singh et al., 2010).

EXTRACELLULAR LIPASE ASSAY

Titrimetric assay of lipases
0.5 ml of culture supernatant was added to assay substrate in test tube that contained 1 ml of 10% (v/v) homogenized olive oil in 10% (w/v) gum acacia, and 0.5 ml of phosphate buffer (pH 8.0). The enzyme substrate mixture was incubated at 37°C for one hour. Then 2 ml of alcohol: acetone (1:1) mixture was added to the reaction mixture to stop the further activity. With the help of 0.05N NaOH using phenolphthalein as an indicator liberated fatty acids were titrated. The light pink color was the end point.

Lipase unit calculation: Lipase activity = ΔV x N/ V (sample) x 1000 / 60

ΔV= V₂ – V₁
V₁= Volume of NaOH used against control flask
V₂= Volume of NaOH used against experimental flask
V = (Sample) = Volume of the extracted enzyme
N= Normality of NaOH

The units of extracellular lipase activity were measured as units per ml (U/ml) while for intracellular activity the units were measured as per gram (U/g)

One unit of lipase is defined as “the amount of enzyme that releases one micro mole fatty acid per minute under specified assay conditions”.

90 ml of the minimal media was transferred in each 250 ml flask and 10 g of the desired substrate was weighed and suspended in them separately. The flasks were then autoclaved for sterilization for 20 minutes.
Protein estimation
Bradford method (1976) was used to determine the total protein in the crude enzyme. Test tube containing 0.1 ml of enzyme extract was added with 5 ml of Bradford reagent. The absorbance was noted using spectrophotometer at 595 nm after allowing the test tube to stand for 5 minutes. For the preparation of blank reagent 5 ml of Bradford reagent was taken in a test tube along with 0.1 ml distilled water and allowed to stay for 5 minutes. The blank was used as a control. Standard curve of bovine serum albumin (BSA) was used to estimate the total protein.

To make the standard curve of BSA, Stock solution of 0.1 % having 100 μg BSA/ml of distilled water was used. Using the stock six different dilutions were made in separate test tubes having 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg of BSA in 1 ml of distilled water. In different test tube 5 ml of Bradford reagent was taken and 0.1 ml from dilutions was added into the test tubes and allowed to stay for 5 minutes. Then absorbance was taken at 595 nm and standard curve was prepared using different absorbance values.

RESULTS

In the present study locally isolated *Bacillus* species were used to produce the lipase. The isolation of the *Bacillus* spp. was done from the soil samples taken from the vicinity of the G. C. University, Lahore. After isolating a *Bacillus* spp. that had the maximum lipase activity, the production of the enzyme was enhanced by optimization of various parameters such as temperature, pH, inoculum size and nutritional parameters, i.e., carbon sources, nitrogen sources, etc.

Effect of temperature
Temperature is one of the important physical conditions that affect both the bacterial growth rate as well as the enzyme production. The lipase production on different temperatures like 25, 30, 37, 40 and 45°C were calculated. At 25°C the lipase activity was observed as 1.0 U/ml while as the temperature raised up to 37 °C, the activity increased but after 37°C it started decreasing. At 45°C the lipase activity was 0.7 U/ml. The lipase activity was in the range of 0.7-1.9 U/ml.

Effect of pH
The lipase production is very sensitive to pH of the fermentation medium. The effect of pH on the production of extracellular enzyme production was also investigated. The range of pH of cultural medium was from 5–9, and it was concluded that at pH 5 the lipase activity was 0.2 U/ml but as the pH increased up to 8 the activity increased, i.e., up to 1.9 U/ml.

Effect of carbon source
Lipase production was determined using different seed residues like mustard oil cake, wheat barn, coconut oil cake and also nutrient broth as the carbon sources. The maximum lipase production (1.8 U/ml) was noted when mustard oil cake was used while nutrient broth gave 1.3 U/ml.

Effect of nitrogen source
Nitrogen plays an important role in the enzyme production. Many nitrogen sources were used to determine the lipase production, i.e., beef extract, malt extract, peptone, ammonium chloride, yeast extract and ammonium sulfate. Among these sources yeast extract gave the maximum lipase production while ammonium sulfate diminished the production of lipase.

Effects of salt concentrations
Salt concentration was also investigated for the lipase production. Different salt concentrations were used ranging from 0.1%-1.5%. It was concluded that as the salt concentration was increased from 0.5%, the lipase production decreased. Therefore, the optimum salt concentration was considered as 0.5% for maximum lipase production.

Effect of substrate
Different vegetable oils were used as the substrates for the lipase activity. Oils like olive oil, mustard oil and coconut oil were used. It was found that olive oil gave the maximum lipase activity of 1.9 U/ml, while mustard oil gave 1.5 U/ml.

Effect of incubation time
Incubation time of the fermentation medium also affected the lipase production. Lipase productivity was investigated on 24, 40, 48 and 72 hours. It was concluded that lipase activity was increased as the time of incubation increased, but at 48 hours lipase activity was maximum, i.e., 1.8 U/ml. At 72 hours the lipase activity was less as compared to that of 48 hours.

DISCUSSION

The maximum lipase production occurs in the late exponential and early stationary phases of bacterial growth. Different time intervals were
taken for the lipase production. Among them maximum lipase production was achieved at 48 hours of incubation. Lipase production was found to increase after 24 hours of incubation, i.e., the early log phase of bacterial growth curve. After 48 hours only the turbidity of the supernatant increased that was because of releasing of the by-product due to the bacterial cell death. Pogaku et al., (2010) reported 48 hours of incubation as the optimum time of incubation for the maximum lipase production.

Lipase production was also observed on different temperatures and pH values of the fermentation medium. The optimum temperature and optimum pH is 37°C and 8 respectively. These conditions resulted in production of more cell mass and in turn more lipase production. It was also observed that lipase production fluctuated on increasing and decreasing the temperature and pH from its optimum. Lipase production was optimized using different carbon sources. Carbon sources, i.e., wheat barn, nutrient broth and different oil cakes like mustard, coconut were investigated. It was found that maximum lipase production was given by mustard oil cake. It was because of the easy penetration into the cake by bacteria for the lipase production. Therefore, more the oil cake used by bacteria the more lipase synthesis will take place.

Different natural oils were used as the substrate for investigating the lipase activity. Since olive oil and coconut oil contains more number of carbon atoms, they gave better lipase activity than that of mustard oil. Olive oil was considered the ideal substrate because of its already homogenized form and fast conversion into the fatty acids and glycerol by lipase enzyme (Pimentel et al., 2004).

Different nitrogen sources showed varying effects on the lipase production. Organic nitrogen sources gave greater production of lipase as compared to that of inorganic nitrogen sources. The maximum lipase production was observed when yeast extract was used as the nitrogen source while NH$_4$ (SO$_4$)$_2$ gave the least lipase production. Kanimozi et al. 2011 reported the same results using Bacillus species.

The enzyme was partially characterized with reference to the temperature and pH. It was concluded that the enzyme was thermo-stable. Lipase activity was observed up to 60°C of enzyme incubation but maximum lipase activity was observed at 55°C. Lipase activity was also perceived on different pH range of enzyme, i.e., pH 7, 7.5, 8, 8.5 and 9. The maximum lipase activity was observed on pH 8.5 which showed its alkaline nature. For maximum units of enzyme protein estimation was also measured by using common Bradford method.

Two different media were also observed for the lipase production, i.e., minimal fermenting media and enriched fermenting media. It was observed that the enriched fermenting media gave the maximum lipase production (20 U/ml) as compared to that of the minimal fermenting media (2.91 U/ml). Enriched fermenting media support maximum bacterial growth as compared to that of minimal fermenting media. Therefore, more the bacterial growth more will be the enzyme production. After characterization the specific lipase activity was 66.66 U/mg.

CONCLUSION

Physiological changes have a significant influence on the lipase production and also on its activity. By applying optimum conditions to the medium and the organisms, the lipase production and the lipase activity can be achieved at maximum rate. In this study it was observed that mustard oil cake used as the carbon source, olive oil as the substrate yeast extract with pH 8 and the temperature of 37°C were the optimum conditions for the maximum lipase production. The final lipase activity using all optimum conditions was raised from 1.9 to 2.91 U/ml. The protein estimation and characterization of enzyme gave the maximum lipase units (66.66 U/mg).

REFERENCES


Fermentation condition: - “media: nutrient agar; incubation temperature: 37 °C; incubation period: 24h”

“Fig., 1: Screening of bacteria from different soil sources for its lipolytic activity

“All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean”.

“Fig., 2: Effect of temperature on lipase activity

“All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean”.

Fermentation condition: “media: nutrient agar; incubation period: 24h; pH: 8”

Fig., 3: Effect of incubation period on lipase activity

“All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean”.

Fermentation condition: “media: nutrient agar; incubation temperature: 37 °C; pH: 8”

Fig., 4: Effect of pH on lipase activity

“All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean”.

Fermentation condition: “incubation period: 48h; incubation temperature: 37 °C; pH: 8”

Fig., 5: Effect of carbon sources on lipase activity

“All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean”.

Fermentation condition: “incubation period: 48h; incubation temperature: 37 °C; pH: 8”

Fig., 6: Effect of nitrogen source on lipase activity

“All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean”.

Fermentation condition: “incubation period: 48h; incubation temperature: 37 °C; pH: 8”
Fig. 7: Effects of salt concentrations on lipase activity

“All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean”.

Fermentation condition: - “incubation period: 48 h; incubation temperature: 37 °C; pH: 8”

Fig. 8: Effect of substrate on lipase activity

“All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean”.

Fermentation condition: - “incubation period: 48 h; incubation temperature: 37 °C; pH: 8”
Some new remains of Bovini from the Pinjor Formation Khural Sharif (Pleistocene) of the Upper Siwaliks, Pakistan

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ABSTRACT

Fossil remains of Bovini comprising upper and lower dentitions are recovered from the Soan Formation (Pleistocene outcrops) nearby the Khural Shrif Village, Jhelum district, Punjab, Pakistan. The outcrops belong to the Pinjor stage of the Upper Siwaliks. Morpho-metrically, the studied sample quite resembles to Proamphibos (Mammalia, Artiodactyla, Bovidae). The early bovine is widespread in the Pleistocene sediments of the sub-continental Siwaliks and the fossil material is discovered from the sediments belonging to the deposits of 2.58 to 0.6 Ma in age. The detail description of Proamphibos kashmiricus is given in this paper.

Key words: Dentition, Soan Formation, Bovidae, Fossils, Siwaliks.

INTRODUCTION

The material comes from the Khural Sharif fossil site belonging to the upper Soan Formation (Pinjor) of the Upper Siwaliks. The Khural Sharif fossil site (33° 05' 247" N, 73° 34' 826" E) is situated about 10 km NE to Dina town and 25 km W of Jhelum city in the Jhelum district, Punjab, Pakistan (Fig. 1). The Formation ranging in age from 2.58 to 0.6 Ma has a youngest fauna of the Siwalik Group, and after 0.6 Ma there is no record of this fauna from the foothills of Himalaya (Ghaffar et al., 2012). The Pinjor mammalian fauna marks the end of the record of the Siwalik vertebrate faunal succession since the overlying Lei Boulder Conglomerates, the youngest formation of the Siwalik Group, is devoid of fossils. Nanda (2002, 2008) has noted that Pinjor fauna, on species level, shows gradual extinction and not the massive migration. However, the upper Soan mammalian fauna is very rich and diversified in Pleistocene (Table 1). The outcrops consist of brown to grayish brown, fine / medium, and coarse-grained sandstones, multistoried sandstones, pebbly sandstones, pedogenic and non-pedogenic over bank facies probably deposited by high gradient low sinuosity stream (Shah, 2009). The Boulder Conglomerates are transitional; alterations of mudstones, sandstones and conglomerates transitionally pass into thick and massive Boulder Conglomerate. A braided river channel and proximal alluvial fan depositional environment was suggested for the upper Soan Formation (Kumaravel et al., 2005; Shah, 2009).

More recently, Ghaffar et al. (2012) discovered a cervid antler from this site. The bovid sample is also collected with the antler from the same site but they are unpublished yet. In the present paper, the new bovine remains are described and discussed. Nevertheless, the site was not visited by the previous researchers (e.g. Pilgrim, 1910, 1913, 1937, 1939; Lydekker, 1876, 1884; Brown, 1926, Colbert, 1935).
Fig. 1: Map of the studied section showing the fossil locality.

Table 1: The mammalian fauna of the upper Soan Formation (Pilgrim, 1910, 1913; Keller et al., 1978; Barry et al., 1982; Hussain et al., 1992, Nanda, 2002, 2008; Ghaffar et al., 2012).

<table>
<thead>
<tr>
<th>Class</th>
<th>Species</th>
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<tbody>
<tr>
<td>Primates</td>
<td>Procynocephalus pinjorii (?)Homo erectus</td>
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<tr>
<td>Rodentia</td>
<td>Rattus sp.</td>
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<tr>
<td></td>
<td>Mus cristata</td>
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<tr>
<td></td>
<td>Cremnomys cf. C. blanfordi</td>
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<tr>
<td></td>
<td>Dilatomys sp.</td>
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<tr>
<td></td>
<td>Tatera pinjoricus</td>
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<td></td>
<td>Rhizomys pinjorensis</td>
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<td></td>
<td>Hystrix leucurus</td>
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<td>M. flynni</td>
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<td>Golunda sp.</td>
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<td></td>
<td>Hadromys ieujacobsi</td>
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<td></td>
<td>Bandicata sp.</td>
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<tr>
<td>Carnivora</td>
<td>Canis pinjorensis</td>
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<tr>
<td></td>
<td>Crocuta feline</td>
</tr>
<tr>
<td></td>
<td>Panthera cristata</td>
</tr>
<tr>
<td></td>
<td>Mellivora sivalensis</td>
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<tr>
<td></td>
<td>C. colvini</td>
</tr>
<tr>
<td>Proboscidea</td>
<td>Pentalophodon sivalensis</td>
</tr>
<tr>
<td></td>
<td>Elaphas hysudricus</td>
</tr>
<tr>
<td></td>
<td>Stegolophodon stegodontoides</td>
</tr>
<tr>
<td></td>
<td>E. platycephalus</td>
</tr>
<tr>
<td>Lagomorpha</td>
<td>Caprolagus sp.</td>
</tr>
</tbody>
</table>
Equidae
   Equus sivalensis
   (?) E. namadicus

Rhinocerotidae
   Coelodonta platyrhinus
   Rhinoceros palaeindicus
   R. sivalensis

Suidae
   Potamochoerus theobaldi
   Sus falconeri
   Hippohyus sivalensis
   S. choprai

Anthracotheriidae
   Merycopotamus dissimilis

Cervidae
   Rucervus simplicdens
   Axis punjabiensis

Giraffidae
   Sivatherium giganteum

Bovidae
   Sivacapra subhimalayaensis
   Sivacobus palaeindicus
   Hemibos acuticornis
   H. triquetricornis
   H. antilopinus
   Babalus palaeindicus
   B. platyceros
   Leptobos falconeri
   Bison sivalensis
   Bos acutifrons
   Proamphibos lychrimans
   P. kashmiricus

MATERIALS AND METHODS

The specimens were recovered from the sediments of the upper Soan Formation (Upper Siwaliks), Pakistan. Piercing instruments like chisels and geological hammers were employed for the excavation of the partially embedded fossils. Careful measures were taken so as to prevent the fossils from disintegrating during excavation. Some fossils were exposed and easily available for the collection. Each specimen was wrapped with a cotton piece to avoid the shocks of the transportation. Eventually the collected specimens were brought in the laboratory for the taxonomic and morphological analysis.

In order to remove dust particles and prepare the specimens for the clear observation, the specimens were carefully washed and cleaned in the Palaeontology Laboratory of the Zoology Department, GC University Faisalabad. The clay and other hardly adjoined sedimentary particles were removed with the help of fine needles and brushes. Accidentally broken fragment of the specimens were rejoined by using gums and resins such as Magic, Elfy and Fixings. A hand lens was used for keen observation of very small and ambiguous morphological character.

All the specimens were carefully observed for the description of morphological characters along with discussion of their systematic determination. Measurements were taken with the help of a vernier caliper and expressed in millimeters (mm). Upper case letter is used for upper teeth and lower case letter for lower teeth. The catalogue number of the specimens consist of yearly and a serially catalogue numbers, so the number on the specimen represents the collection year (numerator) and the serial number (denominator) of that year. The terminology of the tooth crown elements and manners of measurements follow Gentry et al. (1999) and Gentry & Hooker (1988).

SYSTEMATIC PALAEONTOLOGY

Order Artiodactyla Owen, 1848
Suborder Ruminantia Scopoli, 1777
Infraorder Pecora Linnaeus, 1758
Superfamily Bovidae Simpson, 1931
Family Bovinae Gray, 1821
Subfamily Bovinae Gray, 1821
Tribe Bovini Simpson, 1945
Genus Proamphibos Pilgrim, 1939

Type species: Proamphibos kashmiricus Pilgrim, 1939.
Generic diagnosis: Upper molars, extremely hypsodont, longer than broad, enamel moderately thick and rugose, with traces of cement, basal pillars well developed and complicated, extended transversely, styles and ribs very strong, central cavities wide and deep, not complicated in outline; upper premolars not reduced P4 is shorter than P3, lower molars slender without goat fold, stylids and ribs are strong, m3 is with moderately short and slender talonid (Pilgrim, 1939).

Distribution: Proamphibos is an Asiatic genus and is recorded only from the Upper Siwaliks of Pakistan, India and Nepal (Pilgrim, 1939; Khan et al., 2009; Khan & Akhtar, 2011).

Proamphibos cf. kashmiricus Pilgrim, 1939
(Fig. 2)

New material: PC-GCUF 10/20 - left maxilla fragment with M1-M3 and unerupted P4, PC-GCUF 10/20 - an isolated right lower m2, PC-GCUF 10/19 - an isolated right lower m3.

Locality: Khural Sharif Village (upper Soan Formation, Upper Siwaliks), Jhelum district, the Punjab province, Pakistan.

DESCRIPTION

Upper dientition: The upper dentition includes a fragment of right maxilla with molar series (Fig. 2 (1)). The fourth premolar is an unerupted tooth but visible due to the fragile condition of the fragment. The premolar crown height is lower than the molars. The first molar is in an early stage of wear. The entostyle is strongly developed. The parastyle and the metastyle are broken. The metacone is broken at the apex. The mesostyle is well developed. The posterior median rib is more developed than the anterior one. The 2\textsuperscript{nd} molar show the morphological features of large size bovids. The paracone and the metacone are higher than the protocone and the hypocone. The molar is furnished by the vertical grooves labially. The mesostyle is prominent but the parastyle is damaged and the metastyle is missing. The posterior and the anterior median ribs are prominent. The 3\textsuperscript{rd} molar is unworn tooth. The ribs and styles are heavy as in the 2\textsuperscript{nd} molar. The cementation is present in the molars.

Lower dientition: The lower dentition comprises only second and third molars (Fig. 2(2-3). The 2\textsuperscript{nd} molar is in early wear and the anterior transverse flange is present. The ectostylid is well developed. The entostylid is moved outwardly. The median ribs are strong. The enamel is rugose. A cingulum tubercle is present lingually in the vertical furrow. The preprotocristid is longer than the postprotocristid. The conids are sharp and crescentic. The 3\textsuperscript{rd} molar is unworn and the major conids are pointed and equal in height. The premetacristid is larger than the postmetacristid. The postmetacristid is bifurcated into two parts. The pre fossette is less deep than the post fossette. The post fossette is narrow and opens lingually resulting the entoconid is completely separated from the hypoconid. The hypoconulid is ovate in the 3\textsuperscript{rd} molar. The transverse flange is present anteriorly.

DISCUSSION AND ANALYSIS

The large size, crescentic cones/conids, boodontia occlusal pattern, hypsodonty, strong entostyles/ectostylids, extraordinary strong ribs and strong styles/stylids of the specimens associate them to Bovini (Pilgrim, 1939; Gentry, 1978). The presence of the cement, the enlargement of the entostyles/ectostylids and excessive antero-posterior compression are the major characteristics of the bovine. The Siwalik bovines are represented by Hemibos, Bison, Bos, Bubalis and Proamphibos (Pilgrim, 1939). Morphometrically, the specimens resembles with the genus Proamphibos (Pilgrim, 1939). Proamphibos differs from Hemibos in having thin enamel, delicate labial folds, and weak and wide ribs (Pilgrim, 1939). The studied specimens are exactly similar with already described specimens of P. kashmiricus as well as with the type specimen (Table 2). Nevertheless the sample is insufficient for the exact specific determination and it can be assigned to Proamphibos cf. kashmiricus.
Table 2: Comparative measurements (mm) of the studied cheek teeth of *Proamphibos*. Referred data are taken from Pilgrim (1937, 1939), Akhtar (1992), Khan *et al.* (2009).

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Number</th>
<th>Nature/Position</th>
<th>Length</th>
<th>Width</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. cf. kashmiricus</em></td>
<td>PC-GCUF 10/21*</td>
<td>IM1</td>
<td>31.0</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IM2</td>
<td>34.0</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IM3</td>
<td>32.0</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>PC-GCUF 10/20*</td>
<td>rm2</td>
<td>35.3</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>PC-GCUF 10/19*</td>
<td>rm3</td>
<td>40.0</td>
<td>15.0</td>
</tr>
<tr>
<td><em>P. kashmiricus</em></td>
<td>PUPC 84/27</td>
<td>rM1</td>
<td>27.0</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rM2</td>
<td>32.0</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rM3</td>
<td>31.0</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>GSI B561</td>
<td>M1</td>
<td>26.0</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2</td>
<td>31.0</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M3</td>
<td>32.0</td>
<td>24.0</td>
</tr>
<tr>
<td><em>P. sp.</em></td>
<td>PUPC 69/641</td>
<td>IM1</td>
<td>24.0</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IM2</td>
<td>30.0</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IM3</td>
<td>32.0</td>
<td>29.0</td>
</tr>
<tr>
<td><em>P. lachrymans</em></td>
<td>PUPC 68/79</td>
<td>m1</td>
<td>25.0</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m2</td>
<td>28.0</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m3</td>
<td>42.0</td>
<td>16.0</td>
</tr>
<tr>
<td><em>P. dhokawanesis</em></td>
<td>PUPC 69/351</td>
<td>m1</td>
<td>29.0</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m2</td>
<td>35.0</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m3</td>
<td>39.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

The genus *Proamphibos* is known from the Upper Siwalik deposits of the Subcontinent (Pilgrim, 1939), having two valid species of the genus, *P. lachrymans* and *P. kashmiricus* in the Upper Siwaliks. The validity of the genus was doubtful which was confirmed by Geraads (1992). *Proamphibos* (early Soan Formation, Early and Middle Pliocene) follows evolutionary lineage leading in the direction of *Hemibos* which is found in the Plio-Pleistocene of Eurasia and its expansion into Europe might have occurred in earlier times during Pliocene as Lindsay *et al.* (2005) mentioned three major dispersal events of large mammals during the Pliocene. From Pakistan, it is reported from early Soan Formation (Tatrot), Khural Sharif, Dhok Awan and Sardhok of the district Jhelum, Punjab and in India, it is reported from Jammu state as well as from Burma (Pilgrim, 1939).

Bovini were likely present in Southern Asia (Siwaliks) by 9 Ma (Bibi, 2007). After this time, early bovine appeared in Africa and then Europe. The earliest African record of a bovine is at Toros-Menalla, an Upper Miocene locality (Vignaud *et al.*, 2002). The earliest European record of a bovine is in the early Pliocene (Gromoiard, 1980). The water buffalo *Bubalus* most likely emerged from the Pliocene *Proamphibos* (Pilgrim, 1939).

**CONCLUSIONS**

*Proamphibos* was found in the Upper Siwalik sediments of Khural Sharif village with cervids and other bovines. The fresh water outcrops of the Khural Sharif village belong to the upper Soan Formation (Pleistocene) in age. The Khural Sharif village outcrops comprise sand of Pleistocene type as well as variegated clays. The fauna associated with the bovine consists of large mammals such as *Elaphus hysudricus*, *E. platycepalus*, *Anancus sivalensis*, *Coelodonta platyrhinus*, *Rhinoceros palaeindicus*, *R. sivalensis*, *Sus falconeri*, *Hemibos acuticornis*, *H. triquetricornis*, *Babalus palaeindicus*, *B. platyceros*, *Leptobos falconeri* and *Bos acutifrons* that attests to a rather open grassland environment and accompanying forested areas.
Fig. 2: Proamphibos cf. kashmiricus. 1. PC-GCUF 10/21 – a maxillary fragment with M1-M3 and unerupted 4th premolar. 2. PC-GCUF 10/20 – an isolated right lower second molar (m2). 3. PC-GCUF 10/20 – an isolated right lower third molar (m3). a = occlusal view, b = lingual view, c = labial view. Scale bar 10 mm.

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Assessment of Fertility Status of Soil of Botanic Garden, GC University, Lahore

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ABSTRACT

The survey was conducted to evaluate the fertility status of soil of GCU Botanic Garden Lahore. Eleven random sites were selected. Six sites were selected under tree vegetation, three were grass lawns and two were wild areas. Soil samples were collected from 6 different depths (0-150 cm) in each site and their morphological characters like color, calcareousness, presence and abundance of artifacts, lime nodules and roots were determined in the field. Soluble ions like carbonates, bicarbonates, chlorides, sulphates, calcium plus magnesium and sodium were determined in the laboratory. To evaluate the fertility of soil, % calcium carbonate, soil texture, % organic matter, nitrogen, phosphorus, potassium, pH and EC were also determined. The soil of Botanic Garden had dark brown color and uniform texture. The texture was silt loam and soil was moderately calcareous and slightly alkaline. The % of calcium carbonate ranged from 2.45-10.45. Organic matter was found in higher proportion in all the sites except the wild area. Amount of available phosphorus varied from 6.5 ppm to 27.5 ppm. Amount of available potassium varied from 80 ppm to 270 ppm. The pH of soil samples varied from 7.55-8.54 and the EC was 0.625-1.98 dS/m. The uniform texture, brown color and presence of lime indicated that the soil of Botanic Garden was of old river terrace of Indus Plain (Niaz Baig Soil). The analyses showed that the soil of GCU Botanic Garden contained high amount of available potassium and medium to low amount of phosphorus. Amount of Organic matter in soil was good. All the parameters show that soil of Botanic Garden is a fertile soil in which sufficient amount of nutrients are present which are essential for the plant growth.

Key words: Fertile soil, plant growth, nutrients, nitrogen, phosphorus, potassium

INTRODUCTION

Fertility of soil can be defined by physical and chemical parameters of soil. Soil fertility is the ability of soil to give and retain nutrients for plant growth. The soils which are fertile have balanced and adequate elemental supply which is necessary to fulfill plant needs (Foth & Ellis, 1997). The soil which contains a great amount of debris of previous vegetation and have uniform texture and adapt itself to fulfill the requirements of water and air for plants, is called as fertile soil. In such type of soils, a great amount of organic matter is also present in all depths. The nutrients present in soil are available to plants only in small amount. Most of the nutrients present in soil are in the form of organic matter and minerals, so they are not available to plants until they are decomposed. Plants absorb elements in ionic form from soil. Available nutrients may also be present as dissolved ions in soil water, which are most readily available form of plant nutrients. Out of sixteen elements present in soil, thirteen are originated from parent rock material, from which soil develops. These minerals are chemical constituents for the soils in which they are present. Soil concentration of these nutrients and conditions that make them available to plants is of greater importance to growth of plants. Plants take hydrogen, oxygen and carbon from water and air while the soil contains macro and micro nutrients (Miller and Donahue, 1990). Vegetation type, species age, composition density and structure of plant species affect the soil (Zhang et al., 1986).

Unique public green spaces are called as Botanical Gardens. According to Botanical Garden Conservation International (BGCI), Botanical Gardens are the institutions which contain documented living plant collection for display, conservation, scientific research and educational purposes (Wyse Jackson and Sutherland, 2000). Rare plant species are cultivated for conservation purposes in Botanical Gardens (Dosmann, 2006). Botanical Gardens have promoted wild populations of plants (Falk et al., 1996). Botanical Gardens can be appreciated for recreational benefits and physiological restoration, some visitors can have educational experiences and can see rare or unusual flora (Connell, 2004; Ballantyne et al., 2008). Botanical Gardens maintain community
identity and local traditions (Kuzevanov and Sizykh, 2006). Botanical Garden management and staff have vital role in conservation and education (Miller et al., 2003; Pinheiro et al., 2006; Ballantyne et al., 2008).

Botanical Garden G C University Lahore (BGGCU) is located adjacent to Lahore Zoo at Shahra-e-Quaid-e-Azam (The Mall). In 1860 it became a park and a part of Lawrence Garden (Bagh-e-Jinna) which is now adjacent to it. The management of BGGCU is under Department of Botany Government College University, Lahore since 1912.

Very little record is available about BGGCU before 1947. From 1947 and onwards garden was used for instructions and materials for students and researchers of GCU and Punjab University. In mid sixties PU created a new Botanical garden, so BGGCU came under supervision of GCU, Lahore. The area of BGGCU is 7 acres. The main theme of the garden is that of an arboretum i.e. collection of trees and shrubs. The style of garden is just like a typical botanic garden of nineteenth century. The overall themes are that, the trees and shrubs are arranged in such a way that each genus was brought in a given area so that comparison between the trees can be made and their characteristics can easily be estimated. The paths in the garden are named after famous taxonomists as the theme is taxonomic. The soil of BGGCU was first irrigated by Lahore Canal but when supply was cut off, a tube well was installed in early 1970s, which irrigates the whole botanical garden.

The objectives of the study were
- to check the physical and chemical parameters of soil,
- to assess the fertility status of soil and
- to check the effects of different type of vegetations on fertility status of soil of Botanic Garden

MATERIALS AND METHODS

The present study was conducted in Botanic Garden GC University, Lahore. For this survey eleven sampling sites were selected randomly from different plots of Botanic Garden GC University Lahore (BGGCU). Six sites were selected under old trees i.e. Kigelia pinnata DC., Family Bignoniaceae (Plot No.19) , Mimusops elangi L., Family Sapotaceae (Plot No.19), Alstonia scholaris L., Family Apocynaceae (Plot No.19), Olea cuspidata L., Family Oleaceae (Plot No.10) , Mangifera indica L., Family Anacardaceae (Plot No.11) , Bischofia javanica B. j Bl., Family Euphorbiaceae ( Plot NO.12). Three sites were selected from grass lawns i.e. Plot No. 5, 6 and 9 and two sites were selected from bare land. The map of Botanic Garden GCU indicating different sites is given in Fig.1

![Fig. 1: Layout containing plot numbers of GCU Botanic Garden](image)

Soil sampling was carried out at different depths by using auger. The diameter of auger was 7 cm. Auger was marked with cm. It was rotated manually in the soil and when it was filled with soil it was taken out. The samples were collected from different depths by repeating the process again and again. The depths were 0 - 15, 15-30, 30-60, 60-90, 90-120 and 120-150 cm from each site. The samples were preserved in plastic bags and labeled. After collecting each sample, their morphological characters were observed. Soil color, texture, pH, calcareousness, presence of artifacts, roots and lime nodules and their sizes and abundance were examined. Soil texture was checked directly in the field by feel method. It was checked by crushing and rolling of dry soil samples between fingers. Then it was wetted with water and moist sample was crushed and rolled between the fingers and paste was formed. Soil color was noticed from Munsell soil chart which contained 196 color chips. Soil pH was noted by using thymal blue indicator and from pH color chart. Calcareousness was noted with 10% HCl. Samples were then taken to the laboratory for further analyses. The procedure for soil analyses (Organic matter, pH, EC, Chlorides, Calcium & Magnesium, Sulphates, Carbonates, Bicarbonates and Calcium Carbonate)
were adopted from Technical Guide for Chemical Analysis of Soil and Water revised by Khan & Rafiq, (1980). The procedure for soil texture was followed from Guidelines for Soil Texture Determination written by Ali & Rafiq, (1978). Phosphorus was determined by colorimetric method. Sodium and potassium were determined by flame photometer (AFP 100).

RESULTS AND DISCUSSION

The importance of soil fertility and plant nutrition to the health and survival of all life cannot be understated. With increase in human population, the rever increasing load on earth’s ecosystem to produce food and fiber greatly rely on soil to supply essential nutrients. Therefore it is critical that we increase our understandings of physical, chemical and biological properties and relationship in the soil-plant-atmosphere continuum that control nutrient availability (Havlin et al., 2003).

Fig., 2: Percentage of sand, silt and clay in various soil sampling sites

Soil Texture

The results show that the percentage of sand of all the soils ranges between 16-23 except in wild areas where it is 43% and 37% in the surface area. Percentage of silt in all the sites is more than 50 and percentage of clay varies between 10 to 30. The percentages of sand, silt and clay show that the soil is silt loam in texture. From fertility point of view silt loam texture is regarded as very good texture (Fig. 2). Shetty et al., (2008) also conducted a study on fertility status of garden of Karnataka and found that the soil of garden was sandy loam to sandy clay loam. Sharma et al., (2008) assessed the soil of Amritsar District and soil texture was found as silt loam in that area. Predominance of silt percentage in all the soils indicates the uniformity of the soil material which is silt loam in all the profiles. Furthermore dark yellowish brown (10YR 4/4) and yellowish brown (10YR5/4) dominant colors in all profiles also indicate uniformity of the soil scattered material. The typical dark color of many soils is caused by organic matter and may facilitate warming. Lime nodules are also present in all the profiles. Silt loam texture, yellowish brown color and presence of scattered lime nodules suggest that the soils under study pertain to old river terrace of the Indus Plain (Ali et al., 1968).

pH and Electrical Conductivity

As the texture of the soil under study is uniformly silt loam and uniformly moderately calcareous, the pH and electrical conductivity of the soils would largely depend upon leaching regime of the soils. The leaching regime of the soils under study may vary according to the vegetation on them. pH influences ion activities that affects nutrient availability or element toxicity like manganese and aluminium. Availability of nitrogen is greater between pH 6 and 8 because nitrogen mineralization is maximum in this range. In acid soils availability of soil nutrients and plant uptake of phosphorus are reduced due to precipitation and aluminium adsorption. In calcareous soils the solubility of calcium phosphorus compounds is reduced due to adsorption of calcium carbonate on surfaces or increase in activity of Ca²⁺. These examples show that nutrient availability is highly affected by pH (Foth & Ellis, 1997).

Fig., 3: pH and EC of various soil sampling sites

The pH of the soils, on the whole, varies from 7.55 to 8.54. This means that the soils are slightly to moderately alkaline (Fig. 3). A general trend of higher pH is found under grass vegetation. Sharma et al., (2008) found that soils are neutral to alkaline which is similar to the results in this study. Electrical conductivity of the soil samples varies from 0.625 to 1.98dS/m which falls in slightly alkaline range (Fig. 3). However there is an
increasing trend in the values of electrical conductivity down the profiles in all the sites. Surface soils in all the sites have values of electrical conductivity less than 1 except one which is 1.715 dS/m, thus the surface soil is non saline which is very good from fertility point of view. Similar results were also shown by Sharma et al., (2008).

**Soluble Ions**

Soluble ions are used only to calculate the SAR and ESP values of saline sodic soils and their gypsum requirement for reclamation. As the values of pH are less than 8.5 and the values of electrical conductivity are less than 4 dS/m, of the samples of soil under study, so the soil is non saline and non sodic. That’s why the soluble ions are not important for the soil under study.

**Organic Matter, Percentage Nitrogen and Calcium Carbonate**

Soil productive capacity or its potential can be influenced by many interrelated factors that are present in soil. These properties of soil are collectively called as soil quality. Most critical property among these is organic matter (OM) because of its influence on many chemical, biological and physical characteristics in a productive soil (Foth & Ellis, 1997; Havlin et al., 2003).

Soil organic matter is very important soil component. It is the source of 90 to 95% of nitrogen in unfertilized soils. Percentage of Nitrogen in the surface soils under study varies from 0.02 to 1.5 (Fig. 5). It can be a major source of both available phosphorus and available sulphur when it is more than 2%. The soils that are derived from material which is transported, having fine grained and uniform texture, contains particles of coarser order than clay so that water can easily move by capillary action and can freely traverse by water and air, are the soils which show greatest fertility. In such type of soils, a great amount of organic matter is present, in all depths and it helps to maintain a uniform soil pH and increases soil CEC from 20-70% of CEC of many soils. Total average water is lost by leaching (Foth & Ellis, 1997; Havlin et al., 2003).

Soils of Indus Plains, of which the study area is a part, generally have organic matter less than 1% on the surface layer due to hot, arid/semi-arid climate which does not favor its accumulation on the surface. According to the results organic matter contents of soil surface layer are more than 1% except in one site in wild area (Fig. 4). As the soil under study has percentage of organic matter more than 1 in surface layer so the soil is satisfactory to good according to Pakistani standards.

If we view the accumulation of organic matter in upper 30 cm of soil under study, there is a definite higher proportion of organic matter in the soils under grass vegetation than those under tree vegetation or wild area. This is due to the fact that more organic matter accumulates under grasses than under trees and wild areas over time. This fact is in perfect agreement with general concepts of organic matter production in the soils. Grasses have greater biomass below ground than above ground so they humify the soil to greater depths (Foth & Ellis, 1997; Havlin et al., 2003).

The soils of Indus Plains are predominantly calcareous because of their calcareous parent materials and arid climate in most of the areas. Presence of free lime is mainly responsible for alkaline reactions in the normal soils which restrict the availability of some macro and many micro nutrients to the plants. Percentage of calcium carbonate of soils under study varies from 2.45 to 10.45 which falls under category of moderately calcareous (Fig. 4). The amount of lime is relatively less in soils under trees followed by soils under...
grasslands and maximum in wild areas. This may attribute to more effective leaching under the tree canopies and less effective leaching under grasses and wild areas.

Sharma et al., (2008) found low amount of calcium carbonate. The values of calcium carbonate in the soils of Botanic Garden are relatively high than the normal soils on the old river terrace in Lahore area. According to Soil Survey investigations (Ali et al., 1968), such soils correspond to the eroded edges of the old river terrace (Niaz-Baig Soil Series).

Available Potassium

The amount of available potassium in the surface soils under study varies from 80ppm to 270ppm (Fig. 7) which is in the range of fertile soil according to standards of Pakistan. The amount of available potassium in the soils of Indus Plains is generally sufficient except where they are intensively cultivated and are less fertilized. Soils of Indus Plains, being younger, are rich in weatherable minerals particularly mica which are a constant source of available potassium in soil conditions.

In the soils, under study, since no crop is removed so they favor accumulation of nutrients than their depletion. Similar results were found by Shetty et al., (2008) and found medium to high value of potassium in the soils of garden. High value of potassium was also observed by Sharma et al., (2008) in soil of Amritsar. Benjaminsen et al., (2010) also concluded that fertility of soil is highest in areas that are not cultivated.

CONCLUSION

As the soil of GCU Botanic Garden has percentage of silt more than 50, the texture of soil is silt loam. Due to uniform texture, yellowish brown color and presence of lime indicates that soil of botanic garden is of old river terrace of Indus Plain (Niaz Baig Soil). Soil of botanic garden is moderately calcareous and slightly alkaline. Available potassium is high in the soil while amount of phosphorus is medium to low. The amount of organic matter present in soil is good. All these parameters show that soil of botanic garden is a fertile soil. Sufficient amount of nutrients is present in the soil of botanic garden. As the age of botanic garden is more than 100 years, the vegetation has a good influence on the fertility of soil. One of the greatest challenges of our generation is to develop and implement soil, crop and nutrient management technologies that enhance plant productivity and the quality of soil, water and air.

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Parasitic Infection of an Ornamental fish, Shubunkin Carassius auratus L. imported to Pakistan

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ABSTRACT

The present study was aimed to investigate parasitic infection of an imported ornamental fish shubunkin, a variety of goldfish, Carassius auratus. The body weight and total length of fish ranged from 1.2-11.8 g and 3.3-11.6 cm respectively. Thirty fishes were examined, 19 fishes were found infected with one or more species of parasites. The prevalence was 63.33%. Five species of parasites belonging to three groups were observed from the fish and identified. These parasites were; monogenean, Dactylogyrus extensus. Gyrodactylus sp., -protoazon-, Trichodina sp. Ichthyophthirius multifiliis, crustacean parasite, Argulus foliaceus. The fishes were heavily infected with D. extensus (prevalence 63.3%, mean intensity 22.31). There was significant difference in the infection of left and right gill by D. extensus (χ²=4.28; p = 0.05). The secondary lamellae were severely damaged due to high infection by D. extensus. Gyrodactylus sp. prevalence was 46.6% and mean intensity 27.07. There was significant difference in the infection of skin and fins by Gyrodactylus sp. (χ²=5.29; p = 0.05). The tips of caudal fins were eroded. The infection of Trichodina sp., on gills as well as fins while Ichthyophthirius multifiliis on gills was 16.6% and 20% respectively. Argulus foliaceus had very low prevalence (6.66%) and mean intensity 1.0. Dactylogyrus extensus is found to be the most pathogenic parasite affecting shubunkin compared to other parasite species. These parasites may have escaped detection while sending fish consignment to Pakistan. May be the presence of developmental stages of the parasites has been ignored by the consignment dispatchers. The present situation points our attention to one point that the import of ornamental fishes must be done under strict regulations. The imported fishes must be checked at landing sites to stop introduction of pathogenic parasites into Pakistan.

Key words: Shubunkin, imported fish, parasitic fauna, gills, fin infection.

INTRODUCTION

Ornamental fish keeping is a popular hobby worldwide. The ornamental fish trade is a multimillion dollar industry. The world export of ornamental fish was at its peak amounting to 282.6 million US$ in 2006 (FAO, 2007). Goldfish and some of its varieties such as; Telescope eye, Lion head, Celestial, Comet, Fantail, Veiltail, Shubunkin, Bubble eye, Pearl scale, Red cap Oranda and Black moor (Ahilan et al., 2009) are the most popular ornamental fish, kept as pet world over. It was reported that 20 species of ornamental fishes are imported live into Pakistan from Southeast Asian countries (Ahmed, 1996) and sold at the pet shops as disease free fishes to the hobbyists. The occurrence of parasites on ornamental fishes and their transport to other countries has been reported worldwide: Australia (Evans and Lester, 2001), Korea (Kim et al., 2002), Sri Lanka (Thilakaratne et al, 2003) and Brazil (Pizza et al., 2005; Tavares-Dias et al., 2010). The most common parasites of freshwater fishes are two types of monogeneans which are typically parasites of gills and skin and are generally host specific. Dactylogyrids are gill parasites while gyrodactylids live on skin and fins. Some ectoparasites of freshwater fishes are species of protozoans such as: Ichthyophthirius multifiliis and Trichodina sp. Lernaea cyprincea, a copepod parasite is common on freshwater fishes and is distributed in Asia and Africa (Bond, 2004). Argulus foliaceus, a crustacean parasite has been reported to parasitize many freshwater fishes (Rasouli et al., 2012). The present study was aimed to investigate parasitic infection of an imported freshwater ornamental fish shubunkin, a variety of goldfish and identify various parasites.

MATERIALS AND METHODS

The experimental fish was obtained from pet shop in Lahore from June to October 2012. Fishes were brought live in sterilized polyethylene bag and kept in aquarium in aerated water in Fish Disease and Health Management Lab. The fish was examined
within 2 hours of arrival in the Lab. The fishes were weighed, measured and examined by standard Parasitological methods (Bauer et al., 1973; Kabata, 1985; Roberts, 1989, Noga, 2010). The body, skin, fins, head and gills were thoroughly examined for parasitic infections. The wet mount preparation / biopsy procedure was applied to observe the parasites (Noga, 2010). The site of infection varied for each species of parasite. The prevalence, mean intensity and total number of parasite of each species are given in Table 1.

Table 1: Parasites recorded from various sites of shubunkin, *Carassius auratus*.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parasites</th>
<th>Fish infected</th>
<th>Prevalence(%)</th>
<th>Total parasites</th>
<th>Range</th>
<th>Mean intensity</th>
<th>Site of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gills</td>
</tr>
<tr>
<td>1</td>
<td><em>D. extensus</em></td>
<td>19</td>
<td>63.66</td>
<td>420</td>
<td>1-110</td>
<td>22.10</td>
<td>187+233</td>
</tr>
<tr>
<td>2</td>
<td><em>Gyrodactylus</em> sp.</td>
<td>6</td>
<td>3.33</td>
<td>60</td>
<td>1-35</td>
<td>10.00</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>13.33</td>
<td>319</td>
<td>1-83</td>
<td>39.87</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td><em>Trichdina</em> sp.</td>
<td>1</td>
<td>3.00</td>
<td>3</td>
<td>3-12</td>
<td>7.0</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>9.16</td>
<td>28</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td><em>I. multifiliis</em></td>
<td>6</td>
<td>55</td>
<td>5-13</td>
<td></td>
<td>9.16</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td><em>A. foliaceus</em></td>
<td>2</td>
<td>10.00</td>
<td>2</td>
<td>1.0</td>
<td>1.0</td>
<td>2</td>
</tr>
</tbody>
</table>

The highest prevalence 63.3% was observed for *D. extensus* and mean intensity 22.10. The maximum parasite recorded from one fish was 110. Out of 420 total parasites recovered from gills, 187 were found attached to left side gills and 233 to right side gills in all infected fishes. There was significant difference in infection site in both gills (χ²=4.28; p = 0.05). The pathological changes in the gills caused by *D. extensus* were very prominent and serious. In heavily infected fishes, the gills were damaged, the secondary lamellae were eroded (Fig. 1). In low infection the tips of secondary lamellae were damaged (Fig. 2). In mild and fresh infection there was slight damage to the gills. *Gyrodactylus* sp. infection in shubunkin was high (46.6%) and the mean intensity was the highest (27.07). There were two patterns of infection such as, 1) single site infection on fins only. 2) Concurrent infection of the parasite at two sites at the same time such as, skin and fin. Mean intensity of infection in single site infection was 10.0. In concurrent infection the mean intensity of infection was 39.87. The maximum number of parasites in one fish was 83. *Gyrodactylus* sp. recorded from skin was 95.9% and fins 3.1%.

RESULTS

A total of 30 fishes were examined for parasitic infection. The body weight and total length of the fish ranged from 1.2-11.8 g and 3.3-116 cm. Out of the total 30 fishes, the number of infected fishes varied for each type of parasitic infection (Table 1). Five species of parasites belonging to three groups were observed to infect these fishes. These were: monogenean, *Dactylogyrus* *extensus*, *Gyrodactylus* sp., protozoans, *I. multifiliis*, *Trichdina* sp., Crustacean, *Argulus foliaceus*. The site of infection varied for each species of parasite. The prevalence, mean intensity and total number of parasite of each species are given in Table 1.

![Fig. 1: Severe damage of secondary lamellae due to heavy infection by *D. extensus* in shubunkin](image)
Trichodina sp. was recorded from 5 fishes, with prevalence 16.66% and mean intensity 6.2. Three infection cases were observed from caudal fins (no. of parasites 3, 6, 12), one case from dorsal fin (7 parasites) and one case from gills (3 parasites). The mild Trichodina sp. infection was not pathogenic to fishes as no inflammatory response was noticed on fishes.

Ichthyophthirius multifiliis was found on gills of 6 fishes and prevalence was 20%, with mean intensity 9.16. The parasite number ranged from 5-13. The varied size trophonts were observed from gills. The crescent shaped macronucleus was very clear in mature trophonts. The secondary lamellae were abnormal, swollen at the point of penetration of the parasites (Fig. 5). Ichthyophthirius multifiliis infection on gills of shubunkin showed two pattern of infection. Such as; 1) high infection of Itch versus low or no infection of D. extensus, 2) low infection of Itch versus high infection of D. extensus (Table 2). Argulus foliaceus was recorded from caudal fins of two fishes, prevalence 6.6%, and mean intensity 1.0. The infection was very low and not pathogenic and serious to these fishes.

Table 2: Concurrent infection of I. multifiliis and D. extensus on gills of shubunkin

<table>
<thead>
<tr>
<th>S. No of Fish in sample</th>
<th>D. extensus</th>
<th>I. multifiliis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right gill</td>
<td>Left gill</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>34</td>
<td>28</td>
</tr>
<tr>
<td>22</td>
<td>33</td>
<td>77</td>
</tr>
<tr>
<td>23</td>
<td>22</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>91</td>
<td>135</td>
</tr>
</tbody>
</table>

Fig., 2: Mild damage at the tips of gill filaments due to D. extensus infection in shubunkin

Fig., 3: Gyrodactylus sp. infection on caudal fin of shubunkin, tips of fin are eroded

Fig., 4: Gyrodactylus sp. attachment to the caudal fin of shubunkin. No inflammatory response shown by the fish after

Fig., 5: Concurrent infection of many D. extensus and a single I. multifiliis on gills of shubunkin
The species wise parasitic attack on fish was variable. There were 7 cases of single species infection on a fish; 5 cases were of *Dactylogyrus extensus*, one case of *Gyrodactylus* sp. and one case of Itch. Two parasites species infection on one host was higher (11) including; 8 cases of *Dactylogyrus extensus* and *Gyrodactylus* sp., one case each of *Dactylogyrus extensus* and Itch, *Dactylogyrus extensus* and *Trichodina* sp.

*Gyrodactylus* sp. and Itch. Four parasite species infection was also observed with two combinations; *Dactylogyrus extensus* - *Gyrodactylus* sp.

*Trichodina* sp. - *Argulus foliaceus*; *Dactylogyrus extensus* - *Gyrodactylus* sp. - *Trichodina* sp. - Itch (Table 3). It is very clear that *Dactylogyrus extensus* parasitic load was maximum followed by *Gyrodactylus* sp. and Itch, *Trichodina* sp and *Argulus foliaceus*.

Table 3: Infection of various parasites species per fish in shubunkin (Rg-Right gill, Lg-Left gill).

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parasite sp./fish</th>
<th>No. of Fish</th>
<th><em>Dactylo</em>.</th>
<th><em>Gyrodac</em>.</th>
<th><em>Trico</em>.</th>
<th><em>Argulus</em></th>
<th>Itch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rg.</td>
<td>Lg.</td>
<td>Skin fins</td>
<td>Gill fins</td>
<td>Rg.</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>8</td>
<td>71</td>
<td>82</td>
<td>3</td>
<td>139</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>12</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3</td>
<td>4</td>
<td>1</td>
<td>12</td>
<td>6</td>
<td>1</td>
<td>58</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>88</td>
<td>135</td>
<td>7</td>
<td>145</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>28</td>
<td>2</td>
<td>-</td>
<td>55</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>187</td>
<td>233</td>
<td>366</td>
<td>3</td>
<td>28</td>
<td>2</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the present study shubunkin, a variety of goldfish, was examined for parasitic infection. Three groups of parasites; monogenean, protozoans and crustacean were observed. High prevalence and mean intensity of monogenean; *D. extensus*, and *Gyrodactylus* sp. were observed, which is due to their high reproductive rates. *Dactylogyrus extensus* is oviparous and *Gyrodactylus* sp. is viviparous and their short direct life cycle facilitates them to spread rapidly in fish rearing facilities. The transmission of monogenean has been reported to increase under poor pond management conditions (Woo, 2006, Thilakaratine et al., 2003). *Dactylogyrus extensus* is known as gill fluke and is common parasite on gills of fishes, feed on dermal and gill debris (Post, 1987). The damage caused by *Dactylogyrus* to gill epithelium make opening for secondary bacterial, fungal, protozoan infection on gill surface. Heavy infection result trauma and injuries on gill surface, lamellar hyperplasia, excessive mucus production. The ultimate effects are the impairment of respiration by gills and fish become heavily stressed (Post, 1987). *Gyrodactylus* sp. or skin fluke is less pathogenic compared to *Dactylogyrus* sp. Damage to scales and epithelium may be mild. The fish infected with *Gyrodactylus* sp. may develop white to grey whitish area of thick mucus on skin. Our results are comparable to Tavares-Dias et al., (2010) and Chanda et al., (2011).

*Trichodina* sp. was found with low prevalence. This parasite is neither host nor site specific (Thilakaratine et al., 2003). The high mean intensity of *Trichodina* sp. is associated with its reproduction by binary fission (Ogut & Palm, 2005). Thilakaratine et al., (2003) and Tavares-Dias et al., (2010) reported *Trichodina* sp. infection in ornamental fishes. *Ichthyophthirius multifiliis*, was only found on the gills of the fish and mean intensity was high. This parasite is wide spread and has low host specificity (Tavares-Dias et al, 2010). Heavy gill infection results in severe gill damage and respiratory stress (Raissy et al., 2010). Tavares-Dias et al., (2010) and Chanda et al., (2011) also recorded high mean intensity of *I. multifiliis* in ornamental fishes. *Argulus foliaceus* is a common parasite of cyprinids and very low infection was observed in shubunkin. *Argulus foliaceus* infection
produce ulceration on the skin of the host and facilitate other pathogens such as; bacteria, fungi and viruses to attack the host through ulcers. Iqbal et al., (2013) have reported low prevalence and mean intensity of *A. foliaceus* from two ornamental fishes; black moor *C. auratus* (12.5%, 4.6) and koi *Cyprinus carpio* (13.6%, 8.6). High infection up to 800-1000 parasites /fish may cause mortality in *C. carpio* and other carps (Pekmezic et al., 2009; Chanda et al., 2011).

The variations in the infection levels of different species of parasites in shubunkin may be associated to the susceptibility of host to various parasite species. High infection of monogenean and low infection of protozoans on this fish may be due to their reproductive rate and their presence on the host fish before shipment. Low infection of *A. foliaceus* may be associated with the, accidental escape of infected fish into the shipping consignment and remained unnoticed till air lifted. Furthermore, checking of fishes for transport does not normally include rigorous efforts to locate parasites. This is the first report of introduction of parasites along with its host fish, shubunkin imported into Pakistan. It is very necessary to improve the fish health management practices at the fish farms before shipment to overseas importers. The import of infected ornamental fish may create dangerous situation for the local fish species. Hence, strict rules on import of live fish are required to be implemented. Health certification for import of ornamental fishes into the country may be made mandatory.

**ACKNOWLEDGEMENT**

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Effect of Dung, Leaf Litter and Urea on Growth of VA Mycorrhizae in *Lens culinaris* Medik. CV. Massur 95 Grown under Field Conditions

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*Department of Botany, GC University Lahore, Pakistan
**Department of Botany, University of Balochistan, Quetta, Pakistan

ABSTRACT

In the present study, the effects of dung, leaf litter and urea were examined in the development of mycorrhizal associations under field conditions in Lentil (*Lens culinaris* Medik.) cv. masur 95. Various growth parameters, fresh and dry weights of the plant, root-length and shoot-length were determined. In the final yield, the percentage of general infection, number of arbuscules, number of vesicles, number of pods per plant, as well as the number of seeds per plant, 100 seeds weight were recorded over a period of six weeks to ascertain the effects of the mycorrhizal associations. The control plot (T3) showed normal growth while experimental plot, especially amended with leaf litter (T2), showed the greatest mycorrhizal infection as compared to the soils amended with urea (T1) and dung (T1). This is because urea and dung contain high amounts of nitrogen and phosphorus which suppress the mycorrhizal infection that is why the plots T3 and T1 showed stunted growth. The number of arbuscules was maximum during vegetative growth, i.e., elongation and branching. Similarly, vesicles were less at the initial and the final stage but high at the stage of flowering.

Key words: arbuscules, fertilizers, mycorrhizae, nitrogen, phosphorus, vesicles

INTRODUCTION

Mycorrhizae are mutualistic associations between the fungus and roots of higher plants. Such associations enhance the absorption of required nutrients which are abundantly available in the soil (Mack & Rudgers, 2008; Smith et al., 2004). Mycorrhizal infestation generally results in substantial increase in the absorptive surface area of the root as well as the soil volume available for absorption, both of which cause a correlative increase in nutrient and water uptake by the host plant (Allen et al., 2003). In addition, mycorrhizae provide resistance to pathogens by direct competition for resources and provide stabilization to environmental stresses (Graham 2001; Whittfield 2007 English 2009). However, the benefits of this mutualistic approach are minimized when certain fertilizers, especially those rich in P and N, reallocate the soil conditions and make the soil unfit for the growth of the mycorrhizal infection (Smith et al., 2004). Moreover, under certain conditions, mycorrhizae can become parasitic as well (Mack & Rudgers, 2008). Jamal et al. (2002) found that certain arbuscular mycorrhizal (AM) fungi also enhance the uptake of zinc and nickel from contaminated soil by lentil and soybean. Lentil was more dependent on mycorrhizae than wheat and responded to AM fungi inoculants even more in soil containing high levels of indigenous AM fungi (Xavier & Germida, 2002).

Lentil is an important legume crop of Pakistan, and serves as a major protein source for humans. It is grown as a major winter (Rabi) crop in rain-fed tracts of Pakistan (Shah et al., 2000). The overall yield of legumes in Pakistan is 0.5-0.6 tons per hectre which is lower than in many other countries (Aslam et al., 2000). Low moisture, low soil fertility and poor weed management are the main causes of its low yield. In 2006 lentil was grown on 43, 4000 ha with 25, 9000 tones production and its average yield was 597 kg/ha (MINFAL, 2006).

In the present research, the effects of various fertilizers were investigated on the growth of mycorrhizae and ensuing yield of lentil crop. The hypothesis behind this investigation was that some synthetic fertilizers suppress the growth of mycorrhizae, resulting in the stunted growth and reduced crop yield. It is because mycorrhizae are generally considered as natural manure for the crops (Smith et al., 2004). Growth performance of lentil crop was estimated according to local agricultural protocol throughout its life cycle.

MATERIALS AND METHODS

The seeds of Lentil were obtained from Seed Certification Department 4, Lytton Road,
Lahore. The pH of the soil was determined at the start and in the end of the experiment as well, there was no salinity or sodicity problem in the selected fields. Seeds of lentil were sown in the plots selected in the Botanical Garden, GC University, Lahore. Urea, leaf litter and dung were applied immediately after germination in a single split. There were four experimental fields in total; the length and width of each field was 30 square feet. One plot was kept as control and other three were treated as experimental. Control plot was denoted by T0, while the remaining three plots labelled as T1, T2 and T3 were amended with Dung, leaf litter and urea, respectively. T2 was amended with 500g of leaf litter, which was obtained from GCU Botanic Garden. The leaf litter was composed of leaves from Oak and *Ficus religiosa* L. The T3 was amended with 500g of urea, which is a synthetic fertilizer containing 46% nitrogen.

The parameters measured to assess the mycorrhizal infection in response to fertilizers were shoot fresh weight, root fresh weight, total fresh weight, shoot oven dried weight, root oven dried weight, and total oven dried weight, shoot length, root length, and total plant length. Parameters for final yield were General infection (GI), percentage of arbuscules, percentage of vesicles, number of pods per plant, weight of seeds per plant, and 100 seeds weight.

Statistical analysis of the data was performed by using Costat version 3.03. The improved method of Philips & Haymann (1970) was used to determine the percentage infection of mycorrhizae.

**RESULTS**

**Vegetative Growth**

It is apparent from Table 1 that the plants of T2 were the healthiest ones as compared to the plants of control (T0) and their other counterparts, i.e., T1 and T3. It was also noted that the shoot and root systems of T2 were comparatively well-developed as compared to the plants of control and other treatments.

**Plant Fresh Weight**

The Table 1 showed that the total highest fresh weight was found in the plants of T2, treated with leaf litter followed by a progressive reduction in the weight in T3, T1 and T0, respectively. The plants of T2 were the healthiest ones as the plants of T2 exhibited high shoot and root fresh weight as compared to the plants of control and other treatments. The statistical analysis also showed the significant difference between plants of T2 and plants of control and other fertilizer treatments.

**Plant Dry Weight**

The results of plant dry weight analyses showed that the shoot dry weight, root dry weight as well as total dry weight also followed the same pattern as fresh weight (Table 1). The highest total dry weight was of the plants of T2, followed by a progressive decrease in dry weight of T1, T0 and T3, respectively. The difference is statistically significant between control and T2. The difference is also statistically significant between control and T3, while it is statistically non-significant between control and T1.

**Plant length**

The data on plant length indicates that the plants of T2 have highest length, while the plants of T3 have the lowest length (Table 1). The plants of T1 are also taller than plants of control. The plants of T2, having leaf litter treatment, showed the healthiest growth throughout the growth season (Fig. 1.). Table 1 showed statistically significant difference between plants of control and plants of T2, and also between plants of control and T1.

![Fig. 1: Changes in the Height of the plant in different treatments with respect to age of the plant in weeks.](image)

**Reproductive Growth**

The final yield was obtained at maturity. Plants at this period were totally dried up. It appears that plants of T2 treatment looked much healthier than those of the control and other treatments.
Different parameters of growth were observed between the plants. The differences were calculated at the final yield and the differences were

Table 1: Effect of different fertilizers on vegetative growth of Lentil cv Masur 95 after 8 weeks mid-term harvest

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot fresh weight (g)</th>
<th>Root fresh weight (g)</th>
<th>Total fresh weight (g)</th>
<th>Shoot dry weight (g)</th>
<th>Root dry weight (g)</th>
<th>Total dry weight (g)</th>
<th>Total Plant length (cm)</th>
<th>Root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>19.94 ±2.32</td>
<td>1.86 ±0.08</td>
<td>19.00 ±0.00</td>
<td>4.5 ±0.42</td>
<td>0.86 ±0.19</td>
<td>6.34 ±0.60</td>
<td>49.9 ±0.95</td>
<td>14.19 ±0.88</td>
</tr>
<tr>
<td>T₁</td>
<td>15.74 ±2.32</td>
<td>2.02 ±0.20</td>
<td>17.97 ±2.51</td>
<td>2.83 ±0.65</td>
<td>0.76 ±0.15</td>
<td>5.79 ±0.81</td>
<td>44.75 ±1.16</td>
<td>12.0 ±2.12</td>
</tr>
<tr>
<td>T₂</td>
<td>22.26 ±2.00</td>
<td>2.84 ±0.19</td>
<td>23.81 ±1.96</td>
<td>5.36 ±0.45</td>
<td>1.26 ±0.13</td>
<td>7.43 ±0.41</td>
<td>47.95 ±1.05</td>
<td>16.76 ±1.60</td>
</tr>
<tr>
<td>T₃</td>
<td>12.91 ±0.54</td>
<td>1.06 ±0.25</td>
<td>15.18 ±0.79</td>
<td>3.23 ±0.22</td>
<td>0.56 ±0.06</td>
<td>4.79 ±0.22</td>
<td>41.75 ±1.40</td>
<td>11.43 ±1.93</td>
</tr>
<tr>
<td>LSD</td>
<td>2.64 ±0.44</td>
<td>0.44 ±0.25</td>
<td>2.91 ±1.06</td>
<td>0.24 ±0.13</td>
<td>1.24 ±0.38</td>
<td>0.38 ±3.44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means are significantly different at P = 0.05 according to Duncan’s new multiple range test, ±: standard error; LSD: least significant difference

Table 2: Effect of different levels of urea on reproductive growth of Lentil at the final stage of experiment

<table>
<thead>
<tr>
<th>Treatments</th>
<th>General Infection per plant (%)</th>
<th>No. of arbiacules (%)</th>
<th>No. of Vesicles (%)</th>
<th>No. of pods per plant</th>
<th>Wt of seeds per plant (g)</th>
<th>100 seeds weight (g)</th>
<th>Total Seed yield per plant (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>68.22 ±0.282</td>
<td>49.03 ±0.14</td>
<td>9.25 ±0.13</td>
<td>11.57 ±0.15</td>
<td>3.14 ±0.15</td>
<td>3.52 ±0.15</td>
<td>2.40 ±0.134</td>
</tr>
<tr>
<td>T₁</td>
<td>66.05 ±0.282</td>
<td>21.22 ±0.325</td>
<td>9.36 ±0.09</td>
<td>11.43 ±0.35</td>
<td>4.15 ±0.24</td>
<td>3.05 ±0.49</td>
<td>2.22 ±0.25</td>
</tr>
<tr>
<td>T₂</td>
<td>78.43 ±0.141</td>
<td>65.32 ±0.325</td>
<td>9.67 ±0.14</td>
<td>12.00 ±0.15</td>
<td>5.35 ±0.30</td>
<td>3.92 ±0.61</td>
<td>3.06 ±0.36</td>
</tr>
<tr>
<td>T₃</td>
<td>38.10 ±0.141</td>
<td>21.30 ±0.282</td>
<td>8.88 ±0.39</td>
<td>11.17 ±0.42</td>
<td>2.66 ±0.25</td>
<td>2.13 ±1.73</td>
<td>2.12 ±0.04</td>
</tr>
<tr>
<td>LSD</td>
<td>0.22</td>
<td>1.01 ±0.38</td>
<td>0.61 ±0.55</td>
<td>3.91 ±0.47</td>
<td></td>
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</table>

Means are significantly different at P = 0.05 according to Duncan’s new multiple range test, ±: standard error; LSD least significant difference
Percentage of General Infection (GI)

The results indicate that the percentage of GI is high in T2 treatment (Table 2). However, the results in T0, T1, T2, and T3 treatments are significant at the initial growth stage, but at the stage of physiological maturity their growth became non-significant. Comparatively, the percentage of GI was high when the age of the plant reached 10 weeks in T0, T1, T2, and T3 treatments, after which no increase or decrease in infection was observed. In T0 and T1 treatment, the results are highly non-significant at the maturity of the plant.

Percentage of Arbuscules

The results indicate that the percentage of arbuscules was high in T2 treatment (Table 2). The plants of T0 also showed greater number of arbuscules as compared to the plants of T1 and T3. The results showed statistically significant differences between plants of control and plants of T3, and also between plants of control and T1.

Percentage of vesicles

The results indicate that the percentage of vesicles was high in T2 treatment and the results are significant (Table 2). In T1 treatment, the results are non-significant as compared to T3 treatment in which the percentage was very low. In T0 treatment, the results are non-significant throughout the growing period.

Seed Yield

The data of seed yield, given in the Table 2, shows that the number of pods per plant and the weight of the seeds per plant are maximum in the plants of T2. The differences between the number of pods per plant of control and T2, as well as between control and T1, and control and T3 are statistically significant. There was a significant difference of 100-seed weight in T2 and T3. In case of treatments, the T2 showed significantly higher yield (3.06 g) than its counter treatment having an average yield of 2.12 g per plant.

DISCUSSION

Plants exchange their nutrients with mycorrhizae only that support up a mutualistic relationship (Bever et al., 2009), hence the Lentil showed beneficial relationship with leaf litter in T2 and found a low relationship with dung and urea. Competition for available resources between the fungi and the plant may have ensued leaving less available nutrients for the seedlings (Allen 2001; Bronstein, 1994).

The data recorded showed different results in T0, T1, T2, and T3 treatments. As dung (T1) contains high amount of phosphorus and nitrogen which retard the mycorrhizal infection so the plants show less growth, less number of leaves and less percentage of infection. Vesicular arbuscular mycorrhizal (VAM) infection is related with phosphorus content of the soil and plant (Nelsen et al., 1999).

Dung (T1) provides a source of slow releasing nutrients and is used as natural fertilizer in rural areas. Due to this, the release of ammonium ions, increases soil pH and their absorption in excess retard the plant growth when dung is applied (Siqueira et al., 2002). Phosphorus and mainly the nitrogen fertilizer increase the accumulation of N, P, K, Ca, Mg, and C in plants (Santos & Morais, 2004). On the other hand, phosphorus is responsible for better root growth in forage crops (Andrade et al., 2004). Finally, it is concluded that soils amended with farmyard manure had no effect on the shoot and root growth (Bajwa et al., 2002).

The number of arbuscules and vesicles increases when leaf litter (T2) was added in the soil, as the amount of phosphorus is less in the soils amended with leaf litter. The relationship between the soil phosphorus concentration and AM colonization has been reported to be negative in P-rich soil and positive in P-deficient soils (Khan, 2002), and therefore the number of arbuscules and vesicles increases to satisfy the P requirements of the plants. It is concluded that nutrients obtained from leaf litter improves the growth and affects both Ectomycorrhizae and community structure in dipterocarp species (Bever et al., 2009).

The plot amended with urea (T3) showed very poor growth. This is because urea contains 46% of nitrogen that hinders mycorrhizal infection that is why mycorrhizal infection was very low in this treatment. Hence, the plants showed stunted growth. As mycorrhizae do not flourish in nitrogen rich soils, the number of arbuscules and vesicles is dramatically decreased in the soil amended with urea. The urea dramatically increases the concentration of exchangeable ammonium and water-soluble phosphorus, which ultimately decreases the root colonization (Sylvia & Neal, 1990). Similar results then also been obtained by other researchers (Achakzai et al., 2012).

The above-mentioned results clearly indicates that the observations made on all parameters do not differ as much as the variables of age and percentage of infection. However, in all the plots of lentil (Lens culinaris Medik) as the
plant age increases, the length and the number of leaves also increases which ultimately increases the yield of the plant so VAM (vascular arbuscular mycorrhizae) is beneficial for enhancing the growth of lentil.

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Enhanced Seed Germination and Callogenesis under long days using Leaf Disc as Explant in Guava cultivars

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ABSTRACT

Elite guava (Psidium guajava L.) cultivars Round and Pyriformed were explored for in vitro seed germination response to acid treatment and regeneration from leaf disc and other vegetative tissues. Hydrochloric acid (HCl) treatment of seed significantly enhanced in vitro and in vivo seed germination (97% and 80%, respectively) compared with sulfuric acid (H2SO4) treatment. Seed germination was found to be genotype dependent in both acid types. Among vegetative tissues employed for callus induction, only leaf disc responded to higher callus induction percentage i.e. (90%) on B5 Gamborg’s media supplemented with either 2,4 dichlorophenoxy acetic acid (2,4-D) or Naphthalene acetic acid (NAA) under long days (LD, 16/8 hr photoperiod) compared with dark conditions. Cultivar Round produced more calli compared with Pyriformed under LD. The induced calli proliferated well on the fresh media for several weeks. The B5 medium devoid of growth hormones (control medium) and reduced basal salt strength (starvation media) did not induce embryogenesis in the calli, hence, further studies are suggested.

Keywords: Callus induction, Guava, Gamborg B5 media, In vitro, Leaf disc, Seed germination.

INTRODUCTION

Guava (Psidium guajava L.) is a nutraceutically important fruit crop. India and Mexico are the leading fresh fruit producers in the world (Watson & Dallwitz, 2007). In Pakistan, guava stands as the fourth most important fruit crop and 0.54 million tons are produced annually. Punjab province is the main contributor with 77% share in guava production in the country (Anonymous, 2011). Commercial guava cultivars include different selections viz. Gola and Surahi (Round and Pyriformed) while other varieties like Allahabad, Karela, Red fleshed and Apple color are less frequently cultivated. Yield gap compared with leading guava producers range from 3-5 tons per hectare mainly due to lack of major guava crop improvement programs. Pakistani guava industry is seedling based since no asexual propagation method has yet been commercialized (Ali et al., 2003; Usman et al., 2012). Seed germination in guava is inhibited by presence of tannins in plants leading to poor, uneven and delayed seed germination thus making guava difficult to propagate sexually (Doijode 2001; Ali et al., 2003). In vitro raised seedlings act as source of sanitized plant material for healthy clonal propagation, thus efficient seed germination, by breaking seed dormancy and enhancing seed coat permeability, is highly desirable in guava. In vitro plant propagation is rapid, cost-effective, provides disease free and vigorously growing plant material in bulk (Liu & Yang, 2011).

Somatic embryogenesis has emerged as the most accepted regeneration method for fruit plants micropropagation, genetic transformation and other genetic improvement studies (Rai et al., 2007; 2009; Li et al., 2008; Dhekney et al., 2009; Nicholson et al., 2012). Among different explant sources used for fruit crop improvement in vitro and genetic transformations, leaf disc is preferred over other tissues since it ensures better transformation efficiency provided efficient regeneration protocols are available (Rai et al., 2007; Nicholson et al., 2012; Khan et al., 2012). There are reports of in vitro propagation in guava using direct or indirect organogenesis in cultivars like Allahabad Safeda (Singh et al. 2002), Pakistani Round and Pyriformed cultivars (Usman et al., 2012), Pineapple guava (Stefanello et al., 2005; Dal Vesco et al., 2005; Cangahuala-Inocente et al., 2007) and Mango (Usman et al., 2005). None of these studies in guava used leaf disc tissue for direct or indirect regeneration. Therefore, this study was aimed to enhance in vitro seed germination using acids and exploring leaf disc and other vegetative tissues to induce embryogenic calli in guava cultivars utilizing different media, plant growth regulators and culture conditions.

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MATERIALS AND METHODS

Media preparation and sterilization procedures

Murashige & Skoog (1962) medium was used as basal media for raising guava seedlings in vitro. MS media and Gamborg’s B5 medium (Gamborg et al., 1968) were supplemented with different levels of either 2,4-Dichlorophenoxyacetic acid (2,4-D) or Naphthalene acetic acid (NAA) at concentrations 0, 0.5, 1, 1.5, 2, 3, 5 mg L⁻¹ for callus induction (CI). Sucrose (Phytotech Lab., USA) was added at 40 g L⁻¹ as carbon source in plant multiplication media and 30 g L⁻¹ in CI media. Medium pH was adjusted at 5.7 using 1M KOH and 8 g L⁻¹ of phyta agar (Phytotech Lab., USA) was added as a solidifying agent in the media. Ten and thirty ml medium was dispensed in each test tube and Petri plate, respectively. Media were sterilized using autoclave for 20 minutes at 121°C and 15 psi.

Seed extraction and acid scarification

The fully ripe guava fruits of two commercial cultivars Pyriformed and Round (locally called as Surahi and Gola, respectively) were collected from Experimental Fruit Garden Institute of Horticultural Sciences, University of Agriculture, Faisalabad (Pakistan). Fruits were horizontally cut, seed cores excised and agitated for 30 min in tap water to wash the pulp. Guava seeds have very hard seed coat that gives sparse and delayed germination therefore the seeds were pretreated with 15%, 20% and 25% (v/v) of either hydrochloric acid (HCl) or sulfuric acid (H₂SO₄) for scarification for 12 hours on orbital shaker.

Seed sterilization and in vitro germination

The acid treated seeds were washed with sterile water to remove excessive acid and were surface sterilized with 70% Ethanol (v/v) + 1-2 drops of Tween-20 detergent for 3-5 minutes followed by 2-3 rinses with sterile distilled water. Seeds were dipped in 5% NaOCl solution for 10 min. on orbital shaker followed by 3-4 rinses with sterile distilled water. These seeds were cultured on MS medium for germination.

Explant sources for callus induction, embryogenesis and culture conditions

Hypocotyl (2-3 mm) and cotyledonary leaves (2x2 mm size) were taken as explants from young seedlings and placed on callus induction media for callus induction and embryogenesis. In vitro raised seedlings were multiplied using stem cuttings (SC; 3-4 mm in size) in glass jars following Usman, et al. (2012) to have sterile expanded leaves as explants for callus induction and embryogenesis. The young expanding leaves from 3rd node from top of the seedlings were selected for callus induction. These leaves were cut into sections (2x2 mm in size) and placed on callus induction media. Cultures were placed under long days (LD: 16 hrs light: 8 hrs dark) in the growth room facilitated with 50-60µEm⁻²sec⁻¹ light intensity using white fluorescent light and continuous dark conditions (D) to observe response of explants for callogenesis and embryogenesis or shoot regeneration on modified MS and B5 media. Growth room temperature was maintained at 25 ± 1°C. Calli induced were sub-cultured for plant regeneration on different media such as starvation media (¼ salts, ½ salts B5 media) under LD and control media (full strength B5 salts).

Experimental layout

The experiments were laid out in Completely Randomized Design (CRD) and replicated thrice with at least 20 explants per treatment. Data were analyzed using MSTAT-C and significance among treatment means were compared using Duncan’s Multiple Range (DMR) test (Steel et al., 1997).

RESULTS

Acid scarification enhanced guava seed germination in vitro

Seed germination was found to be dependent upon genotypes in response to acid types used. Fifty percent seedlings emerged after 10-12 days of treatment in both the cultivars. Seed treatment using 15%-20% HCl significantly (P<0.05) enhanced germination in Round (98.9%) and Pyriformed (97.66%) cultivars compared with H₂SO₄ (Figure 1A,B; 2A,B). Cultivar Pyriformed showed higher germination, 96% at higher concentration of H₂SO₄ as compared to cv. Round. Further increase in the acid concentration of both acid types significantly reduced seed germination suggesting that 15-20% acid treatment is optimal to enhance seed germination up to 37% compared with control medium MSO. Overall among cultivars, cv. Round showed mean seed germination enhancement by 97.45% compared with cv. Pyriformed by 95.63%.

Callus induction under long days on B5 media

Among different explants used for callus induction on different types of media, only leaf disc explants induced calli on B5 medium supplemented with 2,4-D or NAA. Significantly (P<0.001) higher callus induction was observed in cv. Round on both PGRs under LD conditions compared with D (65%-75%, respectively, Figure 1C,D). In cv. Pyriformed, callus induction was not significantly different under
LD and D (Figure 3B). Amongst PGR's, callus induction frequency was significantly higher on NAA under LD across genotypes whereas callus induction response against 2,4-D was not significantly different under LD and D. Maximum callus induction was observed on 1.5 mgL⁻¹ of 2,4-D (88.67%) whereas NAA induced more calli at 2 mgL⁻¹ (89.33%, Figure 1E). Both PGRs showed decline in callus induction on higher levels suggesting 1.5-2 mgL⁻¹ as the optimal level (Figure 3A). Other explants including hypocotyl and cotyledonal leaf did not induce calli on both MS and B5 media under LD and D. This suggests that B5 media is better for callogenic response in leaf disc explants in guava compared with MS media. The induced calli showed proliferation on the respective media after sub-culturing. The developed calli were yellowish to brown in color under D and dark brown colored in light owing to more phenolic exudation. Calli grew and proliferated more under D compared to LD due to reduced release of phenolic contents by tissues and more water contents (Table 1). The proliferating calli were divided into small chunks and placed on MSO medium and starvation medium to induce embryogenesis, however the induced calli did not show embryogenesis on any media after 4-6 weeks of culture.

**DISCUSSION**

In the present studies the enhanced seed germination results are in accordance with the findings of Ali et al. (2007) who reported higher guava seed germination (85%) using 5% H₂SO₄ for 12 hours while it was (88%) when treated with 10% HCl for 24 hrs. They reported negative effect of increased acid concentration on seed germination. Contrary to their findings we report here seed germination enhanced up to 97-98% using HCl (15%-20%) in Pyriformed and Round cultivars, respectively suggesting HCl acid is better for treatment in both cultivars. The present study showed that higher concentration of HCl treatment for shorter time-12 hours gave better results compared with previous report using lower concentration of HCl and H₂SO₄ for longer time. Further, we observed the optimized treatment response in *in vivo* germination of immature seeds obtained from market maturity fruit that gave little or no germination in field if sown untreated. We obtained >80% seed germination after acid treatment in 25-30 days of seed sowing in field compared with <40% seed germination without treatment in 40-50 days indicating that the optimized system is equally good and applicable *in vitro* and *in vivo* to enhance seed germination (data not shown). In this study, seed germination frequency in field is better compared with Ali et al., (2007) who reported 50% germination in 40 days.

Indirect embryogenesis has been used for genetic improvement in many woody crops. Auxins like 2,4-D, IAA, NAA are usually used alone or in combinations for callus induction followed by subsequent embryogenesis or organogenesis as reported in Eucalyptus (Subbaia & Minocha, 1990). Different explant sources have been used for embryogenesis in guava like mesocarp tissue on MS medium fortified with ascorbic acid, glutamine and sucrose and supplemented with 2,4-D (Chandra et al., 2004); floral tissues and zygotic embryos on LPM basal medium supplemented with 2,4-D (Stefanello et al., 2005; Cangahuala-Inocente et al., 2007), and zygotic embryos on MS medium supplemented with 2,4-D (Raiet al., 2007). We observed significantly higher callus induction response in >80% explants on both 2,4-D and NAA auxins in leaf disc explants on B5 medium compared with MS medium that showed no callus in hypocotyl, cotyledonal leaf and leaf disc explants. These findings are contrary to the previous report of poor callus induction in hypocotyl and leaf disc explants (34% and 23%, respectively) on MS medium supplemented with NAA, 2,4-D and Kin in cv. Pant Prabhat (Singh et al., 2007). The present investigation used different basal media and PGRs for callus induction. Our findings of callus induction on B5 medium containing either 2, 4-D or NAA are better in terms of more calli induced (90%) in leaf segments of guava cultivars compared with Singh et al. (2007). In agreement to previous studies, B5 media has shown better callus induction compared with MS media in woody fruit plants like mango (Usman et al., 2005). Environmental factors and culture conditions like photoperiodic alterations play a significant role in plant tissue behaviour in response to up or down regulation of endogenous hormone production (Lee et al., 2007; Mengxi et al., 2011; Neelakandan & Wang 2012). In the present work significantly higher callus induction occurred in leaf disc explant under LD conditions compared with D. Singh et al. (2007) have not mentioned the culture conditions (light/dark) used for callus induction. The calli induced in the present studies proliferated well; however, it could not regenerate suggesting need for further studies in guava plant regeneration from leaf disc explant.

**CONCLUSIONS**

The present studies have demonstrated enhanced seed germination in response to acid
treatment and callusogenesis behavior of leaf disc explant in guava cultivars in response to photoperiodic alterations. Callus induction response was strongly dependent upon explant types and photoperiodic conditions used in relation to exogenous application of 2, 4-D and NAA and the basal media. These results may contribute to our understanding the process of better callus induction and proliferation from leaf disc explants that may be further useful for the transformation of important biomolecules in guava.

REFERENCES


**Fig. 1:** Seed germination and callus induction from leaf disc explants under light and dark culture conditions on B5 medium. Where figure A & B) shows raising of guava seedlings after scarification in vitro and clonal multiplication, C & D) callus induction from leaf disc explants on B5 media under long days (16:8 hrs photoperiod) in cv. Pyriformed and Round, respectively E) shows callus induction from leaf disc explants on 2,4-D media under dark conditions in cv. Round.

**Fig. 2:** Guava seed germination (%) on MS medium in response to scarification using H_2SO_4 (A) and HCl treatments (B) for 12 hrs on orbital shaker. The vertical bars show standard error and the means are statistically different at probability P<0.05 using DMR test.
Fig. 3: Response of plant growth regulators (PGRs, A) and culture conditions (B) on callus induction in leaf disc explant of Guava cultivars Round and Pyriformed on B5 medium supplemented with 2,4-D and NAA. The vertical bars show standard error and the means are statistically different at probability $P<0.001$ using DMR test.

Table 1: Morphological characterization of calli induced in leaf disc explant under long days (LD) and dark (D) conditions on 2, 4-D and NAA in guava

<table>
<thead>
<tr>
<th>B5 Medium + PGRs (mgL$^{-1}$)</th>
<th>2,4-D</th>
<th>NAA</th>
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<tbody>
<tr>
<td></td>
<td>LD</td>
<td>D</td>
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<tr>
<td>Control</td>
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<tr>
<td>0.5</td>
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<td>++</td>
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<tr>
<td>5.0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

No induction: -, Poor: +, Average: ++, Good: +++, V. Good: ++++, Excellent:+++++
Determination of LD_{50} and Morphometric Analysis induced by Bifenthrin in Developing Fetuses of Mice

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ABSTRACT

The present study was planned to evaluate the teratogenic and embryotoxic effects of bifenthrin in mice (Mus musculus). Based on 48.00 mg/kg body weight LD_{50} value for pregnant mice, three motherly sub-toxic doses were selected as 6.00, 12.00 and 24.00 mg/kg body weight and each sub-lethal dose was administered orally in four different ways (single, double, triple and multiple) to the pregnant mothers during day 6 to 12 of gestation. On day 18 of gestation, the fetuses were recovered for morphological and morphometric studies. The morphometric observations included significant (P<0.001) reduction in body weight, crown rump length, brain circumference, eye circumference, pinna size, snout size, fore limb, hind limb length and tail size of the fetuses as compared to controls. Morphological observations included anencephaly, bulging eye, fore limb micromelia, fore limb dysplasia, hydrocephaly, hind limb dysplasia, haemorrhage spot on head and abdomen, hind limb low arm set, kyphosis, macrocephaly, open eye lid, rough skin, snout less developed, spina bifida, short tail, underdeveloped fore and hind limb dysplasia.

Key words: Bifenthrin, LD_{50}, Morphology, Teratology, Toxicology, Anomalies, Fetal Development

INTRODUCTION

Bifenthrin with biochemical origins in the natural insecticide pyrethrum, an extract of the flower Chrysanthemum cinerariaefolium, is a synthetic pyrethroid insecticide. It is used primarily on turf, in agricultural and for homes applications. Synthetic pyrethroids as a class are in the top ten for usage in the home and garden market (Grube et al., 2011), although have fallen in rank since 2004.

Bifenthrin when ingested with an acute oral LD_{50}, ranging from 53.4 mg/kg to 210.4 mg/kg is moderately toxic to rats (FAO, 2010). Researchers in another study fed bifenthrin to male rats at doses up to 20 or 26 mg/kg and used corn oil as the vehicle to test 1 or 5 mg/kg affected bifenthrin neurotoxicity. At the 1 ml/kg than 5 ml/kg more severe signs were observed. These signs were pawing, elevated body temperature, increased click response, decreased grip strength, motor activity, tremors and increased head shaking (Wolansky et al., 2007).

No toxic effect of bifenthrin is observed on the mother (maternal toxicity NOEL) at the dose 2.67 mg/kg/day for rabbit and 1 mg/kg/day for rat (US EPA, 1987). During development (developmental toxicity NOEL) no toxic effect is observed at the dose 1 mg/kg/day for rats and is greater than 8 mg/kg/day for rabbits (Walker & Keith, 1992).

At the highest levels tested (100 ppm, approximately 5.5 mg/kg/day) in a two generational study in rats, bifenthrin does not demonstrate any teratogenic effects (US EPA, 1988).

Bifenthrin is classified as a class C carcinogen which is a possible human carcinogen by the EPA (Walker & Keith, 1992; US EPA, 1988).

As other pyrethroid insecticides, bifenthrin undergoes similar modes of breakdown within animal systems. Bifenthrin in mammals is readily metabolized and excreted. Within 7 days, rats treated with 4 to 5 mg/kg, excreted 70% in the urine and 20% in the feces. While the remaining bifenthrin was found to be accumulated in tissues with high fat content such as the skin and fat in males and females and the ovaries of females, after 7 days (US EPA, 1987). Yang et al. (2009) found that bifenthrin is efficiently activated rat P×R, while weakly activated in human pregnane × receptor (P×R), suggesting differences in metabolic rates and outcomes in rodents as compared to human that could have implications for potential toxicity.

Unfortunately, no data exist about the embryotoxic and teratogenic effects of bifenthrin. Keeping in view its increasing usage in domestic and agricultural sectors, and a wide range of toxic effects on non-target organisms especially, humans, the present research work has been designed to evaluate its embryotoxic and teratogenic effects in mice.
MATERIALS & METHODS

Swiss Webster strain of albino laboratory mice, *Mus musculus* were kept under the standard protocol of 12-hour day and night (light/dark) cycles, in 12” x 18” steel cages with room temperature 25±2°C. Three females were caged with one male. For the identification of successful coitus, the females were carefully observed daily. Pregnant females were separated from the males with vaginal plug and that was considered day zero of gestation. Under the approved animal treatment conditions, Ethics Committee of University of the Punjab, Lahore, Pakistan, the above cited protocol was used. Bifenthrin under the brand name TALSTAR prepared by FMC United (Pvt.) Pakistan Ltd. was purchased from the local market. Different concentrations of bifenthrin were prepared through dilutions in water in such a way that each 0.1 ml of the solution contained the desired amount of insecticide. These concentrations 125, 250, 500 and 1000 mg/kg body weight of bifenthrin were administered to the experimental animals directly into their gullet to determine the lowest dose value which causes complete mortality.

For the determination of LD₅₀, six appropriate doses i.e. 6.25, 12.50, 25, 50, 100 and 200 mg/kg body weight were given to a group of 16 pregnant females for the determination of LD₅₀ (50% mortality within 48 hours). With the probit analysis procedure “Microsoft Excel regression curve analysis” in line, LD₅₀ values were calculated (Finney, 1971).

The pregnant females were divided into four groups and each group contained forty animals. Then each group was further divided into four different categories, such as single, double, triple and multiple. Doses of bifenthrin dissolved in distilled water containing 6.00 12.00 and 24.00 mg/kg (Corresponding to 12.50, 25.00 and 50.00% of LD₅₀ dose values respectively) were given to experimental animals in Group 1, 2 and 3. While the animals in vehicle treated (control) group received 0.1ml distilled water only.

The treated dams were weighed and anaesthetized with anaesthetic Ether on the day 18 of gestation. By giving surgical incision to the anaesthetized dams, intact gravid uteri were dissected out carefully. After that uteri were opened along the inner curvature and developing fetuses were recovered. Then these fetuses were fixed in Bouin’s fixative for 48 hours (Crookham & Dapson 1991; Carson, 1992) and then these fetuses were preserved in 70% alcohol (Carson 1992; Patki et al., 1992). The preserved fetuses were subjected to morphometric, morphological and histological observations.

The morphometric observations of fetuses involved different parameters recording of fetal weight, head circumference, eye circumference, snout length, tail length, crown rump length, pinna length and length of fore- and hind–limbs. A separate record was prepared for all fetuses recovered from each litter for morphometric measurements. Digital vernier caliper and analytical balance were used to make required measurements except the measurements of head circumference which were carried by “Ellipse Circumference Calculator” of CSG Network; a computer based program that helps to calculate head circumference values (P = mm²) (CSGN, 2006).

For morphometric studies, analysis of variance (ANOVA-1) and Duncan’s Multiple Range Test (DMRT) (Duncan 1955) were applied to check the significance of differences among the subgroups within their respective groups as well as for subgroup wise intergroup comparison (Ahmad & Asmatullah, 2007).

The labelling of deformities was done in Corel Draw 9 and these labelled photographs were pasted in Microsoft Word file of describing and supporting results.

RESULTS

Various doses of bifenthrin (6.25, 12.50, 25.50, 100 and 200 ml/kg body weight) were given to a group of 16 pregnant mice each. Mortality at each of these doses was plotted against a linear regression trend line. For the statistical estimation of the lethal dose for 50% mortality. The calculated LD₅₀ of bifenthrin for the pregnant mice was 48.00 ml/kg body weight (Fig. 1).

In all bifenthrin treated groups the percentage of deformed fetuses generally increased in all the categories as compared to the categories of the control group (Fig. 2).
DETERMINATION OF LD\textsubscript{50} AND MORPHOMETRIC ANALYSIS

Fig., 1: Regression curve analysis in line of LD\textsubscript{50} value of pregnant mothers exposed to different concentrations of Bifenthrin on day 6 of gestation.

Well-developed fetuses recovered from control group showed normal morphological observations of size and organs (Fig. 3a). The fetuses from the group 6.00mg/kg BW showed morphological anomalies like forelimb micromelia and skin hemorrhage (Fig. 3b), hindlimb dysplasia (Fig. 3c). In dose group 12.00mg/kg BW the defects like forelimb dysplasia, bulging out eye and forelimb micromelia (Fig. 3d) were observed, whereas, 24.00mg/kg BW included deformities like anencephaly, forelimb dysplasia, under developed fore and hind limbs dysplasia and short tail (Fig. 3e), hindlimb dysplasia and low set arm (Fig. 3f), macrocephaly, spina bifida and kyphosis (Fig. 3g). A significant (P<0.001) dose dependent decrease as compared to control in fetal body weight, crown rump length, brain circumference, eye circumference, pinna size, snout size, forelimb and hindlimb length and tail size were observed during morphometric analysis (Table 1).
Fig., 3d): 12.00mg/kg BW (s); bulging out eye (boe), forelimb dysplasia (fd) and forelimb micromelia (fm).

Fig., 3e): 24.00mg/kg BW (s); anencephaly (aenc), hind & fore limb malformation (hfm), hind & fore limb dysplasia (hfd) and short tail (st).

Fig., 3f): 24.00mg/kg BW hydrocephaly (hc); hindlimb dysplasia (hd), low set arm (las) and hooked tail (ht).

Fig., 3g): 24.00mg/kg BW (c); macrocephaly (mac), spina bifida (sb) and kyphosis (kyp).

Fig., 3: Macrophotographs of 18 days old mice fetuses recovered from the mothers treated with different doses of bifenthrin on days 6, 9 and 12 of gestation showing wide range of morphological defects.

(s: single, d: double, t: triple and m: multiple).
### DISCUSSION

The present study was aimed to discover the toxicological effects of bifenthrin in relation with pregnancy and to report all sorts of developmental abnormalities at morphological level. There are only a few studies pertaining embryo toxicology with the embryonic exposure of bifenthrin (Uggini et al., 2012).

The estimation of LD$_{50}$ was a very difficult task. However, it is calculated due to the following reasons:

1. Although in literature, the values of LD$_{50}$ of bifenthrin for mice are available but in these reported values there exist a wide disparity such as: 54 mg/kg BW in female mice while 70 mg/kg BW in male mice (US EPA, 1988).

2. Most importantly dealing with the pregnant mice particularly there appears no such value in the available literature.

Keeping in view of all above circumstance for exact decision of the experimental doses, it was decided that a careful estimation of LD$_{50}$ in pregnant mice should be conducted. Results indicate that the value of LD$_{50}$ for bifenthrin in pregnant female mice is 48.00 mg/kg BW (Fig. 1). This is considerably lower than the already reported value that is 54.00 mg/kg BW in female mice (US EPA, 1988).

During the present study acute toxic effects of bifenthrin were seen. Many reports of acute toxicity of bifenthrin in rats and mice such as respiratory distress, swaygait, muscular weakness, prostration, pallor, respiratory failure, clonic convulsions, diarrhea, lack of coordination, tremor, irritability vomiting and salivation are available (US EPA, 1988).

LD$_{50}$ does not specify in the available literature.

### Table 1: Morphometric analysis of 18 days old fetuses of mice recovered from pregnant mothers treated with different doses of bifenthrin on day 6 of gestation.

<table>
<thead>
<tr>
<th>Dose mg</th>
<th>Parameters</th>
<th>0.00</th>
<th>6.00</th>
<th>12.00</th>
<th>24.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>Fetal body weight (mg±S.E.)</td>
<td>1535.22±29.92(a)A</td>
<td>1444.78±31.90(a)B</td>
<td>1269.65±18.43(a)C</td>
<td>1224.76±28.45(a)D</td>
</tr>
<tr>
<td>6.00</td>
<td>Crown rump length (mm±S.E.)</td>
<td>23.55±0.22(a)A</td>
<td>23.00±0.18(a)B</td>
<td>21.94±0.18(a)C</td>
<td>20.33±0.51(a)C</td>
</tr>
<tr>
<td>12.00</td>
<td>Brain circumference (mm±S.E.)</td>
<td>24.67±0.21(a)A</td>
<td>23.34±0.34(a)B</td>
<td>22.02±0.18(a)C</td>
<td>21.10±0.33(a)D</td>
</tr>
<tr>
<td>24.00</td>
<td>Eye circumference (mm±S.E.)</td>
<td>7.20±0.10(a)A</td>
<td>6.83±0.11(a)A</td>
<td>6.02±0.03(a)B</td>
<td>5.66±0.11(a)B</td>
</tr>
<tr>
<td></td>
<td>Pinna size (mm±S.E.)</td>
<td>2.64±0.02(a)A</td>
<td>2.83±0.01(a)B</td>
<td>2.60±0.06(a)BC</td>
<td>2.44±0.10(a)C</td>
</tr>
<tr>
<td></td>
<td>Snout size (mm±S.E.)</td>
<td>3.36±0.03(a)A</td>
<td>3.43±0.05(a)A</td>
<td>2.55±0.10(a)B</td>
<td>2.44±0.07(a)B</td>
</tr>
<tr>
<td></td>
<td>Forelimb size (mm±S.E.)</td>
<td>8.16±0.06(a)A</td>
<td>7.14±0.11(a)BC</td>
<td>7.36±0.11(a)B</td>
<td>6.82±0.19(a)C</td>
</tr>
<tr>
<td></td>
<td>Hindlimb size (mm±S.E.)</td>
<td>9.04±0.01(a)A</td>
<td>8.45±0.11(a)B</td>
<td>8.26±0.04(a)B</td>
<td>8.25±0.26(a)B</td>
</tr>
<tr>
<td></td>
<td>Tail size (mm±S.E.)</td>
<td>11.74±0.05(a)A</td>
<td>11.83±0.10(a)A</td>
<td>11.14±0.24(a)BC</td>
<td>11.20±0.27(a)B</td>
</tr>
<tr>
<td></td>
<td>ANOVA</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

Entries Key: parameter size ± standard error (S.E.).
DMRT (Duncan’s Multiple Range Test) comparison of the different dose groups (intergroup); sharing the same alphabet showing their means in lie in the same range values.
*** Significant difference (P<0.001).
Department of Health and Human Services, 1993; Iyaniwura & Okonkwo, 2004).

A significant decrease in body weight of pregnant mice with the increase in dose concentration was seen which indicate a direct toxicological impact of this insecticide (bifenthrin). Akhtar et al. (1996) and Kaul et al. (1996) supported this view. According to them, body weight was significantly reduced in Talstar treated mice.

On the whole data clearly indicating that bifenthrin is highly embryo toxic. In this context Rutledge (1997) had argued that chemicals adversely affect the early conceptus causing in-utero mortality and developmental abnormalities.

The available literature on the fetomorphologic studies attributable to bifenthrin exposure is relatively skimpy (Uggini et al., 2012). However, morphological deformities including skin haemorrhage, hind and fore limb dysplasia, hind and fore limb malformation, fore limb micromelia, low arm set, short and hooked tail, macrocephaly, spina bifida and kyphosis as compared to the control were observed (Fig. 3a to 3g).

These findings were confirmed by NRC (1993). According to this, developing fetus, infant or child show serious effects when these pesticides are used in even a very small amount, whereas the adult show no effect.

During the present study developmental parameters such as body weight, CR length, Cranium size, eye width, eye length, snout size, forelimb, hindlimb and tail lengths were noted. The body weight and CR length decreased by increasing the dose number of exposures. These results are confirmed by Akhtar et al. (1996) who reported that the body weight was significantly reduced in Talstar treated rats (P<0.01).

CR (Crown Rump) length comparison and measurement of fetal head circumference indicate fetal growth retardation in multiple exposures even at 6.00mg/kg body weight. The results obtained in this regard are comparable with similar previous studies (Ahmad & Asmatullah, 2007).

CONCLUSION

On the basis of these findings, it is concluded that oral exposure of bifenthrin is teratogenic and embryotoxic in pregnant mice especially when given at the time of organogenesis even on dose level as low as 6.00mg/kg/day.

In the light of present observations, this insecticide must be used under strict control and pregnant women and children must be kept away from such places where bifenthrin is in use in a country like Pakistan.

ACKNOWLEDGEMENTS

We are highly thankful to Department of Zoology, University of the Punjab, Lahore and Government College Bosan Road, Multan for providing laboratory, space and miscellaneous facilities to conduct this research work.

REFERENCES


Studies on the Antimicrobial Resistance Pattern of Bacterial Pathogens isolated from Cancer Patients

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ABSTRACT

The present study deals with the antimicrobial resistance pattern of different antimicrobials being used to treat infections in cancer patients. The isolated strains were tested against antibiotics belonging to cephalosporin and aminoglycoside groups. The activity of these drugs was evaluated against 50 bacterial strains isolated from cancer patients undergoing anticancer therapy. The susceptibility was determined by broth dilution method according to British Standard Antimicrobial Chemother (BSAC, 2003) USA guidelines.

The overall Minimum Inhibitory Concentration (MIC) was determined at which 50 and 90 percent of bacterial isolates were inhibited. The resistance of Gram positive isolates against Cefoperazone, Ceftriaxone and Ampicillin was 0%, 30% and 68% respectively. Whereas Pseudomonas aeruginosa among Gram negative bacteria showed resistance against Cefoperazone, Ceftriaxone and Ampicillin which was 0%, 27% and 64% respectively. For Enterobacteriaceae, it was recorded as 0%, 10% and 91% respectively. The order of activity against Gram-negative and Gram positive strains was Cefoperazone> Ceftriaxone >Ampicillin. Overall frequency of isolation of Gram positive bacteria and Gram-negative bacteria was 56% and 44% respectively. The results are useful with reference to the current resistance and susceptibility pattern among isolates against cephalosporin and aminoglycosides.

Keywords: Antibiotics, Resistance, Sensitivity, Cancer, Chemotherapy, Infections and Immune system.

INTRODUCTION

A frequent occurrence of infection is a persistent problem in cancer patients. They are more susceptible to variety of infections due to suppression of immune system as a result of chemotherapeutic agents being used for treatment. Cancer patients are at high risk for a wide variety of bacterial, viral, and fungal infections throughout the phases of immune recovery. These infections can be life threatening and are responsible for the high morbidity and mortality rate among the cancer patients (Pittet et al., 1997; Weinstein et al., 1997). Moreover the frequency of infection is related to the type of underlying neoplastic disease (Saif & Shannon, 2005). Such infections are generally the cause of death in a substantial number of patients (Hsueh et al., 2004).

Studies on Antimicrobial resistance in common bacterial pathogens have revealed that the Frequency of infections by Gram positive bacteria is increasing as compared to that of Gram negative bacteria during the past decade (Koll & Brown, 1993; Viscoli et al., 1988). This increase is mainly caused by the use of catheters i.e., central venous, peripheral and arterial resulting in a parallel increase in catheter-related infections (Butt et al., 2004). Amongst the Gram negative infections P. aeruginosa is a leading infectious agent in the immuno-compromised patients associated with significant morbidity and mortality (Yetkin et al., 2006). P. aeruginosa was responsible for 35% of fatal infections in patients with acute leukemia (Mazzalai, 2009).

The knowledge of antimicrobial resistance pattern is of particular concern in cancer patients where changing spectrum in the incidence and epidemiology of infecting organism has resulted in an increase in resistance to many antibiotic compounds ( Cruciani et al., 2000). The emergence of resistance to beta-lactam antimicrobial agents as a result of the production of type 1 and extended-spectrum beta-lactamases (ESBL) is of great concern (Borg et al., 2006). Rolston (1998) suggested that the widespread use of fluoroquinolone prophylaxis has also resulted in the development of resistance among E. coli and other Enterobacteriaceae.

Amongst the Gram positive infectious bacteria, Staphylococcus is the major cause. A
slight decline in infections caused by *S. aureus* but a considerable increase in the incidence of infections caused by coagulase negative staphylococci has been reported (Koll & Brown, 1993; Viscoli *et al.*, 1988 and Rolston, 1998). The predominant species is *S. epidermidis*, although *S. hominis* and, *S. haemolyticus* are also often isolated.

Antimicrobial agents act on bacteria in different ways, either by killing bacteria due to inhibition of vital activities or inhibition of protein production leading to arrest in bacterial growth and thereby preventing bacteria from reproduction (Cheesbrough, 2000).

In many cancer related infections, the determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients. The emergence of multidrug-resistant microorganisms has intensified the problem (Fluit *et al*., 1999; 2000). Assessment is required to monitor the spread of resistant pathogens throughout the hospital and community. Standard procedures and breakpoints have been defined to predict therapeutic outcome both in time and at different geographic locations.

The aims and objectives of this study are (I) to study the spectrum of bacterial isolates in clinical specimen of cancer patient; (II) to study the antimicrobial resistance pattern of different antimicrobials that are used for treating infections in cancer patients; and (III) to assess the use of new potent antibiotic against infection in cancer patients to reduce the mortality and morbidity due to these infections.

**MATERIALS AND METHODS**

The study was carried out at Institute of Nuclear Medicine and Oncology Lahore (INMOL). Total 50 hospitalized cancer patients undergoing anticancer therapy with suspected blood stream infections were studied. No discrimination was made on the basis of age or gender.

**Bacterial strains and culture conditions**

Bacterial strains were isolated by adding 5 ml blood obtained from peripheral veins of the patients to culture bottles containing brain heart infusion (BHI) broth (Oxoid, Hampshire, UK). The blood culture bottles were incubated at 37°C and regular subculture were made. Single isolated colonies were obtained by streaking the culture on blood agar and MacConkey agar plates by incubating at 37°C for 24 hours. The plates containing purified strains were stored at 4°C till further use for sensitivity testing. The microorganisms were characterized into Gram positive and Gram negative strains by Gram staining method. Each bacterial isolate was further identified by standard biochemical tests according to the manual of clinical microbiology (Cheesbrough, 2000; Greenwood *et al*., 1992).

**Preparation of antibiotic stock solution**

Three antibiotics Ceftriaxone (Roche Fontenay, France) Cefoperazone + sulbactum (High-Q-international) and Ampicillin (sigma, UK) were obtained from local manufacturer. All the stock solutions of antibiotics were prepared in sterilized distilled water as specified by the manufacturer. Different dilutions were prepared for minimum inhibitory concentration (MIC) determination.

**Preparation of inoculum**

Inoculum was prepared by inoculating the single purified colonies from blood agar plates into Mueller-Hinton broth (Oxoid, UK) and incubated at 37°C for 24 hrs. Optical density of inoculum was measured with a Spectrophotometer (Roche, Germany) at 546 nm. The density of the inoculum was adjusted to 10^5 CFU/ml and was used in MIC determination.

**MIC Determination**

Antibiotic sensitivity against cephalosporins (Cefoperazone, Ceftriaxone) and aminoglycosides (Ampicillin) was determined in Gram negative and Gram positive bacterial isolates. MIC was determined in duplicate in Mueller-Highton as outlined by the British Standard Antimicrobial Chemother (2003). Different interpretive resistance and sensitive breakpoints are used for ceftriaxone, BSAC breakpoints of ≤ 1 μg/ml (susceptible) and ≥ 2 g/ml (resistant) were respectively applied for Gram negative and positive bacteria. For cefoperazone BSAC breakpoints of ≤ 4μg/ml (susceptible) and ≥ 8 μg/ml (resistant) was applied. For ampicillin, BSAC breakpoint of 8μg/ml (susceptible) and ≥ 16μg/ml (resistant) were used Gram negative and positive bacteria respectively (Andrews, 2001).

**RESULTS**

During the study period a total of 50 bacterial isolates were collected from blood cultures of cancer patients. Overall frequency of isolation was 56% Gram positive bacteria and 44% Gram negative bacteria (Fig. 1). Among the Gram positive isolates *Staphylococcus aureus* (38%) was most common isolate followed by *Streptococci* (18%). Among Gram negative bacteria *P. aeruginosa* (20%) was the most frequent isolate followed by *E.
coli (10%), Proteus (6%), Klebsiella (4%), Shigella (2%) and Citrobacter (2%) (Fig:2).

The overall susceptibility results of cefoperazone, ceftriaxone and ampicillin against bacterial isolates from blood stream infections of cancer patients are shown in table 1 and 2. In Gram negative bacteria highest in vitro activity against P. aeruginosa strain was observed for cefoperazone. The MIC$_{50}$ and MIC$_{90}$ of cefoperazone was 0.125 µg/ml and 0.25 µg/ml respectively and ranged from 0.125 - 64 µg/ml. Ceftriaxone had three times less in vitro activity against P. aeruginosa isolates than cefoperazone (Table: 1) whereas in vitro activity of ampicillin was least with MIC$_{50}$ and MIC$_{90}$ of 4 µg/ml and 64 µg/ml respectively. In case of Enterobacteriaceae, cefoperazone was found more effective than ceftriaxone and ampicillin (Table,1). Therefore, the order of activity of the antimicrobials in Gram negative isolates was found in the order of cefoperazone> ceftriaxone> ampicillin.

Among Gram positive bacterial isolates cefoperazone is most effective with MIC$_{50}$ of 0.125 µg/ml as compared to Ceftriaxone (Table.2). It inhibited 50% of isolates at 1 µg/ml concentration. For ampicillin 50% and 90% isolates had MIC of 16µg/ml and 64 µg/ml, respectively. Therefore, the order of activity of cephalosporins and aminoglycosides in Gram positive bacterial isolates was cefoperazone> ceftriaxone> ampicillin.

The percentage sensitivity and resistance of Enterobacteriaceae, P. aeruginosa and Gram positive bacteria against Ampicillin, Ceftriaxone and Cefoperazone was compared. High resistance was observed in Enterobacteriaceae against ampicillin that showed 91% resistance whereas only 9 % strains were susceptible. The sensitivity against ceftriaxone and cefoperazone was 90% and 100%, respectively whereas only 10% strains were resistant to ceftriaxone. No resistance strains were found against cefoperazone (Fig., 3). In P. aeruginosa resistance against ampicillin, ceftriaxone and cefoperazone was 64%, 21% and 0% respectively. Higher susceptibility rates 79% and 100% were observed against ceftriaxone and cefoperazone respectively than against ampicillin where susceptible strains were only 36% (Fig., 4). Similar trend was observed in Gram positive bacteria where 68%, 30% and no resistance was observed against ampicillin, ceftriaxone and cefoperazone while 32%, 70% and 100% sensitivity for ampicillin, ceftriaxone and cefoperazone was observed, respectively (Fig.5).
Fig. 3: Comparison of sensitivity and resistance of Enterobacteriaceae against Ampicillin, Ceftriaxone and Cefoperazone.
Abbreviations: S = sensitive, R = resistant, Amp = Ampicillin, Ctx = Ceftriaxone, Cfz = Cefoperazone.

Fig. 4: Comparison of sensitivity and resistance of P. aeruginosa against Ampicillin, Ceftriaxone and Cefoperazone according to BSAC susceptibility and resistance breakpoints (BSAC, 2003).
Abbreviations: S = sensitive, R = resistant, Amp = Ampicillin, Ctx = Ceftriaxone, Cfz = Cefoperazone.
Fig. 5: Comparison of sensitivity and resistance of Gram positive bacteria to Ampicillin, Ceftriaxone and Cefoperazone according to BSAC susceptibility and resistance breakpoints (BSAC, 2003). Abbreviations: S= sensitive, R=resistant Amp=Ampicillin, Ctx=Ceftriaxone, Cfz=Cefoperazone

Table 1: Antimicrobial activity of cephalosporins and aminoglycosides against Gram negative bacterial pathogens isolated from cancer patients.

<table>
<thead>
<tr>
<th>Antimicrobial class and agent tested</th>
<th>Activity against $P. aeruginosa$</th>
<th>Activity against $^b$Enterobacteriaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC Range μg/ml</td>
<td>MIC$_{50}$</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>0.125-64</td>
<td>0.125</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.125-64</td>
<td>1</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.125-64</td>
<td>4</td>
</tr>
</tbody>
</table>

$^a$MIC$_{50}$ and MIC$_{90}$; MICs at which 50 % and 90 % of the isolates, respectively, were inhibited. The unit for all MICs are microgram per milliliter. % S, % R ; percent of isolates susceptible and resistant per BSAC criteria (2003).
Table 2: Antimicrobial activity of Cephalosporin and Aminoglycosides against Gram positive bacterial pathogens isolated from cancer patients.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MIC range µg/ml</th>
<th>MIC₅₀</th>
<th>MIC₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoperazone</td>
<td>0.125-64</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.125-64</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.125-64</td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

MIC₅₀ and MIC₉₀: MICs at which 50% and 90% of the isolates, respectively, were inhibited. The unit for all MICs are microgram per milliliter. %S; %R; percent of isolates susceptible and resistant per BSAC criteria (BSAC, 2003).

**DISCUSSION**

The potential for antimicrobial resistance is an important concern for clinicians treating patients with confirmed or suspected bacterial infections as they are often resistant to a broad range of antimicrobial agents. Detection of microorganisms in blood cultures is considered as indicator of infection and has been shown to be a valid marker for surveillance of bloodstream infection (Pittet et al., 1997). It is noted that they cause extensive changes in the microbiology, epidemiology, clinical and prognostic significance of positive blood cultures over a period of 20 year (Zinner, 1999; Saif & Shannon, 2005; Schimanski et al., 2006). During last thirty years most of the infections in cancer patients were reported to be caused by aerobic Gram negative bacilli. A shift in the bacterial spectrum towards Gram positive cocci has been reported in the western countries over the last twenty years. Although the exact cause of this shift is not known, long-dwelling intravascular devices, fluoroquinolone prophylaxis and chemotherapy-induced mucositis have been considered as important factors (Donowitz et al., 2001). This trend however is not prominent in the developing world (Pizzo, 1993).

In our study although Gram negative bacilli (44%) were the frequent isolates but more than half (56%) of the patients were infected with Gram positive cocci and S. aureus was the most common (38 %). Among the isolates a definite shift towards Gram positive microorganisms has been observed in our study. Similar shift has also been noted by Karamat et al, 1993.

In the United States and Europe, S. aureus and E. coli were identified as the two most common blood culture isolates from hospitalized cancer patients (Fritsche et al., 2003; Sader et al., 2002). Similar results have been reported by SENTRY for laboratories in the United States, Canada, Latin America, and Europe. Among the Gram negative bacteria, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumonieae are the common pathogens (Diekema, 1999). These pathogens were also common isolates with P. aeruginosa as the predominant isolate in our study. The results of the present study indicated the in vitro activities of cephalosporins and aminoglycosides against blood culture isolates of Gram positive and negative bacteria. It is clear that among cephalosporins, cefoperazone has greater in vitro activities against Gram negative bacterial isolates than ceftriaxone where 100% and 79% strains were susceptible to cefoperazone and ceftriaxone in case of P. aeruginosa and 100% and 90% sensitive strains in case of bacterial isolates belonging to Enterobacteriaceae (Fig., 3).

Increasing antimicrobial resistance has been reported for Klebsiella pneumonieae and Enterobacter by the Canadian Antimicrobial Resistance Study Group (Toye, 1993). Cefoperazone and ceftriaxone (third generation of cephalosporins) are susceptible to both Gram positive and Gram negative bacteria. Cefoprazone are highly active against bacterial pathogens isolated from cancer patients. High rate of sensitivity to Ceftriaxone and Cefoperazone (third generation of Cephalosprins) in Gram positive bacteria was also reported by (Viscoli et al., 1988).

For aminoglycosides (Ampicillin) used in this study, high resistance rates were observed in both Gram negative and positive strains. The resistance against P. aeruginosa and other gram negative bacteria was 64% and 91% respectively. In Gram positive bacteria 68% resistance was observed. High resistance to ampicillin in Gram
positive bacteria was also reported by Brinkmann et al., (2005); Zinner (1999). Ampicillin is thus not found suitable for infection treatment in cancer patients due to its high resistance rates. High rate of extended spectrum B lactamases by most of strains were reported to be major cause of high resistance.

The present study provides important information on the current resistance pattern among bacterial isolates against cephalosporins and aminoglycosides. Resistance against cephalosporins and fluoroquinolones appears to be increasing more rapidly with an increase in their use for treatment. Antimicrobial resistance rates require vigilance with respect to both the appropriate use of antimicrobial agents and continued surveillance for changes in rates of resistance among bacterium infections.

With continued antibiotic selective pressure in clinical settings, particularly in hospitals, pathogens resistant to these agents have emerged and pose further problems beyond the lack of available antimicrobial therapy. A careful monitoring of antimicrobial use in hospitals is required to identify situations in which prescription patterns are contributing to the development of resistance. The lack of any new compounds in the near future indicates that there is need of constant monitoring at national and regional level, as these surveillance efforts are imperative to provide clinicians with information for choosing empirical treatment regimens. Moreover bacterial strains resistant to most classes of antibiotics will continue to arise unless the inappropriate use of these drugs is curtailed.


REFERENCES


Role of Plant Growth Regulators in Improving Oil Quantity and Quality of Sunflower Hybrids in Drought Stress

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ABSTRACT

The present study was conducted to investigate the role of salicylic acid and ascorbic acid in alleviating the adverse effects of drought stress on oil yield and fatty acid profile of two sunflower hybrids (Hysun-33 and Hysun-38). Varying levels of salicylic acid and ascorbic acid (0, 100mg L\(^{-1}\) and 200mg L\(^{-1}\)) were applied via foliar spray and root treatment. Drought stress was imposed at vegetative and reproduction growth stages. Results revealed that imposition of drought stress at growth stages of both hybrids significantly reduced oil, stearic acid, oleic acid and linoleic acid percentages, while non-significant reduction was noted in palmitic acid. Further, rate of reduction was significantly higher at reproductive stage. Both hybrids differed significantly from each other. Hysun-38 had a maximum decrease than Hysun-33. However, application of all the concentrations (0, 100mg L\(^{-1}\) and 200mg L\(^{-1}\)) of salicylic acid and ascorbic acid via foliar spray and root treatment had significantly positive effects on all the attributes, except palmitic acid %. Foliar spray mode of application was significantly effective in respect of all the attributes, except oleic acid % as compared to root treatment. Of both the plant growth regulators, salicylic acid proved extra effective, particularly at reproductive stage. Maximum increase was recorded from 200mg L\(^{-1}\) concentration on Hysun-33 than Hysun-38.

Key words: Salicylic acid, ascorbic acid, sunflower, oil %, fatty acid profile.

INTRODUCTION

Sunflower (Helianthus annuus L) is one of the world’s most important and largest oil seed crop having average oil contents up to 40-50% (Murphy, 1994). Sunflower oil contains four important fatty acids, namely palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids (Baydar & Erbas, 2005), vitamin E, Beta-carotene, vitamin C, micronutrients and antioxidants that protect cells from oxidative damage. The presence of vitamins has further added to its quality and need because vitamin E plays an important role in prevention of heart diseases (Kirshan et al., 2006). It is known fact that environmental stress plays an important role in the economic outcome of an oilseed crop and can affect the oil yield and quality. The growth, development and distribution of plants are severely restricted by a variety of environmental stresses like, extreme temperature, cold, heavy metals, drought and salinity (Athar & Ashraf, 2005). But drought is considered one of the major limiting factors in crop management and limits the growth and distribution of natural vegetation from area to area (Athar & Ashraf, 2005).

Salicylic acid is a natural signal molecule and has been reported to play an important role in regulating a number of physiological processes in plants. Its exogenous application promotes plant performance under biotic and a biotic stress conditions (Senaratna et al., 2003). It provides protection against a number of abiotic stresses such as heat (Date et al., 1998), chilling (Kang & Saltveit, 2001), heavy metal stress (Metwally et al., 2003) and drought stress (Bezrukova et al., 2001). Salicylic acid alleviates the adverse effect of abiotic stress by increasing production of internal growth hormones such as indole acetic acid (IAA) and cytokinins (Shakirova et al., 2003). Ascorbic acid commonly known as (vitamin C) is a non-enzymatic compound that enables plants to resist stresses by reducing oxygenic free radicals constituted stress. Ascorbic acid is one of the abundantly occurring water-soluble antioxidant organic compounds and is mainly distributed in the cytosol of the plant. It is required in trace amount to maintain normal plant growth in higher plants. It is main source of vitamin C for humans and essential compound for plants with important roles as an antioxidant and as a modulator of plant development through hormone signaling (Athar et al., 2008). Ascorbic acid is synthesized in higher plants and affects plant growth and development (El-Kobisy et al., 2005). Ascorbic acid protects plants from harmful effects of higher temperature and positively increases their metabolic processes (Hathout, 1995). Salicylic acid
and ascorbic acid are economical, eco-friendly and induce stress tolerance through metabolic defense mechanisms leading to better plant growth and yields. Therefore, the aim of the present study was to determine the possible role of these plant growth regulators on oil yield and fatty acid profile of sunflower hybrid under drought stress.

**MATERIALS AND METHODS**

**Experimental conditions**

This study was conducted to evaluate the effects of exogenous application of plant growth regulators viz. salicylic acid and ascorbic acid applied via different modes. The experiment was conducted at Baluchistan Agriculture Research Institute, Quetta, Pakistan (latitude = 31°-30’ N, longitude = 73°-10’ E and altitude = 5500 ft).

**Seed material**

Seeds of two sunflower hybrids (Hysun-33 and Hysun-38) were obtained from Oil Seed Department of Baluchistan Agriculture Research Institute, Quetta, Pakistan.

**Nature of experimental field**

Before sowing chemical analysis of the soil of experimental filed was performed and soil was found medium textured (sandy loam) having 8.03 pH.

**Lay out of experimental field**

The main plot was divided into three sub plots and further every subplot into plots having four replications. Row-to-row distance was 75 cm and plant to plant distance was 20 cm. Each replication had two rows having six plants in each. Sowing was done with hand drill using seed rate 10 kg/ha.

**Drought stress treatment:**

After two weeks of seedling emergence, all the three plots were irrigated. Drought stress was then imposed to the plants grown in second and third subplots by missing irrigation at vegetative growth stage (6 week) after emergence and at reproductive growth stage after another (4 week) or (10 week) after emergence of seedlings, while, plants grown in first subplot received normal irrigation.

**Foliar spray application of plant growth regulators:**

Different (0, 100 mg L⁻¹ and 200 mg L⁻¹) concentrations of both the plant growth regulators, salicylic and ascorbic acid were applied as a foliar spray at both the growth stages of both the hybrids which were subject to drought stress.

**Root treatment application of plant growth regulators:**

Two weeks after the emergence of seedlings grown in peat-moss-sand soil filled polystyrene foam trays were irrigated to soften the soil and plants were uprooted and their roots were washed thoroughly with distill water. After this roots of the plant were dipped in the already prepared solution containing different (0, 100 and 200 mg L⁻¹) concentrations of salicylic and ascorbic acid for 6 hours. Then these treated seedlings were transplanted in the field, soon after the transplantation field was irrigated. Plants were also imposed drought stress at vegetative and reproductive growth stages.

**Determination of oil percentage**

Oil contents were determined by Nuclear Magnetic Resonance apparatus (NMR) ModelMQA-7005 Oxford instruments of USA (Granlund & Zimmerman, 1975) by standardizing the equipment with five different oil contents containing samples that had been early analyzed through Soxhletis apparatus. Dried seeds (100 gm) of each replication from each variety and each experiment unit were crushed and fed to Soxhlet extractor fitted with one litter round bottom flask and a condenser. The extraction was executed with 0.5 L of n-hexane on a water bath for six to seven hours. The solvent was distilled off in vacuum under rotary evaporator and percentage of oil was recorded.

**Determination of fatty Acid Profile**

Fatty acid profile (palmitic acid %, stearic acid %, oleic acid % and linoleic acid %) of the oil extracted from five randomly selected plants from each experimental unit was determined by gas liquid chromatography GCL (Banon et al., 1982). A set of standard of fatty acids (saturated and unsaturated) was purchased from Sigma Chemical Company St. Louis, MO 63178, USA. Fatty acid was first converted into methyl esters. For this purpose the oil (ether –extract was mixed with sodium methoxide solution in 10x 75 mm tube). The mixture was allowed to stand for thirty minutes at room temperature (25°C). Then (1M) NaCl was added and mixed. An aliquot (1U) of the resultant methyl esters was analyzed in a Shamadzu Gas Chromatography (GC-9A) equipped with glass column (packed with SP 2% 1300). The operating conditions were temperature, 225 °C, peaks were identified by comparing their retentions times with those of standard fatty acid peaks. The percent composition of fatty acid was recorded by a computing integrator.
**Statistical Analysis**

Four factor completely randomized block design (ANOVA) of the data was computed for all attributes by using the MSTAT Computer Program (MSTAT Development Team, 1989). Four factors were varieties, growth stages, treatments and mode of application of salicylic acid and ascorbic acid. The Duncan's New Multiple Range test at 5% level of probability was used to test the differences among mean values following Steel & Torrie (1997).

**RESULTS**

Variance analysis of data contained in (Table.1) revealed that imposition of drought stress at vegetative and reproductive growth stages of both hybrids induced significant (P<0.001) reduction in oil yield and stearic acid%, while, non-significant (P>0.001) reduction in palmitic acid %. Rate of reduction observed at reproductive stage was significantly higher than vegetative stage. Both the hybrids varied significantly. Hysun-33 showed less decrease compared to Hysun-38. However, varying levels of salicylic acid and ascorbic acid (0, 100 and 200 mg L⁻¹) applied via foliar spray at vegetative and reproductive stages and root treatment had significant increase for oil yield and stearic acid %, while non-significant increase for palmitic acid %. Further, increase observed at reproductive stage was significantly higher than vegetative stage. As for mode of influence evaluation, foliar spray application was significantly effective than root treatment. Moreover, drought stress alleviative effects of salicylic acid were higher than ascorbic acid. Of all the concentrations 200 mg L⁻¹ was found more effective, particularly, when was applied at reproductive stage and on Hysun-33.

### Table 1: Role of plant growth regulators in alleviating the effects of drought stress on oil %, palmitic acid % and stearic acid % when imposed at different critical growth stages.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Foliar spray</th>
<th>Root treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hysun-33</td>
<td>Hysun-38</td>
</tr>
<tr>
<td>Control</td>
<td>41.9a</td>
<td>40.6a</td>
</tr>
<tr>
<td>Irri: missed at V/S</td>
<td>26.4k</td>
<td>24.7l</td>
</tr>
<tr>
<td>Salicylic acid 100 mg L⁻¹ irri: missed at V/S</td>
<td>35.3de</td>
<td>33.1gh</td>
</tr>
<tr>
<td>Salicylic acid 200 mg L⁻¹ irri: missed at V/S</td>
<td>38b</td>
<td>36.2cd</td>
</tr>
<tr>
<td>Ascorbic acid 100 mg L⁻¹ irri: missed at V/S</td>
<td>32.3hi</td>
<td>30.2j</td>
</tr>
<tr>
<td>Ascorbic acid 200 mg L⁻¹ irri: missed at V/S</td>
<td>36cd</td>
<td>34.3ef</td>
</tr>
<tr>
<td>Salicylic acid 100 mg L⁻¹ irri: missed at R/S</td>
<td>33.2lg</td>
<td>30.9ij</td>
</tr>
<tr>
<td>Salicylic acid 200 mg L⁻¹ irri: missed at R/S</td>
<td>37.2bc</td>
<td>35de</td>
</tr>
<tr>
<td>Ascorbic acid 100 mg L⁻¹ irri: missed at R/S</td>
<td>30.1j</td>
<td>27.8k</td>
</tr>
<tr>
<td>Ascorbic acid 200 mg L⁻¹ irri: missed at R/S</td>
<td>35.2de</td>
<td>32.9fg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LSD Variety x Treatment</th>
<th>Treatment 0.8256</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid Percentages</td>
<td>Foliar spray</td>
</tr>
<tr>
<td>Hysun-33</td>
<td>8.5a</td>
</tr>
<tr>
<td>Hysun-38</td>
<td>8.5a</td>
</tr>
<tr>
<td>Treatments</td>
<td>Foliar spray</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>Hysun-33</td>
</tr>
<tr>
<td>Control</td>
<td>9.1a</td>
</tr>
<tr>
<td>Irri: missed at V/S</td>
<td>5.7kl</td>
</tr>
<tr>
<td>Irri: missed at R/S</td>
<td>5.2lm</td>
</tr>
<tr>
<td>Salicylic acid 100 mg L(^{-1}) irri: missed at V/S</td>
<td>7.5ef</td>
</tr>
<tr>
<td>Salicylic acid 200 mg L(^{-1}) irri: missed at V/S</td>
<td>8.5ab</td>
</tr>
<tr>
<td>Ascorbic acid 100 mg L(^{-1}) irri: missed at V/S</td>
<td>6.9gh</td>
</tr>
<tr>
<td>Ascorbic acid 200 mg L(^{-1}) irri: missed at V/S</td>
<td>8.1cd</td>
</tr>
<tr>
<td>Salicylic acid 100 mg L(^{-1}) irri: missed at R/S</td>
<td>7.3fg</td>
</tr>
<tr>
<td>Salicylic acid 200 mg L(^{-1}) irri: missed at R/S</td>
<td>8.3bc</td>
</tr>
<tr>
<td>Ascorbic acid 100 mg L(^{-1}) irri: missed at R/S</td>
<td>6.6ij</td>
</tr>
<tr>
<td>Ascorbic acid 200 mg L(^{-1}) irri: missed at R/S</td>
<td>7.9cd</td>
</tr>
</tbody>
</table>

**LSD Variety x Treatment** 0.5896

Means square values not sharing a common letter in row or column differ significantly.
Statistical variance analysis of data for oleic acid % and linoleic acid % are contained in (Table.2). Result, indicated that imposition of drought stress at both the growth stages of both hybrids caused a significant (P<0.001) reduction in the values of these attributes but reduction observed at reproductive stage was significantly higher than at vegetative stage. Both the hybrids differed significantly from each other. Hysun-33 showed significantly less decrease compared to Hysun-38. However, exogenous application of different concentrations of salicylic acid and ascorbic acid (0, 100 and 200 mg L$^{-1}$) via foliar spray at vegetative and reproductive stages and root treatment proved significantly effective in increasing the values of these attributes but increase observed at reproductive stage was higher. While comparing the mode of influence, foliar spray mode of application showed significantly enhanced production of palmitic acid % than root treatment, whereas, non-significant difference was noted in respect of oleic acid %. Further, higher increases were observed with 200mg L$^{-1}$ concentration of salicylic acid, particularly, when was applied at reproductive growth stage and on Hysun-33.

**Table: 2. Role of plant growth regulators in alleviating the effects of drought stress on oleic % and linoleic acid % when imposed at different critical growth stages.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Oleic acid Percentages</th>
<th>Linoleic acid Percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Foliar spray</td>
<td>Root treatment</td>
</tr>
<tr>
<td></td>
<td>Hysun-33</td>
<td>Hysun-38</td>
</tr>
<tr>
<td>Control</td>
<td>39.6a</td>
<td>38.1ab</td>
</tr>
<tr>
<td>Irri: missed at V/S</td>
<td>25.6ij</td>
<td>24.1jk</td>
</tr>
<tr>
<td>Irri: missed at R/S</td>
<td>23.4jk</td>
<td>21.9k</td>
</tr>
<tr>
<td>Salicylic acid 100 mg L$^{-1}$</td>
<td>31.6ef</td>
<td>29.7gh</td>
</tr>
<tr>
<td>Salicylic acid 200 mg L$^{-1}$</td>
<td>36.5bc</td>
<td>34.8cd</td>
</tr>
<tr>
<td>Ascorbic acid 100 mg L$^{-1}$</td>
<td>29.2gh</td>
<td>27.5hi</td>
</tr>
<tr>
<td>Ascorbic acid 200 mg L$^{-1}$</td>
<td>34.6cd</td>
<td>33de</td>
</tr>
<tr>
<td>Salicylic acid 100 mg L$^{-1}$</td>
<td>30.4fg</td>
<td>28.5hi</td>
</tr>
<tr>
<td>Salicylic acid 200 mg L$^{-1}$</td>
<td>35.5bc</td>
<td>33.3cd</td>
</tr>
<tr>
<td>Ascorbic acid 100 mg L$^{-1}$</td>
<td>27.8hi</td>
<td>25.9jk</td>
</tr>
<tr>
<td>Ascorbic acid 200 mg L$^{-1}$</td>
<td>33.6cd</td>
<td>31.8ef</td>
</tr>
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</table>

LSD Variety x Treatment 1.2456
<table>
<thead>
<tr>
<th>Control</th>
<th>50.5a</th>
<th>48.6ab</th>
<th>50.5a</th>
<th>48.6ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irri: missed at V/S</td>
<td>30.7l</td>
<td>28.5lm</td>
<td>30.7kl</td>
<td>28.6lm</td>
</tr>
<tr>
<td>Irri: missed at R/S</td>
<td>26.7mn</td>
<td>24.5n</td>
<td>26.7mn</td>
<td>24.5n</td>
</tr>
<tr>
<td>Salicylic acid 100 mg L⁻¹ irri: missed at V/S</td>
<td>40.9ef</td>
<td>38.9hi</td>
<td>38.2ef</td>
<td>36.7fg</td>
</tr>
<tr>
<td>Salicylic acid 200 mg L⁻¹ irri: missed at V/S</td>
<td>46.6bc</td>
<td>44.5de</td>
<td>43.9abc</td>
<td>42.2bc</td>
</tr>
<tr>
<td>Ascorbic acid 100 mg L⁻¹ irri: missed at V/S</td>
<td>39.1fg</td>
<td>37ij</td>
<td>36.4gh</td>
<td>34.7ij</td>
</tr>
<tr>
<td>Ascorbic acid 200 mg L⁻¹ irri: missed at V/S</td>
<td>45.5cd</td>
<td>43.3de</td>
<td>42.8bc</td>
<td>41.1cd</td>
</tr>
<tr>
<td>Salicylic acid 100 mg L⁻¹ irri: missed at R/S</td>
<td>38.7hi</td>
<td>36.5jk</td>
<td>36fg</td>
<td>34.7gh</td>
</tr>
<tr>
<td>Salicylic acid 200 mg L⁻¹ irri: missed at R/S</td>
<td>45.6cd</td>
<td>42.8de</td>
<td>42.9bc</td>
<td>41.1cd</td>
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<tr>
<td>Ascorbic acid 100 mg L⁻¹ irri: missed at R/S</td>
<td>36.4jk</td>
<td>34.5kl</td>
<td>33.7ij</td>
<td>32.1jk</td>
</tr>
<tr>
<td>Ascorbic acid 200 mg L⁻¹ irri: missed at R/S</td>
<td>43.6de</td>
<td>41.5ef</td>
<td>40.9cd</td>
<td>39.2de</td>
</tr>
</tbody>
</table>

LSD Variety x Treatment 1.1882

Means Square values not sharing a common letter in row or column differ significantly.

**DISCUSSION**

Drought stress plays a major role with genotype factor in oil production, oil quality and composition of fatty acids determines oil physical and chemical properties and end user. Drought stress strongly decreases production of oil contents (Hussain et al., 2009). It is clear from the results of present study that imposition of drought stress at both the growth stages caused a significant reduction in the oil contents of both sunflower hybrids but exogenous application of salicylic acid and ascorbic acid induced a significant increase. Same are the findings of earlier researchers that salt stress markedly reduced oil contents in two sunflower lines; however, increasing levels of foliarly-applied salicylic acid caused an increase (Noreen & Ashraf, 2010). Results of few earlier researchers who while working with sweet basil (Ocimum basilicum L.) and marjoram (Majorana hortensis) (Fatma, 2007), Mentha arvensisL.var (Abad-Farooq & Misra, 1983) and marjoram (Cheol et al., 2001) also conform that exogenous application of salicylic acid increased seed oil contents respectively. These results are also in accordance with Hendawy & Azza (2010) who while working with (Foeniculum vulgare var. azoricum) and Rawia et al., (2010) (Jasminum grandiflorum L.) found that foliar spray application of various concentrations (50, 100 and 150 ppm) of ascorbic acid produced significantly higher oil yield.

Variation in fatty acids composition is most likely caused by environmental factors, these include salt and drought stress. According to Mikami & Murata (2003) tolerance of plants to salt and drought is strongly dependent on the inheritance of fatty acids levels. Thus results of the present findings clearly indicated that imposition of drought stress caused a decrease in palmitic acid %, stearic acid %, oleic acid % and linoleic acid %, whereas, exogenous application of salicylic acid and ascorbic acid induced increase in all these attributes. Same are the findings of Karker et al., (2007) that foliar spray application of various concentrations of salicylic acid at vegetative stage and reproductive stages of groundnut cultivar GG-7 significantly enhanced free fatty acids but greater improvement was observed when cultivars were sprayed at reproductive stage. Results reported by Faizan & Bano, (2011) and Mona (2012) are also in agreement that foliar spray application of salicylic acid during flowering on safflower (Carthamus tinctorius L.) cv. Thori and two sunflower cultivars increased the production of oleic acid (C18:1) and linoleic acid (C18:2) (%) respectively. In addition, Noreen & Ashraf (2010) mentioned that high doses of salicylic acid caused marked increases in sunflower achene oil content as well as some key fatty acids. The findings of Elnaz & Ahmad, (2012) best support our results that linoleic acid and oleic
acid percentage of sunflower plants was decreased when plants were subjected to salt stress; however, salicylic acid and ascorbic acid application had a positive effect on these variables. Results of an earlier study by Aml et al., (2011) observed that exogenous application of ascorbic acid on two sunflowers (Helianthus annuus L.) hybrids increased monounsaturated fatty acids (palmitic and oleic acid), polyunsaturated fatty acids (linoleic acid) and saturated fatty acids (stearic acid). Much work has been done in evaluating the role of these plant growth regulators on different traits of different crops but very little has been done regarding oil yield and fatty acid profile of sunflower hybrids (Gray, 2004). Therefore, results of present study suggested that use of salicylic acid and ascorbic acid would be highly helpful for increasing oil yield and fatty acid contents in different oil seed crops.

REFERENCES


A contribution to the Ethnobotanical studies of some plants of Loralai District, Baluchistan

*MUHAMMAD AJAIB, QALANDER KHAN & ZAHEER-UD-DIN KHAN

Department of Botany, GC University Lahore, Pakistan

ABSTRACT

A survey of District Loralai, Baluchistan, Pakistan was carried out during 2011-2012 to document the ethnobotanical data on locally found important plants by interviewing the local people through a questionnaire. A total of 28 plant species belonging to 19 families, out of which 1 gymnosperm family and 18 families of angiosperms were documented. The data on plants included botanical names, vernacular names, the parts used and specific purpose of use. Asteraceae was found dominant family with 5 species. The plant species collected were identified mainly with the help of Flora of Pakistan and after mounting on herbarium sheets were submitted to Dr. Sultan Ahmad Herbarium GC University, Lahore after pasting voucher numbers.

Key words: Ethnobotany, Loralai District, Baluchistan

INTRODUCTION

Plants being the primary producers have the capacity to photosynthesize and transfer gasses, minerals and water to other living things. In addition to food, plants also supply human being with fiber, landscape, dyes, building material, cosmetics, medicines, etc. Plants play a significant role in our lives because they help in recycling of functional nutrients, provides us the herbal medicines that have relatively few special effects (Khan et al., 2011). Ethnobotanical search on indigenous plants is continuing at rapid pace for treatment of AIDS, cancer and inflammation (Paye, 2000).

Loralai formerly called District Bori was established on October 1903in the centre of Baluchistan, in the northeast side, lying at an altitude of 4700 feet above the sea level, at 30.333°N and 68.598°E. It is connected by road with Quetta, Ziarat, Killa Saifullah and D.G Khan District of Punjab. The total area of District Loralai is 9,933square kilometer. The District Loralai is bounded Zhob and Killa Saifullah in the North, Pishin and Ziarat in the West, Kohlu and Sibi in the South and Barkhan and Musakhel Districts in the East (Anonymous, 2011). The total population of the District Loralai is estimated to be 327,989 according to census 2005 carried out by Population Census Organization, Islamabad. Over 99% of the population is Muslims. The local inhabitants of Loralai are mainly pushtoons, Marri and Baluch tribes. The climate of the Loralai is dry but varies with changes in elevation. The weather is cold and dry at high altitude. The temperature remains uniform throughout the year, and slightly hot in summer at low altitude especially in south and East (Dukki). Summer is extended from March to October having peak summer temperature upto 40°C. Winter season being very pleasant remains from November to February. The minimum temperature in winter drops up to 5.02 °C (Anonymous, 2012). During the current investigation all the villages of three tehsils of District Loralai including Loralai, Makhtar and Dukki and their villages were chosen where indigenous communities were energetically engaged in gathering and using medicinal herbs for the treatment of human ailments.

MATERIALS AND METHODS

Ethnobotanical Study

The steps taken forethnobotanical study of the plants included:

a. Surveying of the study area
b. Laboratory work
a. Surveying of the study area

The documentation of the ethnobotanical uses on the basis of plant resources of the study area, 60 villages were surveyed to enlist and collect the plants from these areas. Indigenous information on plants was gathered from indigenous people of the area, i.e. hakims, shopkeepers, pansaries, farmers and wood sellers, etc. by interviewing them through a questionnaire. The ethnohtanical
information thus collected included the local name of the plants, their uses, parts used and methods for the preparation of different medicines along with other relevant information. Main areas visited included Nasarabad, Old Dukki, Habib Killah, Kharashang, Nana Sahib, Talal, Rabat, Baghawah, Manditak, Bawar, Katvee, Shahbusai, Lahore Deelye, Allambar, Luni, makhtar, Kangri and Chama Ollang.

b. Laboratory study

The laboratory work included pressing, drying, mounting, identification, labeling and preservation of the plants.

i) Pressing and drying

The plants collected from the field were pressed properly before wilting in between the sheets of newspaper/blotting papers. The newspapers/blotting papers were replaced after every 2 days to remove all the remaining moisture contents of the plants specimens. The plants were then pressed within wooden presser to eliminate all the wrinkles.

ii) Mounting and identification

The plant specimens after drying were mounted on the standard herbarium sheets (11 ½ x 16 ½) with the help of glue and fiber cloth tape to one plant specimen on one herbarium sheet. The mounted specimens were then identified with the help of available literature (Nasir & Ali, 1970-1989; Ali & Nasir, 1990-1992; Ali & Qaisar, 1992-2009). The local name, botanical name, family name, habit, habitat and other appropriate information on the plant specimen was printed on each herbarium sheet.

iii) Preservation

The mounted plant specimens were submitted to Dr. Sultan Ahmad Herbarium, Department of Botany, GC University Lahore after putting the voucher numbers.

RESULTS AND DISCUSSION

In total 28 plant species belonging to 27 genera and 19 families of spermatophytes were recorded. One species belonging to gymnosperms, while remaining 27 to the angiosperms. Family Asteraceae consisted of 5 species, Euphorbiaceae 3, Solanaceae 2, whereas rest of the species to other families, being one species to each family (Table 1).

The people of District Loralai use the plants for many purposes that include thatching, agricultural tools, household articles, honey bee keeping, etc. In the present study, local communities use 28 plant species of 27 genera and 19 families for different purposes. It was noticed that single plant had various local uses an observation which resembles with that of Ajaib et al. (2010 & 2012) who recorded similar results during the ethnobotanical study of shrubs and climbers of district Kotli, Azad Jammu & Kashmir.

The people of the District Loralai live in an area that had a vast diversity of vegetation because of its precipitation. But due to changes in social status, most of the people use allopathic medicines. They have no knowledge about the importance of the plants and therefore, ethnobotanical knowledge is only limited to local hakims and old aged people. People living in remote area; mainly depend on plants especially the nomads who use the plants mainly for their daily needs. The people of the area cut the plant and sell them as fuelwood, medicinal and handcraft as discussed by Zareen et al. 2013 during ethnobotanical evaluation of the shrubs of Central Punjab, Pakistan. Due to poverty in the area, lack of knowledge and ignorance, most of the people in villages still depend on herbal medicines for treating their daily ailments. A similar finding has also been reported by Azaizeh et al. (2003) after working with local Arab practitioners in Middle East region.

Recommendations

For the sustainable use of the plants of Distict Loralai, some important suggestions are as follows:

- Awareness regarding conservation and sustainable uses of plants should be provided to indigenous people.
- Plantation of more plants should be practiced and encouraged.
- There should be alternative ways of earnings for the local people to prevent deforestation.
- There should be well developed methods for the cultivation of plants.

REFERENCES

### Table 1. List of ethnobotanically useful plants of Loralai District

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Local name</th>
<th>Traditional local uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Achyranthes aspera</em> L.</td>
<td>Amaranthaceae</td>
<td>Sahargul, Naru</td>
<td>Stem is used for blood purification. The powder of the burnt plant is mixed with honey and is taken to cure cough. Ash of the plant is given to treat asthma and cough. Decoction of plant is used for skin diseases.</td>
</tr>
<tr>
<td>2. <em>Aerva javanica</em> M. Bieb.</td>
<td>Amaranthaceae</td>
<td>Kerfal, Booh</td>
<td>The decoction of the plant is used to remove swellings. Cotton wool is used to stuff pillows and quilts. Its leaves are used for medicines manufacturing and flower are used for pillow stuffing.</td>
</tr>
<tr>
<td>3. <em>Alhagi maurorum</em> Medic.</td>
<td>Papilionaceae</td>
<td>Zoz, Seez, Janasa</td>
<td>It is used as fodder for camel. It is used for fencing the fields. The decoction of plant is used as blood purifier.</td>
</tr>
<tr>
<td>4. <em>Artemisia sieversiana</em> Ehrh.</td>
<td>Asteraceae</td>
<td>Tarkha, Kaka mush</td>
<td>It is used for blood pressure, intestinal worm and indigestion. The pulp of leaves is used to give freshness to skin. It is used for ornamental and perfume.</td>
</tr>
<tr>
<td>5. <em>Calotropis procera</em> (Ait.) Ait.f.</td>
<td>Asclepiadaceae</td>
<td>Spalmi, Aak, Kuragh</td>
<td>The decoction of the leaves and fruits are used for itching of the body. Leaves are used as purgative. Latex is used for piles, baldness, toothache and as anthelmintic. Latex is toxicant cause’s blindness. Whole plant is boiled and given to buffaloes to treat skin diseases locally called “Zeharwaad”.</td>
</tr>
<tr>
<td>6. <em>Cardaria chalepensis</em> (L.) Hand.-Mazz.</td>
<td>Brassicaceae</td>
<td>Garbust</td>
<td>It is cooked as vegetable. The leaves are grind and applied on skin disease called Mamur in local language.</td>
</tr>
<tr>
<td>7. <em>Carthamus oxyacantha</em> M.Bieb.</td>
<td>Asteraceae</td>
<td>Poli</td>
<td>Root is used to cure piles. Plant without spine is given to buffalo to increase milk production. Seeds are supposed to prevent from cancer and for this purpose, seeds are slightly brown into fire and used orally.</td>
</tr>
</tbody>
</table>


8. *Citrullus colocynthis* (L.) Schard.  
Cucubitaceae  
Tuma. Kortuma  
Powdered plant is used in digestion of man and cattle. Root is used as tooth sticks to relieve toothache. The fruit is processed into sweet dishes (Locally called as *Murabba*) which are prescribed to the patients of constipation, gas troubles and liver diseases and treat remove abdominal worms.

Menispermaceae  
Zahmar  
Plant is soaked in water and decoction is used for motion and dysentery. Powder of leave is mixed with small amount of milk and is applied on the eye for freshness.

Asteraceae  
Tik, Baski  
The plant extract is very useful for wound healing, expectorant and tonic.

11. *Euphorbia helioscopia* L.  
Euphorbiaceae  
Dhadarboodi  
Milky sap is used as an ointment on the ring worms.

12. *Euphorbia hirta* L.  
Euphorbiaceae  
Washah  
Plant is used as fodder for cattle. Leaves are used as vegetable and to cure constipation.

Urticaceae  
Spandha, Malkangi  
Leaves extract is used for blood tonic and diuretic.

Cupressaceae  
Aspurse, Saroz  
Plant used for burning and its ash used for niswar. The aqueous extract from crushed fruits is anthelmintic. It is considered as one of the best timber woods of the area due to its durability. About 95% of the houses are constructed by this wood. It is also useful for hedging houses.

15. *Kickxia incana* (Wall) Pennell  
Scrophulariaceae  
Bohar  
Fruits and leaves are used as antidiabetic, laxative and tonic.

Celasteraceae  
Hekel, Malkangi  
Leaves extract is used for dysentery. Oil from seed is used medicinally.

17. *Nannorrhops ritchiana* (Griffin.) Atchison Palmae  
Pathe, Pish  
Fruits are edible. Leaves are used to make hand fans, mats, container for bread, baskets and prayer mat. The leaves are used for making rope used for weaving bed steed, hand fan, small prayer mat, large prayer mat, grain bins and for store of grain.

Labiatae  
ChingomButi, Simsok  
Seed oil is given for the treatment of rheumatism after delivery to the women.

19. *Peganum harmala* L.  
Zygophyllaceae  
Harma  
Seeds are grind to make powder and used for pain of legs where as seeds are chewed for stomach pain. The plant is burnt and the smoke is considered antiseptic and insect repellent. Leave and seeds used for treatment of asthma, jaundice and high temperature fever.

20. *Phyllanthus urinaria* L.  
Euphorbiaceae  
Saresh  
It is used as fodder for cattle. The paste of whole plant is mixed with milk and is given to the buffaloes as anthelmintic.

21. *Plantago lanceolata* L.  
Plantaginaceae  
Bar-e-Tang, Brohi Barz, Purhat  
Leaf extract is used for wound healing and seeds are used for constipation abdominal problems, eye redness and for washing hairs.

22. *Prosopis cineraria* (L.) Druce  
Mimosaceae  
Kandi, Jand  
The plant is used as fuel wood. It is also used for fencing of fields. The bark is used as a remedy for rheumatism. Women eat the flowers during pregnancy to safeguard them against miscarriage. The ashes are rubbed over the skin to remove hair. The natives eat mealy pulp
<table>
<thead>
<tr>
<th>No.</th>
<th>Genus</th>
<th>Family</th>
<th>Common Names</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td><em>Rhyzia stricta</em></td>
<td>Apocynaceae</td>
<td>Zeer Gull, Sihar</td>
<td>Plant leaves extracts are used for chronic diseases. Seeds after grinding are mixed with the rice water to control menstruation. Its stem used for burning purposes.</td>
</tr>
<tr>
<td>24</td>
<td><em>Solanum elaeagnifolium</em></td>
<td>Solanaceae</td>
<td>Sheen-e-Gulli</td>
<td>It is poisonous and mainly found in wheat fields.</td>
</tr>
<tr>
<td>25</td>
<td><em>Taraxacum officinale</em></td>
<td>Asteraceae</td>
<td>Kanhul</td>
<td>It is useful in dropsy and obstruction of the liver. It is very popular in local area for hepatic congestion associated with dyspepsia and constipation.</td>
</tr>
<tr>
<td>26</td>
<td><em>Trianthema portulacastrum</em></td>
<td>Aizoaceae</td>
<td>Kharphary, Teervik</td>
<td>It is used as fodder for cattle, goat and sheep. Roots are used in cough, asthma and fever.</td>
</tr>
<tr>
<td>27</td>
<td><em>Withania coagulans</em></td>
<td>Solanaceae</td>
<td>Khamazura, Panir</td>
<td>Fruit of plant used for diuretic, jaundice and cooling agent.</td>
</tr>
<tr>
<td>28</td>
<td><em>Xanthium strumarium</em></td>
<td>Asteraceae</td>
<td>Brat, Geskay</td>
<td>Leaves extract is used for long standing malarial fever and skin diseases.</td>
</tr>
</tbody>
</table>
Evaluation of Treated Textile Effluent for Irrigation and its Effects on Growth of Zea mays L. CV C1415

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1Sustainable Development Study Centre GC University Lahore, 2Institute of Industrial Biotechnology GC University Lahore, 3Botany Department GC University Lahore

ABSTRACT

Conventional methods used for treating textile effluent are very expensive, whereas biological treatment is cost-effective, efficient and environment friendly, but its evaluation is needed. For this purpose in the present study, treated and untreated textile effluent along control were applied to the maize (Zea mays L. CV C1415) crop to check their effects on its growth. Plant height, number of leaves, number of nodes and internodes were monitored. Photosynthesis, transpiration rate and fresh and dry weights were also measured. The results clearly indicated that all the parameters were significantly affected by untreated textile effluent as compared to control but treated effluent improved all the parameters. This study proves that treated water may be used as irrigation water on large scale to overcome water crisis.

Key words: Biotreated, Textile dyes, Maize crop, Irrigation, Physiological growth

INTRODUCTION

Textile industrial effluents are causing a detrimental effect on the living systems (Gomes et al., 2013; Hayyat et al., 2013). Textile processes release large volume of waste water, its disposal without treatment to environment causes adverse effects to terrestrial life, aquatic biota, crops and livestock. Untreated textile effluents in water bodies cause serious environmental and health hazards. Land irrigated with textile effluents act as a sink for heavy metals and other resistant chemicals consequently reducing soil fertility. Pollutants leach down and cause contamination of ground water. These contaminants enter in the food chain and become health risk for plants, animals and ultimately to humans (Ross, 1994; Jadhav et al., 2010). Crops irrigated with water containing textile effluents show remarkable reduction in yield (Hayyat et al., 2013).

Conventional methods (ozonation, photooxidation, electrocoagulation, adsorption, activated carbon, froth flotation, reverse osmosis, ion exchange, membrane filtration and flocculation) used in past (Daneshvar et al., 2004) to treat textile effluents are now proved insufficient and are very expensive (Sagehashi et al., 2009). Presently, biological treatment has gained much importance and is cost-effective, efficient and environment friendly, but the suitability of the treated effluents in this way is still to be ascertained finally for the crops. Thus, it is of great concern to assess the phytotoxicity of the textile effluents before and after treatment. Phytotoxicity tests in relation to different plants demonstrated that the biodegraded products did not interfere with the germination of plant seeds. The phytotoxicity studies have revealed that the metabolites generated after the biodegradation are non toxic than the original dye (Telke et al., 2010; Saratale et al., 2013). Researchers around the globe indicated the importance of effluent's phytotoxicity (Tigni et al., 2011). Effects of effluents and detoxified textile dyes have been studied on germination of crops (Saratale et al., 2009; Khandare et al., 2013). But for the assessment of comprehensive consequences, there is a need to check the toxicity of treated effluents on complete life cycle of crops. The objective of the current study was to use the textile effluent treated with consortium BMP1/SDSC/01 and to investigate its effects on growth of maize in order to find the efficiency of treated effluents.

MATERIALS AND METHODS

Sample collection

Textile effluents were collected in screw capped sterilized plastic cans from main channel of Nishat Mills Pvt. Limited 5Km Off - 22Km Ferozepur Road Lahore, Pakistan (APHA, 2005; Mahmood et al., 2012). The treated effluents by consortium BMP1/SDSC-01 (Mahmood et al., 2013) were collected from Sustainable Development Study Centre, GC University Lahore.
Effect of treated and untreated textile effluents on physiological growth parameters of Maize

Experiment was conducted at Botanic Garden, GC University Lahore on Zea mays L. CV C1415 (Maize) to check efficacy of treated textile effluents. Thoroughly cleaned and polyethylene lined earthen pots of 30 cm diameter filled with botanic garden soil were used in experiment. The holes at the bottom of the pots were closed with pebbles in order to prevent excessive drainage. Overnight soaked seeds of maize (4 in each pot) were grown. After one week of germination, two plants of same size in each pot were selected after thinning. Experiment comprised of three replicates for each: control, untreated and treated textile effluent. After one week of thinning, plants were irrigated with untreated and treated textile effluent while the control plants were irrigated with tap water for whole experiment at regular time intervals. Plant height, number of leaves and number of nodes & internodes were monitored on weekly basis for a period of 12 weeks. Photosynthesis and transpiration rates were also monitored monthly and recorded by IRGA (Infra Red Gas Analyzer LCA4). At the time of harvest the plant fresh and dry weights were also determined (Aslam et al., 2007).

Statistical analysis
Data was analyzed by one-way analysis of variance (ANOVA) using software package Co-stat version 3.03 to check the significance of treatments (Steel et al., 1997).

RESULTS

Effect of treated and untreated textile effluents on plant height of maize

Effect of biotreated and untreated textile effluents on plant height of maize was monitored for a period of 12 weeks. The plants irrigated with untreated effluents showed significantly less growth as compared to the control as after one week of the experiment the height of former plant was 57.51% less than the later plants, whereas the plants irrigated with biotreated effluents showed significantly more growth than those irrigated with untreated effluent. The height of former plants was 72.32% more than the latter plants, respectively but as compared to control plants the biotreated plants had 26.78% less height after the same time period. Similar trend was observed throughout the experiment (Table 1).

Effect of treated and untreated textile effluents on number of leaves of maize

Effect of biotreated and untreated textile effluents on number of leaves of maize was also observed throughout the monitoring period on weekly basis. The results clearly indicated that the highest number of leaves was present in control plants which were significantly reduced (P ≤ 0.05) in plants irrigated with untreated textile effluent, while in plants irrigated with biotreated effluents the number of leaves was almost same as in case of control. At the end of monitoring period, it was noticed that 11.49±0.721, 7.62±0.176 and 9.41±0.862 number of leaves were present on control plants, plants irrigated with untreated and plants irrigated with biotreated textile effluent respectively (Table 2).

Effect of treated and untreated textile effluents on number of nodes and internodes of maize

Effect of biotreated and untreated textile effluents on nodes and internodes of maize was observed for 12 weeks. It was found that number of nodes in first week 30.59% less in plants irrigated with untreated effluents as compared to plants irrigated with control whereas plants irrigated with biotreated effluents showed 23.22% more. But the number of nodes in plants irrigated with biotreated effluents was 14.47% less than that of plants irrigated with control. Similar trend was observed throughout the experiment. Similarly, the number of internodes was 32.06% less in plants irrigated with untreated effluents than that of plants irrigated with control. And improvement in number of nodes was 43.63% in plants irrigated with biotreated effluents than that of plants irrigated with untreated effluents. The results indicated maize plants growing in textile effluents showed decrease in number of nodes and internodes throughout the growing season (Table 3 & 4).

Effect of treated and untreated textiles effluent on transpiration and photosynthesis rate of maize

Photosynthesis rate in maize plants irrigated with untreated effluents was reduced to 53.32% than that of plants irrigated with control. Whereas photosynthesis rate was improved to 86.05% in plants irrigated with biotreated effluents. Likewise in transpiration rate 57.29% was reduced in plants irrigated with untreated effluents than that of plants irrigated with control. Whereas, it was improved to 104% in plants irrigated with biotreated effluents but it was 12.5% less than plants irrigated with control (Figure 1 a & b).

Effect of treated and untreated textile effluents on fresh and dry weight of maize

Total fresh and dry weight in maize was reduced to 41.2% and 23.60% of plants irrigated with untreated effluents than that of plants irrigated...
with control, respectively. While on applying biotreated textile effluents fresh and dry weights of plants were improved (Figure 2a). The results indicated that textile effluent had toxic effects on maize. Results denoted the reduction in dry and fresh weight of root, shoot and leaves of plants treated with textile effluent in contrast to control. Biological treatment of effluent indicated significance in results of dry and fresh weights of root, shoot and leaves (Figure 2b).

**DISCUSSION**

Water scarcity has a great impact on human life as it becomes one of the most pressing problems. The global challenges to meet future demand are constrained by sustainable freshwater availability (Manez et al., 2012). Reuse of treated effluents in irrigation is a good idea. The untreated textile effluents impose high load of complex dyes which diminishes the quality of water bodies used for irrigation. This practice reduces the soil fertility and crop yield (Khan et al., 2010). The toxicity of effluents in plants causes growth inhibition, reduces photosynthesis and transpiration, decreases roots and shoots length (Mallick et al., 2010). Stomatal conductance in most of plants is affected by toxic pollutants present in effluents. It was also reported that toxic effluents reduced fresh and dry weight of plant (Hayyat et al., 2013; Singh et al., 2013).

The textile effluents treated by isolated indigenous bacteria may lead to the generation of variety of products (Saratate et al., 2013). Therefore, it is important to study the toxicity impact of these degraded products on the life stages of crops in order to overcome yield reduction. Phytotoxicity impact of textile effluents and treated effluents on plant height, number of leaves, photosynthesis and transpiration rate and biomass of Zea mays L. CV C1415 (Maize) a common crop of Punjab, Pakistan were studied. The result clearly indicated that treated textile effluents are less toxic as compared to untreated effluents which are in agreement with the results reported by Phugare et al. (2011). The study revealed that reuse potential of effluents for irrigation as current source are depleting day by day, but it requires regular monitoring (Augustine, 2009). The results have highlighted the benefits of reuse of textile effluents after treatment. This study proves that treated water may be used as irrigation water at large scale to overcome water crisis.

**ACKNOWLEDGEMENTS**

The authors admiringly acknowledge the Sustainable Development Study Centre, Government College University Lahore for providing the support to carry out this research.

**REFERENCES**


Table 1: Effect of treated and untreated textile effluent on plant height (cm) of Zea mays L. CV C1415

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1\textsuperscript{st} week</th>
<th>2\textsuperscript{nd} week</th>
<th>3\textsuperscript{rd} week</th>
<th>4\textsuperscript{th} week</th>
<th>5\textsuperscript{th} week</th>
<th>6\textsuperscript{th} week</th>
<th>7\textsuperscript{th} week</th>
<th>8\textsuperscript{th} week</th>
<th>9\textsuperscript{th} week</th>
<th>10\textsuperscript{th} week</th>
<th>11\textsuperscript{th} week</th>
<th>12\textsuperscript{th} week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33.26 ± 0.374a</td>
<td>44.10 ± 1.265a</td>
<td>56.96 ± 0.049a</td>
<td>67.37 ± 0.883a</td>
<td>79.51 ± 0.721a</td>
<td>89.18 ± 1.152a</td>
<td>102.27 ± 1.803a</td>
<td>108.11 ± 1.251a</td>
<td>115.48 ± 0.735a</td>
<td>121.11 ± 1.258a</td>
<td>127.17 ± 1.654a</td>
<td>131.47 ± 0.671a</td>
</tr>
<tr>
<td>Untreated</td>
<td>14.13 ± 1.223c</td>
<td>23.4 ± 0.848b</td>
<td>32.03 ± 1.371c</td>
<td>40.95 ± 1.477c</td>
<td>54.11 ± 1.576c</td>
<td>59.97 ± 0.035c</td>
<td>65.85 ± 0.212c</td>
<td>73.77 ± 0.319c</td>
<td>80.51 ± 0.142b</td>
<td>87.47 ± 0.7b</td>
<td>89.98 ± 0.393c</td>
<td>93.2 ± 1.781c</td>
</tr>
<tr>
<td>Treated</td>
<td>24.35 ± 0.912b</td>
<td>37.99 ± 1.407a</td>
<td>47.84 ± 0.219b</td>
<td>57.93 ± 1.506b</td>
<td>71.51 ± 2.13b</td>
<td>82.39 ± 2.276b</td>
<td>91.33 ± 0.947b</td>
<td>101.15 ± 1.195b</td>
<td>108.37 ± 0.883a</td>
<td>114.34 ± 0.933a</td>
<td>117.61 ± 0.979b</td>
<td>124.41 ± 2.00b</td>
</tr>
<tr>
<td>LSD</td>
<td>3.65</td>
<td>6.19</td>
<td>3.10</td>
<td>1.51</td>
<td>3.06</td>
<td>4.82</td>
<td>6.13</td>
<td>2.25</td>
<td>7.34</td>
<td>7.91</td>
<td>8.72</td>
<td>3.07</td>
</tr>
</tbody>
</table>

Mean followed by different letters in the table are significantly different at \( P=0.05 \) according to Duncan’s multiple range test, ± standard deviation, LSD: least significance difference.

Table 2: Effect of treated and untreated textile effluent on number of leaves of Zea mays L. CV C1415

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1\textsuperscript{st} week</th>
<th>2\textsuperscript{nd} week</th>
<th>3\textsuperscript{rd} week</th>
<th>4\textsuperscript{th} week</th>
<th>5\textsuperscript{th} week</th>
<th>6\textsuperscript{th} week</th>
<th>7\textsuperscript{th} week</th>
<th>8\textsuperscript{th} week</th>
<th>9\textsuperscript{th} week</th>
<th>10\textsuperscript{th} week</th>
<th>11\textsuperscript{th} week</th>
<th>12\textsuperscript{th} week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.74 ± 0.226a</td>
<td>3.86 ± 0.084a</td>
<td>4.88 ± 0.021a</td>
<td>5.48 ± 0.169a</td>
<td>6.17 ± 0.106a</td>
<td>5.94 ± 0.926a</td>
<td>7.23 ± 0.190a</td>
<td>8.77 ± 0.233a</td>
<td>9.66 ± 0.098a</td>
<td>10.65 ± 0.205a</td>
<td>10.97 ± 0.176a</td>
<td>11.4 ± 0.721a</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.86 ± 0.084b</td>
<td>2.63 ± 0.098b</td>
<td>3.34 ± 0.197b</td>
<td>3.94 ± 0.219b</td>
<td>4.27 ± 0.176b</td>
<td>4.82 ± 0.169a</td>
<td>5.14 ± 0.219b</td>
<td>5.53 ± 0.091c</td>
<td>5.96 ± 0.19c</td>
<td>6.36 ± 0.042b</td>
<td>7.13 ± 0.176b</td>
<td>7.62 ± 0.176b</td>
</tr>
<tr>
<td>Treated</td>
<td>2.03 ± 0.091b</td>
<td>2.78 ± 0.155b</td>
<td>3.59 ± 0.156b</td>
<td>4.47 ± 0.240b</td>
<td>5.17 ± 0.197a</td>
<td>5.36 ± 0.190b</td>
<td>6.03 ± 0.077b</td>
<td>6.44 ± 0.205b</td>
<td>6.95 ± 0.233b</td>
<td>±0.247b</td>
<td>7.72 ± 0.862ab</td>
<td>9.41 ± 0.862ab</td>
</tr>
<tr>
<td>LSD</td>
<td>0.67</td>
<td>0.57</td>
<td>0.77</td>
<td>0.75</td>
<td>0.92</td>
<td>2.40</td>
<td>1.02</td>
<td>0.46</td>
<td>0.96</td>
<td>1.08</td>
<td>0.65</td>
<td>3.42</td>
</tr>
</tbody>
</table>

Mean followed by different letters in the table are significantly different at \( P=0.05 \) according to Duncan’s multiple range test, ± standard deviation, LSD: least significance difference.
Table 3: Effect of treated and untreated textile effluent on number of nodes of *Zea mays* L. CV C1415

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; week</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; week</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; week</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>5&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>6&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>7&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>8&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>9&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>10&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>11&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>12&lt;sup&gt;th&lt;/sup&gt; week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.04±0.077 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.01±0.127 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.14±0.197 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.73±0.240 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.10±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.00±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.61±0.155 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.13±0.190 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.48±0.028 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.01±0.155 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.38±0.021 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.89±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Un-treated</td>
<td>2.11±0.155 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.47±0.035 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.09±0.148 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.04±0.056 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.53±0.098 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.74±0.219 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.03±0.091 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.47±0.056 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.76±0.240 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.17±0.014 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.09±0.176 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.62±0.017 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Treated</td>
<td>2.60±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.14±0.077 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.44±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.30±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.69±0.049 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.33±0.113 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.98±0.091 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.27±0.106 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.43±0.162 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.02±0.098 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.21±0.098 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.57±0.098 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LSD</td>
<td>0.57</td>
<td>0.74</td>
<td>0.79</td>
<td>0.87</td>
<td>0.68</td>
<td>0.82</td>
<td>0.57</td>
<td>0.21</td>
<td>0.14</td>
<td>0.19</td>
<td>0.45</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Mean followed by different letters in the table are significantly different at P=0.05 according to Duncan’s multiple range test, ± standard deviation, LSD: least significance difference

Table 4: Effect of treated and untreated textile effluent on number of internodes of *Zea mays* L. CV C1415

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; week</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; week</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; week</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>5&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>6&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>7&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>8&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>9&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>10&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>11&lt;sup&gt;th&lt;/sup&gt; week</th>
<th>12&lt;sup&gt;th&lt;/sup&gt; week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.04±0.077 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.01±0.127 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.14±0.197 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.73±0.240 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.10±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.00±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.61±0.155 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.13±0.190 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.48±0.028 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.01±0.155 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.38±0.021 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.89±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Un-treated</td>
<td>2.11±0.155 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.47±0.035 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.09±0.148 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.04±0.056 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.53±0.098 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.74±0.219 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.03±0.091 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.47±0.056 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.76±0.240 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.17±0.014 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.09±0.176 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.62±0.017 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Treated</td>
<td>2.60±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.14±0.077 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.44±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.30±0.148 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.69±0.049 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.33±0.113 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.98±0.091 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.27±0.106 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.43±0.162 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.02±0.098 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.21±0.098 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.57±0.098 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LSD</td>
<td>0.57</td>
<td>0.74</td>
<td>0.79</td>
<td>0.87</td>
<td>0.68</td>
<td>0.82</td>
<td>0.57</td>
<td>0.21</td>
<td>0.14</td>
<td>0.19</td>
<td>0.45</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Mean followed by different letters in the table are significantly different at P=0.05 according to Duncan’s multiple range test, ± standard deviation, LSD: Least Significance Difference
Fig. 1: Effect of treated and untreated textile effluent on transpiration (a) and photosynthesis (b) of Zea mays L. CV C1415

Fig. 2: Effect of treated and untreated textile effluent on total fresh and dry weights (a), root, shoot and leaves fresh and dry weights (b) of Zea mays L. CV C1415
Preliminary effect of sub-lethal dose of BPA on Biochemical Profile of Grass Carp (*Ctenopharyngodon idella*) fry

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**ABSTRACT**

Bisphenol A (BPA, 4,4-isopropylidenediphenol) belongs to the family of endocrine disrupting chemicals and is commonly used in the production of epoxy resins and polycarbonate (PC) plastics. The aim of present study was to determine the acute toxicity of BPA to Grass carp fry and its effect on biochemical constituents. The 96-hours LC50 of BPA for grass carp fry (*Ctenopharyngodon idella*) was calculated as 3.44mg/l using Finney probit analysis. The effect of sub-lethal concentration of BPA on biochemical constituents like cholesterol, glycogen, total proteins and lipids was evaluated at 95% confidence interval.

**Keywords:** *Ctenopharyngodon idella*, acute toxicity, LC50, Bisphenol A.

**INTRODUCTION**

Bisphenol A (BPA) an important industrial chemical, primarily used as an intermediate in the production of polycarbonate plastics and epoxy resins. Resins containing BPA are usually used in coating metal products such as food cans, beverage cans, bottle caps and water supply pipes (Soares et al. 2008). From production and processing facilities and sewage treatment plants, known releases of small amounts of BPA enter the environment (Staples et al., 1998; Cousins et al., 2002). As the demand for polycarbonates and epoxy resins is increasing, the production of BPA is also increasing (Morgan, 2006).

The annual global production of BPA reached 640,000 tons in 1993, and estimated 0.017% was released in to the environment (Benjonathan & Steinmetz, 1998; Staples et al., 1998). Its distribution in the environment has been estimated as, 24% in soil and 43% in water bodies (TemaNord, 1996). Acute toxicity bioassays (LC50 and lethal concentration) are used for the evaluation of toxicity and assessment of fish susceptibility to toxicants (Abdullah et al., 2007). Acute toxicity tests provide rapid assessment of impact of toxicants on organisms (Azmat et al., 2011).

Fish are most at risk because their habitat receives the greatest input of anthropogenic pollution, including xenoestrogens (Kime, 1998). Fish accumulate large amount of toxicant in its body and act as good indicator of pollution (Olaifa et al., 2004).

The objectives of present study was to determine the acute toxicity of grass carp fry and determine the preliminary effects of BPA on basic biochemical profile of grass carp fry.

**MATERIALS AND METHODS**

The fish fry of Grass carp (*Ctenopharyngodon idella*) obtained from Fisheries Research and Training institute, Manawan, Lahore were acclimatized for a week in glass tanks before using in the experiment.

**Acute study**

Acute BPA toxicity tests were conducted with grass carp (*Ctenopharyngodon idella*). A group of 60 fish fry were taken and divided into 10 subgroups. 06 fish fry per group were placed in glass aquaria having 70 L of dechlorinated tap water. Dissolved oxygen, temperature and pH of water were maintained in suitable range. These fish fry were exposed to different concentrations of BPA (0.5mg/l-5mg/l). Toxicant solution was replaced after 24 hours and percentage mortality was calculated till 96 hours (APHA, 2005). LC50 (conc in which 50% of fish die) was calculated through probitregression analysis, using SPSS (Finny, 1971).

**Sub lethal studies**

For sub lethal studies, a group of 40 fish fry were taken and divided into 4 treatment groups. (10 fry per group). One group served as control and three served as experimental groups.

The median lethal dose of BPA for grass carp (*Ctenopharyngodon idella*) for 96 hours was found to be 3.44 mg/l. From this dose, non-lethal...
A dose of 0.344 mg/l and 1.72 mg/l, (1/10th, and ½ of LC₅₀) were selected and fish fry were exposed to 3.44 mg/l, 0.344 mg/l and 1.72 mg/l for 96-hours. After 96 hours of exposure, 05 fish fry were randomly selected from control and experiment groups for the protein, glycogen, cholesterol and lipid analysis.

Biochemical analysis

Proteins were determined by method of Lowery et al., (1951) as modified by Schacterle & pollak (1973). Total lipids were determined by using the method of Woodman & Price (1971). Glycogen content was determined using method of Lone & Matty (1980). Cholesterol was determined by Bowman & Wolf (1962) method.

Statistical procedure

Probit-regression analysis was done using SPSS-15. Data was analyzed to evaluate statistically significant result using student t-test. Minitab 15 was used for data analyses.

RESULTS AND DISCUSSION

Acute toxicity studies of BPA on the grass carp fry showed significant changes in the biochemical constituents. According to probit-regression analysis, 96-h LC₅₀ of BPA for fry of grass carp was 3.44 mg/l with 95 % lower and upper confidence limits as 2.962mg/l and 3.716mg/l respectively. This finding is supported by data of acute toxicity on other fish. Chen et al., (2012) calculated the 72-h LC₅₀ for zebra fish embryos and larvae as 1.8222mg/l and 10.943 mg/l respectively. Faheem & Lone (2013) reported 96-h LC₅₀ for Cirrhinus mrigala fingerlings as 3.67358 mg/l.

As the exposure time and dose increase the fish showed increased surfacing behavior and loss of balance. Physiological changes include loss of body color near fins and eyes become bulgy and pale in color (Fig:1).

Table 1: Probit-regression analysis of grass carp (Ctenopharyngodon idella) for against Bisphenol A at 96 hours Post exposure (SPSS-15).

<table>
<thead>
<tr>
<th>Conc. mg/l</th>
<th>Total no. of fish</th>
<th>Observed responses</th>
<th>Expected responses</th>
<th>LC₅₀ mg/l</th>
<th>Lower confidence limit</th>
<th>Upper confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>6</td>
<td>0</td>
<td>0.083</td>
<td>3.44</td>
<td>2.96</td>
<td>3.716</td>
</tr>
<tr>
<td>3.0</td>
<td>6</td>
<td>0</td>
<td>1.192</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>6</td>
<td>4</td>
<td>3.705</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>6</td>
<td>6</td>
<td>5.410</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>6</td>
<td>6</td>
<td>5.909</td>
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<td></td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>6</td>
<td>5.990</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values of lipid increased as the dose and exposure time increased but this increase is not significant at P=0.05. This increase may be due to the fact that BPA mimic estrogen like activity and estrogen is a known obesogen (Schneider et al., 1979). Phrakonkham et al. (2008) reported that dietary xenoestrogens increased the expression of adipocyte differentiation genes. It was observed that BPA also enhanced glucose transport in adipocytes (Sakurai et al., 2004), which in turn may contribute to lipogenesis.
Table 2: Changes in biochemical constituents of the fish fry, (Ctenopharyngodon idella) exposed to the 1/10th of LC$_{50}$, ½ of LC$_{50}$ and LC$_{50}$ of BPA

<table>
<thead>
<tr>
<th>Biochemical constituents</th>
<th>Exposure for 96 hour</th>
<th>Control</th>
<th>1/10$^{th}$ of LC$_{50}$ (0.344mg/l)</th>
<th>½ of LC$_{50}$ (1.72mg/l)</th>
<th>LC$_{50}$ (3.44mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Protein mg/100ml</td>
<td></td>
<td>0.127±0.017</td>
<td>0.533±0.20 n.s</td>
<td>1.646±0.017</td>
<td>2.604±0.498</td>
</tr>
<tr>
<td>Total Lipids mg/100ml</td>
<td></td>
<td>0.551±0.28</td>
<td>1.648±0.24 n.s</td>
<td>1.88±0.14 n.s</td>
<td>2.88±0.80 n.s</td>
</tr>
<tr>
<td>Total Glycogen mg/100ml</td>
<td></td>
<td>6.92±0.219</td>
<td>26.25±1.250 n.s</td>
<td>41.425±1.42</td>
<td>51.215±7.28</td>
</tr>
<tr>
<td>Total Cholesterol mg/100ml</td>
<td></td>
<td>3.215±0.575</td>
<td>3.82±0.019 n.s</td>
<td>4.42±0.139 n.s</td>
<td>10.16±0.296 n.s</td>
</tr>
</tbody>
</table>

Values expressed as Mean ±S.E.M and Data analyzed using t-test at 95% confidence interval. P=0.05 n.s= non-significant, *=significant

Protein levels differ significantly in fish exposed to LC$_{50}$ and ½ LC$_{50}$, as compared with control. This increase may because of the fact that BPA and other endocrine disrupting chemicals cause up regulation of proteins. Lemos et al., (2010) found BPA induce differential protein expression in a terrestrial isopod. Present study showed that glycogen level increased significantly at 1/10$^{th}$ exposure of BPA while cholesterol level increase was not significant.

Published experimental work on acute toxicity of BPA and its effects on fish biochemistry are very limited. Further work with BPA acute toxicity testing in fish and its effects on biochemistry will be very useful in accessing possible ecological risk of BPA.

REFERENCES


Soluble protein contents from *in-vivo* and *in-vitro* sources of *Brassica juncea*, var. poorbiraya, under salt stress

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Department of Botany, Govt. College University Lahore

ABSTRACT

Soluble protein contents, produced under salt stress, from in vivo and in vitro sources of *Brassica juncea* were evaluated. Salt stress was created by NaCl alone and by combination of NaCl + CaCl₂ 2H₂O + MgSO₄.7H₂O ranging from (control) 0 to 200mM equimolar strength. Results indicated a gradual increase in soluble protein contents in in vitro samples while in vivo samples showed gradual decrease in protein contents under increasing salt stress conditions. In vitro studies might establish possible co-relation, between salt stress, water stress, proteins and antioxidant.

Key words. *Brassica juncea* var. poorbiraya, soluble proteins, salt stress, water stress, tissue culture, antioxidant.

INTRODUCTION

Salinity and drought are the twin environmental stresses which are badly affecting plant life (Kavi-Kishor *et al*., 1995, Skirver & Mundy 1990). Up to 20% of irrigated land in arid and semiarid regions is affected by salt; the area is expanding with every passing day (Mühling & Läuchli, 2003). Of the two factors salinity is the most important factor which is badly hampering plant productivity and thus survival of the plants (Eker *et al*., 2006). Furthermore the salt tolerance studies, in plants had shown a close relationship in salt and water stress phenomena (Munir & Aftab, 2009). Under salt stress plants had to manage the stress imposed by low external water potential and high ion toxicity, due to accumulation of ions, inside the plants (Romero-Aranda *et al*., 2006). It has been observed that salt stress leads to oxidative stress resulting in the accumulation of Reactive Oxygen Species (ROS) and free radicals (Azevedo-Neto *et al*., 2006; Ashraf 2009), not only increasing the antioxidant contents but also its activity (Frary *et al*., 2010).

High salt concentration either causes an increase in the N-contents and high protein content in some glycophytic plants (Abed El- Baki, 1996) or an increase in soluble proteins (Shaddad *et al*., 2005). It has also been reported by Kuznetsov *et al*., (2007) that under environmental stress number of N-containing compounds accumulates in plants, amino acids like proline, asparagine and amino butyric are produced which can play important roles in osmotic adjustment of plant under saline conditions (Gilbert *et al*., 1998).

Brassica juncea* var. poorbiraya, is a major oil seed crop of Indo-Pak subcontinent. In vitro studies for salt tolerance of *Brassica juncea* has been reported by Jains *et al*., (1991 a, b). The salt tolerance mechanisms exhibited by cells towards salinity have been shown associated with a number of factors, which includes ion exclusion, ion compartments, favourable ion balance, proline accumulation (Daines & Gould, 1985, Shah *et al*., 1990, Yang *et al*., 1990) production of certain polyanimes and rapid release of stress ethylene (McCue & Hanson, 1990) by changing the hormonal balance (Nilsen & Orcutt, 1996) as growth in saline soils is controlled by hormonal signals rather than water relations (Munns, 2002). The levels of several polypeptides especially some basic proteins had been reported to be stimulated by addition of NaCl to culture medium (Yen *et al*., 1997).

Study was an attempt to develop cell lines of *Brassica juncea* var poorbiraya, which can endure the stress of salt for cultivation in the salt claimed soils of Punjab.

MATERIALS AND METHODS

Seeds of *Brassica juncea* var. poorbiraya were collected from NARC (National Agriculture Research Council) Islamabad and were germinated aseptically in Petri plates. The plumule, hypocotyls, root and leaf were obtained from fifteen days old seedling. Each ex-plant was incubated on MS (Murashige & Skoog 1962) medium supplemented with 3 mg/L NAA + 1 mg/L BAP in the presence of various concentrations (0, 10, 25, 50, 100, 150, 200 mM) of NaCl alone or a combination of NaCl + CaCl₂ 2H₂O + MgSO₄.7H₂O in equimolar strength.

Physical conditions of cultures were maintained at 26 ± 1°C under 16 hours photoperiod.
at 3K lux of cool white light provided by florescent tubes. Explants and calli were sub-cultured after regular interval of 4 to 6 weeks. Protein estimation was carried out on 12 weeks old callus after Roenson & Johnstone (1961).

RESULTS AND DISCUSSION

In-vivo soluble protein contents of leaf decreased gradually with increasing salt stress (Table 1) while in-vitro results showed gradual increase in soluble proteins. Salt stress created by combination of salts were more stress inducing as compared to when single salt was used in spite of the fact that both were used in equal strength. Same trend was observed in plumule, hypocotyl and root. In all cases in-vivo reading showed reduction in soluble protein formation while in vitro results showed gradual increase in the soluble protein contents.

At the molecular level one of the most extensively characterized stress response in higher plants is the synthesis of stress shock proteins (SSP) as was also observed in this study. The proteins, being capable of creating more stability in the presence of high concentration of Na⁺ in the cytoplasm, are reported to be synthesized under a variety of stresses such as high temperature, desiccation, heavy metals, chilling, anoxia and salinity (Uma et al., 1995).

Various plant species, even the different parts of the same plant are reported to differ in adopting different strategies for response to different levels of salt stress in relation to protein accumulation (Yen et al., 1997). Thus metabolic changes make plants respond differently to the stress. It was observed in Bruguiera parviflora, that total protein contents of leaf gradually decreased with increasing NaCl concentration (Parida et al., 2004). In the same fashion, total protein contents of tomato cultivars decreased by increasing salt stress (Zeynep et al., 2010). Same trend was also observed in Phaseolus vulgaris, but in Phaseolus acutifolius increasing NaCl concentration does not affect relative water or protein contents (Yurekli et al., 2004).

<table>
<thead>
<tr>
<th>Strength equimolar</th>
<th>Salts used</th>
<th>Proteins mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L</td>
</tr>
<tr>
<td>Control 10</td>
<td>A</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.50</td>
</tr>
<tr>
<td>25</td>
<td>A</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.41</td>
</tr>
<tr>
<td>50</td>
<td>A</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.32</td>
</tr>
<tr>
<td>100</td>
<td>A</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.25</td>
</tr>
<tr>
<td>150</td>
<td>A</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.12</td>
</tr>
<tr>
<td>200</td>
<td>A</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.02</td>
</tr>
</tbody>
</table>

A = NaCl, L = Leaf, P = Plumule, H = Hypcotyl, Rc = Root callus
B = NaCl + CaCl₂ + MgSO₄, Lc = Leaf callus
Pc = Plumule callus
Hc = Hypcotyl callus

This variation in metabolic response can be attributed to the kind and distribution of various endogenous and/or exogenous hormones. A correlation might exist between stress and hormone distribution or vice versa. Increasing salt stress was observed to increase abscisic acid (Sibole et al., 1998), IAA level in leaves of Lycopersicum pennelli behaved similarly (Yurekli, 2004). Moreover as it
was reported by Hare et al., (1997) that application of cytokinin activated transcription of stress – inducible genes in plants. It seems that under stress genetically modified plants (GMPs) with desired genes could be promising solution for biotic and abiotic stresses (Mohamed et al., 2010), which are affecting food production so badly. In vivo and in vitro studies showed considerable difference in their response. This might be due to the application of exogenous hormones and response of individual cells of callus (in vitro) rather than response of cells of organs (in vivo). For stress studies biotic or/and abiotic it seems desirable to use in vitro cell source instead of in vivo source. This might establish possible co-relation, between salt stress, water stress, proteins and antioxidant.

REFERENCES


**Iris aitchisonii** (Bakar) Boiss.: A potential source of Natural Antioxidants

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& SABAHAT ZAHRA SIDDIQUI²

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²Department of Chemistry, GC University, Lahore, Pakistan

**ABSTRACT**

The organic and aqueous plant extracts of *Iris aitchisonii* (Bakar) Boiss. were obtained in petroleum ether, chloroform, methanol and water and were tested for their antioxidant potential, using four techniques, i.e. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity, total antioxidant activity, ferric reducing antioxidant power (FRAP) assay and ferric thiocyanate assay along with the determination of their total phenolic contents. The results revealed that among these fractions the chloroform soluble fraction showed highest DPPH radical scavenging activity, i.e. 92.17±1.25% inhibition of DPPH radical at a concentration of 130 µg/ml with IC₅₀ value 48.55±1.08 relative to butylated hydroxytoluene (BHT), having IC₅₀ of 12.52 ± 0.89 µg/ml. Methanol extract showed highest total antioxidant activity, i.e. 1.18±0.09 as well as highest FRAP value, i.e. 142.33±0.96 TE µM/ml. Chloroform and methanol extract showed considerable amounts of total phenolic contents, i.e. 122.33±0.12 and 121.83±0.85 GAE mg/g respectively. Chloroform extract showed good value of inhibition of lipid peroxidation, i.e. 51.61±0.64.

**Key words:** Antioxidant activities, *Iris aitchisonii* (Bakar) Boiss., FRAP assay, Total phenolic contents, Total antioxidant activity, IC₅₀

**INTRODUCTION**

Plants have been a source of medicine for thousands of years, and phytochemicals continue to play an essential role in medicine (Aggarwal et al., 2003). Medicinal plants are in greater demand due to their increased popularity and it is being suggested by a large number of conservation groups, that wild medicinal plants should be brought into cultivation. Numerous medicinal plants as well as their purified components have shown beneficial therapeutic potentials. Various herbs and other plant species are reported to show antioxidant activity. Majority of the antioxidant potential is due to the presence of flavones, flavonoids, isoflavones, anthocyanin, lignans, coumarin, catechins and isocatechins in plants (Aqil et al., 2006). Antioxidant-based drug products are being used for the treatment and prevention of complicated diseases like atherosclerosis, diabetes, stroke, Alzheimer’s disease, and cancer (Devasagayam et al., 2004). In living organisms, free radicals are produced as a result of the normal metabolic process, and also free radical chain reactions normally occurring as respiratory chain reaction in the mitochondria, through xanthene oxidase activity, liver mixed function oxidases, atmospheric pollutants and from the transitional metal catalysts, xenobiotics, and drugs. In addition to this chemical mobilization of the fat stores in different conditions such as lactation, fever, exercise, infection, and even fasting, may result in enhanced radical activity, and damage. Oxidative injury or free radicals now appears as the fundamental mechanism, causing a number of the human neurologic and many other disorders. Peroxidation of lipids can be initiated by the oxygen free radical, which in turn stimulates the glycation of protein, inactivation of some enzymes, and alteration in the function and structure of collagen basement and a few other membranes, and also play a role in chronic complication of diabetes (Ara & Nur, 2009). *Iris aitchisonii* (Bakar) Boiss. belongs to a monocotyledonous family Iridaceae and is locally used to treat various diseases. It is a herb up to 35cm in height. The plant is common in grassy fields of Brooth near Khuiratta, flowering during March-April. Locally this plant is called Sanp Buti and is used as diuretic, cathartic and antidote for snakebite. It is a toxic plant and is used very carefully (Ajaib, 2012).

**MATERIALS AND METHODS**

**Plant Material**

The plant *Iris aitchisonii* (Bakar) Boiss. was collected from District Kotli, Azad Jammu & Kashmir during April 2011, identified and deposited in Department of Botany, GC University, Lahore as a voucher specimen no. GC.Bot.Herb.0701.
**Test organisms:**
Gram –ve, Gram +ve bacteria and fungi were obtained from PCSIR Laboratories Lahore, as test organisms.

**Extraction and Fractionation of Antioxidants**
About 250 gm shade-dried and well ground whole plant was extracted successively with non-polar and polar solvents, like petroleum ether, chloroform, methanol and water using maceration technique. The extracts, concentrated on rotary evaporator, were used to evaluate their in vitro antioxidant potential.

**Antioxidant Assays**
The DPPH radical scavenging activity of various extracts was examined by comparison with that of a known antioxidant, butylated hydroxytoluene (BHT) using the reported method of Lee et al. (2001). The percent of DPPH decoloration of the samples was calculated according to the formula:

\[
\text{Antiradical activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

The total antioxidant activity of various extracts was evaluated by phosphomolybdenum complex formation method following Prieto et al. (1999). The FRAP assay was according to Benzie and Strain (1996) while Total phenolics of various extracts were determined after Makkar et al. (1993). The antioxidant activity of various extracts on inhibition of linoleic acid peroxidation was investigated by thiocyanate method following Valentao et al. (2002). Each sample was assayed in triplicate and mean values were calculated for statistical analysis.

**RESULTS AND DISCUSSION**

**DPPH radical scavenging activity**
The various extracts of *Iris aitchisonii* tested for their percent of DPPH radical scavenging activity indicated that activity was increased by increasing the concentration of the fractions in the assay. The various concentrations of chloroform extract exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 92.17±1.25% inhibition of DPPH radical at a concentration of 130µg/ml. The \( IC_{50} \) values were also calculated (Table 1). Lowest the \( IC_{50} \) value, greater was the DPPH radical scavenging activity. Chloroform extract showed lowest \( IC_{50} \) value, i.e. 48.55±1.08 relative to \( IC_{50} \) 12.52 ± 0.89µg/ml of butylated hydroxytoluene (BHT). Methanol extract showed moderate activity (\( IC_{50} \) 189.10±2.36) while petroleum ether and aqueous extracts showed no significant activities.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Sample?</th>
<th>Concentration in assay (µg/ml)</th>
<th>% scavenging of DPPH ± S.E.Ma)</th>
</tr>
</thead>
</table>
| 1.      | Petroleum ether extract | 1000  
500  
250  
130 | 72.59±1.39  
60.03±1.28  
50.0±1.19  
42.46±0.91 |
| 2.      | Chloroform extract | 130  
60  
30 | 92.17±1.25  
52.41±1.06  
43.01±1.13 |
| 3.      | Methanol extract | 500  
250  
130  
60 | 69.08±1.97  
59.04±1.86  
48.37±1.05  
36.44±0.94 |
| 4.      | Aqueous extract | 1000  
500  
250  
130 | 73.79±1.63  
52.71±1.58  
48.49±1.61  
34.33±0.89 |
| 5.      | BHTb) | 60  
30  
15  
8 | 92.46 ± 0.25  
74.57 ±0.39  
49.61 ± 0.55  
28.33 ± 0.83 |

a) All results are presented as mean ± standard error of three assays.  
b) Standard antioxidant.

**Total Antioxidant Activity by Phosphomolybdenum Complex Method**
The total antioxidant activities of the extracts were measured and compared with the standard antioxidant BHT (Table 2). It was revealed that methanol extract showed highest total antioxidant activity, i.e. 1.182±0.09 as compared to other fractions. The chloroform fraction also showed reasonably good value, i.e. 0.951±0.05. Petroleum ether and aqueous extracts showed very less values (0.432±0.03 and 0.139±0.02 respectively). The results were compared with BHT, a reference standard having total antioxidant activity 1.293 ± 0.09.
The FRAP assay of the methanol extract showed highest value, i.e. 142.64±0.96 TE μM/mL. Chloroform extract also showed a good value of 127.33±0.69 TE μM/mL while petroleum ether and aqueous extracts showed poor FRAP values. High FRAP values may be ascribed partially to the presence of phenolic and flavonoid contents. The results were compared with the blank having value 10.73.

**Total Phenolic Contents**

Table-2 shows the phenolic contents in the studied fractions of plant extracts in milligrams of gallic acid equivalents (GAEs) per gram of fraction. Among them, chloroform and methanol extract showed good total phenolic contents, having values very near to each other, 122.33±0.12 and 121.83±0.85 GAE mg/g respectively. Petroleum ether and aqueous extracts showed poor values. The results were compared with the blank having a value, 12.68.

**Ferric Thiocyanate (FTC) Assay**

It was observed from the results (Table 2) that that chloroform extract showed highest value for % inhibition of lipid peroxidation, i.e. 51.61±0.64%. Methanol extract showed moderate activity (27.99±0.69%) while petroleum ether and aqueous extracts showed no significant activities in this assay. The results were compared with BHT, i.e. 62.93 ± 0.78%.

**DISCUSSION**

The model of scavenging the stable DPPH radical is very widely used to evaluate antioxidant activities in a relatively short time. The addition of extracts to the DPPH solution caused a rapid decrease in the optimal density at 517nm. The degrees of decoloration indicated the scavenging capacity of the extracts. Free radicals cause autoxidation of unsaturated lipids in food. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical scavenging activity. Antioxidants cease the free radical chain of oxidation to donate hydrogen from the phenolic hydroxyl groups. Hence 92.17±1.25% inhibition of DPPH radical at a concentration of 130μg/ml of chloroform extract exhibited significant percent inhibition of DPPH radical as compared to the inhibition by other fractions as recorded by Abbasi et al. (2012). The significant total antioxidant activity in phosphomolybdenum method, i.e. 1.182±0.09 and 0.951±0.05 of methanol and chloroform extracts, respectively, confirms the presence of ascorbic acid, some phenolics, tocopherols and carotenoids. The presence of such compounds has already been indicated by Prieto et al. (1999). Ferric Reducing Antioxidant Power (FRAP) assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions and the reducing power of methanolic extract showed good FRAP value, i.e. 142.64±0.96 TE μM/mL while chloroform extract also possessed good FRAP value.
value, i.e. 127.33±0.69 TE μM/mL. These results were similar to those of Ajaib et al. (2013), but on different plant species, such as *Echinochloa colona* (Linn.) Link and *Sporobolus coromandelianus*. Chloroform and methanol extract showed good total phenolic contents having values 122.33±0.12 and 121.83±0.85 GAE mg/g respectively. Similar results were also obtained by Abbasi et al. (2012) and Malik et al. (2012).

REFERENCES


Bacteriology of Sub-Clinical Mastitis in the Dairy Buffaloes Maintained at Private Farms of Yazman, Distt. Bahawalpur.

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* Provincial Diagnostic Laboratory, 16-Cooper Road, Lahore.

ABSTRACT

Mastitis, known to be the most costly disease around the globe and a number of mastitogens belonging to various classes are associated with clinical & sub-clinical mastitis. Of a total of 5461 dairy buffaloes, 1833 buffaloes were found to be positive for mastitis (clinical n=156, sub-clinical n=1677), maintained at private farms located in urban & peri-urban areas of tehsil, Yazman district Bahawalpur. The diagnosis of sub-clinical mastitis was based upon surf field mastitis test (SFMT). Animals suffering from sub-clinical mastitis were divided into four groups (A, B, C, & D) & 200 animals were randomly selected (n=50 from each group). A total of 220 milk samples were collected using standard methods & subjected to isolation & identification. 7 bacterial species were isolated out of 243 isolates recovered. Coagulase negative Staphylococci spp. were found to be most prevalent (32.51%). Among other bacteria, Staphylococcus aureus was 18.11%, Escherichia coli 15.23%, Streptococcus agalactiae 13.17%, Streptococcus dysgalactiae 9.47%, Streptococcus pyogenes 6.99%, & Corynebacterium bovis 4.53%.

Key Words: Sub-clinical mastitis, Bacteria, Buffaloes

INTRODUCTION

Mastitis has been known to be the most costly disease of dairy animals around the globe & has been ranked at first position according to Field Surveys of Major Livestock Diseases of Pakistan (Hussain et al., 2005). This disease is associated with huge economic losses as it affects both the quantity & quality of milk. On an average, in dairy buffaloes, mastitis has been reported to shorten the lactation period by 57 days as well as reduction of 438 kg of milk production per lactation (Cady et al., 1983). In Pakistan, economic losses due to mastitis are not available yet; however, in Punjab only, it accounts for a total of Rs. 240 millions per year (Chaudhry & Khan, 1978). These losses are due to: 1) discarding the milk, 2) loss of milk production, 3) culling of animals & replacements, & 4) decline in quality of milk & milk products (Khan & Khan, 2006).

Mastitis is categorized as clinical & sub-clinical. Clinical mastitis is characterized by abnormalities in udder (swollen, painful, & reddened udder) & milk (clots & flakes in milk) whereas sub-clinical mastitis is characterized by loss of milk production without having any visible sign of abnormalities in udder as well in milk (Khan & Khan, 2006). Sub-clinical mastitis is a disease of high economic importance due to the fact that it is 15 to 40 times more prevalent than its clinical form & its presence usually remains hidden as well (Shearer & Harris, 2003). It prevails for a longer duration. The animal suffering from sub-clinical mastitis is usually a source of infection for other animals within a herd. Mastitis occurs as a result of interaction between the host & pathogens (mastitogens). These mastitogens are classified as contagious, environmental, minor, & uncommon (Radostits et al., 2000).

The present study was largely aimed to explore the bacteria associated with sub-clinical mastitis which will help in the treatment and the control strategy based upon rational approach could be devised.

MATERIALS & METHODS

Diagnosis of Mastitis & Collection of Milk Samples:

Of a total of 5461 dairy buffaloes, 1833 buffaloes were found to be positive for mastitis (clinical n=156, sub-clinical n=1677), maintained at private farms located in urban & peri-urban areas of tehsil Yazman. The diagnosis of sub-clinical mastitis was based upon surf field mastitis test (SFMT) demonstrated by Muhammad et al. (1995) whereas clinical mastitis was diagnosed by abnormalities in milk & udder. Animals suffering from sub-clinical mastitis were divided into four groups (A, B, C, & D) based upon location of farms & 200 animals were randomly selected (n=50 from each group). The farms present in close vicinity were categorized in
the same group. A total of 220 milk samples were collected from the selected animals, using standard aseptic method prescribed by National Mastitis Council Inc., USA (Anonymous, 1990). The collected milk samples were stored & then subjected to isolation & identification.

Isolation & Identification of Bacteria
Each milk sample was subjected to isolation & identification after culturing using the procedures described by National Mastitis Council Inc., USA (Anonymous, 1987) except for the isolation of Staphylococcus aureus for which milk samples were cultured on Staph. 110 medium (Oxoid, UK). The isolated S. aureus was then subjected to standard biochemical testing recommended by Cowan & Steel (1965).

RESULTS
A total of 243 isolates comprising of 7 bacterial species were recovered (Table 1) in which Coagulase negative Staphylococci spp. were found to be most prevalent accounting for 32.51% of all isolates. Among other bacteria, Staphylococcus aureus were 18.11%, Escherichia coli 15.23%, Streptococcus agalactiae 13.17%, Streptococcus dysgalactiae 9.47%, Streptococcus pyogenes 6.99%, and Corynebacterium bovis 4.53% (Fig. 1).

Among the groups (A, B, C, & D), the highest number of isolates n=78 (32.10%) were recovered from group C (Table 2), followed by group A (24.69%), group B (22.22%), & group D (20.99%). The highest prevalence of S. aureus (45.45%), Streptococcus agalactiae (50%), & E. coli (62.16%) was also observed in group C. On the other hand, coagulase negative staphylococci & Streptococcus pyogenes were highest in group B, accounting for 37.97% & 47.06% respectively (Fig. 2). Corynebacterium bovis was prevalent only in group B (81.82%) & C (18.18%) whereas Streptococcus dysgalactiae was the most prevalent in group D (39.13%).

DISCUSSION
Mastitis has been known as the inflammation of parenchyma of udder occurring due to variable etiology including pathogens. Among the pathogens, bacteria are regarded as the most frequently occurring mastitogens. In our present study, coagulase negative Staphylococci spp. was most frequently isolated bacteria followed by S. aureus, E. coli, Streptococcus agalactiae and so on. Coagulase negative Staphylococci spp. have been observed to be prevalent in dairy herds where mastitis control practices recommended by mastitis monitoring bodies are adopted (Barlett, 1992). However, the prevalence of two contagious bacteria (S. aureus & Streptococcus agalactiae) in our study, collectively accounting for 31.28% in addition to coliform bacteria (E. coli 15.23%) reveals that no control practices have been adopted in this area which is in agreement with Ali et al. (2008). Among the groups, the highest prevalence of bacterial species was observed in group C due to the fact that the highest number of milk samples was obtained from the animals of this group. More than one teat (either 2 or 3) were mastitic in 17 buffaloes. Therefore, high number of milk samples resulted in highest number of isolates in group C.

CONCLUSIONS
In the present study, it is clear that contagious as well as coliform bacteria are highly prevalent. A regular surf field mastitis test should be practiced for a timely diagnosis of sub-clinical mastitis so that proper treatment may be initiated. The treatment program should be devised after monitoring the sensitivity of bacteria prevalent in this area.

REFERENCES


Table 1: Frequency distribution of bacterial isolates recovered from dairy buffaloes at private farms of Yazman

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Species</th>
<th>No. of isolates</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coagulase negative Staphylococci spp.</td>
<td>79</td>
<td>32.51</td>
</tr>
<tr>
<td>2</td>
<td>Staphylococcus aureus</td>
<td>44</td>
<td>18.11</td>
</tr>
<tr>
<td>3</td>
<td>Escherichia coli</td>
<td>37</td>
<td>15.23</td>
</tr>
<tr>
<td>4</td>
<td>Streptococcus agalactiae</td>
<td>32</td>
<td>13.17</td>
</tr>
<tr>
<td>5</td>
<td>Streptococcus dysgalactiae</td>
<td>23</td>
<td>9.47</td>
</tr>
<tr>
<td>6</td>
<td>Streptococcus pyogenes</td>
<td>17</td>
<td>6.99</td>
</tr>
<tr>
<td>7</td>
<td>Corynebacterium bovis</td>
<td>11</td>
<td>4.53</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>243</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 1. Distribution of bacteria at private farms in urban & peri urban of Yazman. Coagulase –ve Staphylococci spp.= coagulase negative Staphylococcus spp.; Str. agalactiae = Streptococcus agalactiae; Str. dysgalactiae = Streptococcus dysgalactiae; Str. pyogenes = Streptococcus pyogenes; C. bovis = Corynebacterium bovis

Fig. 2. Distribution of bacterial pathogens among the groups associated with sub-clinical mastitis at private farms in urban & peri urban areas of Yazman. Coagulase –ve Staphylococci spp.= coagulase negative Staphylococcus spp.; Str. agalactiae = Streptococcus agalactiae; Str. dysgalactiae = Streptococcus dysgalactiae; Str. pyogenes = Streptococcus pyogenes; C. bovis = Corynebacterium bovis
Table 2: Frequency distribution of bacterial isolates among the groups (A, B, C, & D) of dairy buffaloes suffering from sub-clinical mastitis in Yazman

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of Isolates among the groups</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (%)</td>
<td>B (%)</td>
</tr>
<tr>
<td>Coagulase negative Staphylococi spp.</td>
<td>30 37.97</td>
<td>19 24.10</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>07 15.91</td>
<td>09 20.45</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>04 10.81</td>
<td>07 18.92</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>05 15.62</td>
<td>03 09.38</td>
</tr>
<tr>
<td>Streptococcus dysgalactiae</td>
<td>06 26.09</td>
<td>05 21.74</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>08 47.06</td>
<td>02 11.76</td>
</tr>
<tr>
<td>Corynebacterium bovis</td>
<td>0 0.00</td>
<td>09 81.82</td>
</tr>
<tr>
<td>Total</td>
<td>60 100.0</td>
<td>54 100.0</td>
</tr>
</tbody>
</table>
Nutritional comparison of three fish species co-cultured in an earthen pond

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ABSTRACT

The aim of this study was to investigate proximate composition of the muscles of the three commonly demanded freshwater carp species, raised under the semi-intensive culture conditions. Twenty seven specimens of three Indian major carps, Labeo rohita (n=9), Catla catla (n=9), Cirrhinus mrigala (n=9) were evaluated for proximate composition. The muscles of Labeo rohita were highest in crude protein (19.3%) and fat (2.7%) contents but lowest in carbohydrates (1.9%) and moisture (75%). In Cirrhinus mrigala crude protein was less (17.37%), carbohydrates were highest (2.8%) and ash contents (0.96%) were lowest. Conversely, Catla catla had highest moisture (77.3%) and ash (1.4%) but lowest crude protein (16.9%). In conclusion, all the three co-cultured carp fish species are ranked as lean. Labeo rohita showed significantly higher muscle protein contents than C. mrigala and C. catla. Moreover, this is the highly liked and greatly demanded fish locally. Hence, L. rohita is nutritionally better than two other species.

Key words: Fish farming, Indian major carps (Labeo rohita, Cirrhinus mrigala, Catla catla), proximate composition.

INTRODUCTION

Freshwater fishes constitute a great food potential for human population. Fish products comprise an important ingredient in the human diet to upgrade their nutritional standards. While nutritional value of fish obviously depends on their biochemical composition (Prado, et al., 2009). Fish is widely used throughout the world as besides being a good source of biologically high valued protein, it also provides other benefits such as lowering of blood cholesterol level. Fish contain significant amounts of essential amino acids, especially lysine which is low in cereals. Therefore, fish protein may be used to complement important amino acids and also to improve overall protein quality of a mixed diet (FAO, 2005).

Freshwater fishes are not only a rich source of high quality protein, minerals and vitamins but they also contain nutritionally valuable lipids and fatty acids. Owing to their rapid growth, size, taste and nutritional quality, Cirrhinus mrigala, Labeo rohita and Catla catla are economically important freshwater fish species in Pakistan. Healthy fishes’ fat contains essential polyunsaturated fatty acids like arachidonic acid (ω 6), eicosapentaenoic acid (EPA= ω 3) and docosahexaenoic acid (DHA= ω 3) which are not synthesized in human body. But being essential for the growth and development, these fatty acids (FA) must be supplied in the diets like the case of essential amino acids (Ismail, 2005). Several studies have shown significant changes in whole body, specific organs or muscle or other tissues’ composition of fishes responding the variations in feeding frequency, ration, starvation, age, migration, sex and temperature etc. (Millikin, 1982; Weatherley & Gill, 1987). Fish have noteworthy position in nutrition, income, employment and foreign exchange earning of a country. The information of fish composition is essential for its maximal utilization (Silva & Chamul, 2000). Fish is safer and healthier to be consumed when compared with goat, mutton, buffalo meat and chicken meat. Compared to other sources of protein, fish are best sources of high valued protein (Louka, et al., 2004).

Knowledge of the proximate analyses of important carp fish species is desirable due to recent dietary and medical emphasis. Proximate body composition is analysis of moisture, crude fat, crude protein and ash and carbohydrate contents of fish (Cui & Wootton, 1988). Percentage of moisture content in fish muscles is good indicator of its relative contents of energy, crude proteins and fat. Lower the percentage of moisture, greater the crude fat and protein contents and higher the energy
density of the fish meat (Dempson, et al., 2004). However, these values vary noticeably within and among species, translated by differences of size, reproductive stage, physical activity and feeding season (Weatherley & Gills, 1987). There is a wealth of literature available on body composition of various fish species (Berg, et al., 2000; Dempson, et al., 2004; Ali, et al., 2005). Main objective of this study was to investigate proximate composition of the muscles of the three commonly demanded freshwater fish species, raised under the same conditions.

MATERIALS AND METHODS

Nine specimens, each of C. mrigala, L. rohita and C. catla were caught from the Punjab University Fish Research Farm (PUFRF), Lahore, Pakistan after the approval of the university ethics committee. After morphometric measurements, the specimens were dissected and muscle of each specimen was sampled and stored at -20°C till further use. Frozen fish muscle samples were carried to the Newcastle University, U.K. and were stored there also at -20°C till further use. The tissues were subsequently freeze dried to determine moisture contents and ground afterwards. The freeze dried muscle samples were analyzed for their crude protein (Kjeldahl nitrogen x 6.25) using CN analyzer, ash by burning samples at 550°C and crude fat through Soxhlet apparatus. Cleaned Soxhlet flasks were placed in an oven at 100°C for 15 min to remove any moisture and then placed in a desiccator for cooling. Each flask was weighed and identified. The thimbles were tarred and known weight of ground freeze dried fish muscle samples was put into them. The Soxhlet extractors with reflux condensers and the previously weighed flasks were fitted on to their stands. The thimbles were placed into the extractors and petroleum ether (boiling point = 40-60°C, Fisher Limited U.K.) was added. The flasks were heated on a uniform heat for 6 hrs to extract the crude fat from the muscles samples. The flasks were removed from the extractors and left in an oven at 60°C for 1 hr to evaporate any excess petroleum ether. The flasks were then cooled in a desiccator and re-weighed. The following formula was used to calculate the percentage of fat extracted:

\[
\text{Fat extracted} \% = \frac{\text{Final wt. of flask with fat} - \text{Initial wt. of flask}}{\text{Wt. of dried muscle sample}} \times 100
\]

Total carbohydrates of fish muscles were determined by subtracting the % values of moisture, ash, crude protein and fat contents from 100.

Statistical analysis

The data were statistically analysed using Minitab software to compare each of the nutrients of the three fish species. These effects were declared significant if P<0.05, very significant if P<0.01 and highly significant if P<0.001. Turkey’s test was used for comparing more than two means for statistical difference at P<0.05.

RESULTS AND DISCUSSION

Twenty seven specimens of, C. mrigala, L. rohita and C. catla with mean total lengths and wet body weights of; 39.92 ± 3.66 cm, and 645 ± 194.71 g; 37.49 ± 3.62 cm and 633 ± 182.01 g and 37.01 ± 3.1947 cm and 624 ± 167.23 g, respectively, were used in this study. The weights and lengths did not significantly differ (P>0.05) among the sampled fish specimens. These biometric data were reported earlier by Shakir, et al. (2010). Overall moisture contents in the sampled fishes’ muscles fluctuated from 74.96 to 77.10% and fell within the ranges observed by other researchers (Sharma, et al., 2010; Memon, et al., 2011). Similar moisture range were recorded in silver and grass carp by Ashraf et al. (2011). The crude fat varied from 1.75 to 2.71% in the sampled fishes’ muscles. While the crude protein contents were highest in L. rohita (19.30%) followed by C. mrigala (17.37%) and C. catla (16.86%). Mehboob, et al. (2003) reported comparable results for fat contents (1.30-2.94 %) of wild L. rohita. On the other hand, Ashraf et al. (2011) reported that farmed culturable grass carp and silver carp are nutritionally better than their wild species. Crude fat in muscles is generally considered as the most important constituent, which determines the quality of fish meat (Love, 1988). Based on fat content and crude protein, all farm fish species of this study were ranked as lean and high protein fish, because fat contents of their muscles were lower than 5% (Rahman, et al., 1995) while the crude protein contents greater than 15% (Stansby, 1976). The low lipid content might be ascribed to environmental factors, species differences and the type of diet the fishes were feed up on. Ash content of the muscles ranged from 0.96 to 1.36%. Normally the ash content range gives an indication that the fish samples may be good sources of minerals such as calcium, potassium, zinc, iron and magnesium (Bolawa, et al., 2011).

Among the studied carp species, L. rohita is rated as good quality fish from the nutritional point
of view. *Labeo rohita* can be referred to as a high protein fish. They can be utilized maximally by food processors for raising added fish products such as fish cake, fish crackers and fish burger. However, all the three carp fish species represent suitable potential industrial material for possible utilization for different products by food industries.

**Table 1: Mean muscle proximate compositions (%) of three carp fish species netted from Punjab University Fish Research Farm, Pakistan.**

<table>
<thead>
<tr>
<th>Composition</th>
<th>Fish species</th>
<th>SEM and significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. mrigala</em></td>
<td><em>L. rohita</em></td>
</tr>
<tr>
<td>Crude protein</td>
<td>17.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crude fat</td>
<td>1.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.71&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>2.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ash</td>
<td>0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Moisture (% wet weight)</td>
<td>77.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.96&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means within the same row with the same letters did not differ significantly (P>0.05)

*Labeo rohita* is highly liked and demanded freshwater fish in Pakistan. This study has indicated that it may be ranked first among the three species in terms of its highest protein and moderate ash contents. While regarding the crude fat contents, nature of the fatty acids is yet to be determined before any dietary recommendation can be made in this regard.

**Acknowledgements**

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Application of Length Categorization System for Endangered Indus Mahseer (*Tor Macrolepis*)

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², ⁴Zoology Department, GC University, Lahore, Pakistan  
³Department of Zoology, PMAS Arid Agriculture University, Rawalpindi, Pakistan

ABSTRACT

Standardized length categories for a species are an important tool used to summarize the length frequency data and deriving it to indices like proportional stock density and relative stock densities which in turn can be used for stock management. In this paper, we developed standard length categories for endangered Indus Mahseer (*Tor macrolepis*) and demonstrated their use for calculation of various stock density indices. These length categories were based upon the maximum length (2,000 mm standard length) reported in literature. We proposed the following length categories: stock = 43 cm (17 in); quality = 76 cm (30 in); preferred = 104 cm (41 in); memorable = 127 cm (50 in); and trophy = 160 cm (63 in). Using these standard length categories, proportional stock density (PSD) was 22 for Indus Mahseer sampled from Mangla Dam.

Keywords: Standardized length categories, Indus Mahseer, *Tor macrolepis*, proportional stock density, Mangla Dam

INTRODUCTION

Mahseer are globally acknowledged as prestigious game and food fish. The Indus Mahseer, *Tor macrolepis*, is found in upper reaches of river Indus and its tributaries (Mirza, 2003; Mirza et al., 2006; Mirza et al., 2011). The catches of this species from wild populations have been decreasing over the last few years (Mirza et al., 2012). Due to fishing pressure, the species has become endangered. Although these dwindling populations are now being augmented with the hatchery reared stock, the success of these measures could not be ascertained due to the lack of biological data on this species.

Fish abundance, size composition and age structure are amongst the primary parameters used to describe the status of fish population. The length frequency data is simple and inexpensive tool commonly used to evaluate the fish stock in an area. The data is used to assess the population parameters like growth rate, mortality rate, length at first recruitment etc. The sport fisheries is often managed to allow for a specifically chosen length range of a species. In spite of wide use, length frequency data is often difficult to interpret the results in terms of chosen standard / length to assess the fisheries. To overcome this difficulty, concept of proportional stock density (PSD= number of fish ≥ specified length/ number of fish ≥ stock length X 100) and relative stock density (RSD= number of fish ≥ specified length/ number of fish ≥ stock length X 100) was introduced by Anderson (1976) using two-cell model (quality length and stock length). Subsequently Gabelhouse (1984) expanded the concept and proposed a five-cell categorization of the length frequency data. As these stock density indices are derived from the number of fish of harvestable size, these provide valuable information to manage the stocks in desired state. That is why this categorization system is now widely used and has become de-facto standard (De Grammont & CuarON, 2006; Flammang et al., 1993; Guy et al., 2006; Hyatt & Hubert, 2001; Milewski & Brown, 1994; Shuman et al., 2006; Vadás, 1990). Inspite of its widespread use, no information is available about the use of this tool on fish populations in Indian subcontinent which highlights that there is an urgent need to define the length categories for the commercial / sport fishes in the region.

The objective of this paper is to develop standard length categories for the Indus Mahseer, *Tor macrolepis*, and demonstrate its practical usage to assess its population status using length frequency data for population from Mangla Dam.

MATERIALS AND METHODS

In order to define the length categories, the method proposed by Gabelhouse (1984) was used. This method requires that standard length
categories should fall within the range of 20-26% (stock length), 36-40% (quality length), 45-55% (preferred length), 59-64% (memorable length), and 74-80% (trophy length) of the world record length of the species. For calculating the length categories for Indus Mahseer, maximum length of 2,000 mm described by Mirza & Bhatti (1993) and Mirza (2004) was used. Indus Mahseer is a slow growing, long lived fish and this fact was kept in view while proposing the length categories. By convention, both Metric and English units have been proposed.

For demonstrating the use of length categorization system to assess the stock indices for Indus Mahseer, the length data of catch was obtained from catches from Mangla Dam during November 2010. The indices were calculated following Anderson et al. (1996) and Shuman et al. (2006) as given below.

\[
\text{Proportional stock density (PSD)} = \frac{\text{Number of fish} \geq \text{quality size}}{\text{Number of fish} \geq \text{stock size}} \times 100
\]

\[
\text{Relative stock density (RSD-}\ P) = \frac{\text{Number of fish} \geq \text{preferred size}}{\text{Number of fish} \geq \text{stock size}} \times 100
\]

\[
\text{Relative stock density (RSD-}\ M) = \frac{\text{Number of fish} \geq \text{memorable size}}{\text{Number of fish} \geq \text{stock size}} \times 100
\]

\[
\text{Relative stock density (RSD-}\ T) = \frac{\text{Number of fish} \geq \text{trophy size}}{\text{Number of fish} \geq \text{stock size}} \times 100
\]

**RESULTS AND DISCUSSION**

The standard length categories proposed for Indus Mahseer are presented in Table 1. These length categories are: stock = 43 cm (17 in); quality = 76 cm (30 in); preferred = 104 cm (41 in); memorable = 127 cm (50 in); and trophy = 160 cm (63 in). The Mahseer is a rheophilic (flow loving) cyprinid (Order Cypriniformes; Family Cyprinidae), slow growing and long lived fish. When these length categories were applied to length frequency data of Indus Mahseer population in Mangla Dam, the stock density indices obtained are shown in Figure 1. Although the length frequency graph shows that the most abundant category is 45-55 cm but it does not convey any information about the quality of the stock which is liked by the anglers. When this sample length frequency data was used to calculate the stock indices, it turned out that the stock had a PSD value of 22 which means that 22% of the sample is larger than the quality stock (76 cm). Similarly the Relative stock density (RSD) values at preferable, memorable and trophy sizes all having value of 1 which means that only 1% of the sampled stock is larger than the preferred size. The results highlight that the proportion of larger / older individuals is scarce in the stock which implies a high fishing pressure. As an example, if the management objective is to maintain quality stock, say at 30%, then appropriate management measures like increasing the minimum length restriction could be imposed so that stock evolves in the desired direction.

The five cell length categorization method was devised keeping in view the length of fish anglers prefer catching. The stock of game fish is often managed to allow for maximum number of fish in the size ranges preferred by the angles. Carline et al. (1984), Serns (1985), and Bettross & Willis (1988) have noted that PSD can vary by seasons owing to the exploitation pattern. If the values PSD and RSD value for different seasons and years are available, information can be derived about the direction of evolution of stock and corrective measures could be taken. The results highlighted in this study suggest that an aggressive scientific management of the stock in Mangla Dam is required to ensure recovery of already imperiled Indus Mahseer populations.

**REFERENCES**


Hyatt, M. W., & Hubert, W. A., 2001. Proposed Standard-Weight (Ws) Equation and


Table 1: Recommended standard length categories for Indus Mahseer based on the Gabelhouse (1984) five-cell model and using maximum fork length of 2,000 mm.

<table>
<thead>
<tr>
<th>Length category</th>
<th>Minimum (mm)</th>
<th>Maximum (mm)</th>
<th>Proposed standards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metric (cm)</td>
<td>English (in)</td>
<td></td>
</tr>
<tr>
<td>Stock</td>
<td>400</td>
<td>520</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quality</td>
<td>720</td>
<td>820</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preferred</td>
<td>900</td>
<td>1100</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Memorable</td>
<td>1180</td>
<td>1280</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophy</td>
<td>1480</td>
<td>1600</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1: Length frequency histogram and stock density indices for Indus Mahseer population sample collected during November 2010 (95% confidence intervals in parentheses when appropriate)
Some Ecological Studies of Saline sodic soil rehabilitated by *Salvadora persica* L. at Harappa Archaeological site

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ABSTRACT

Bioreclamation plays an important role in improving the chemical characteristics of the soil. Vegetation cover enhances the biological activity of the soil, adds organic matter and improves the supply of nutrients. Four years old plantations of *Salvadora persica* L. on two sites of saline-sodic land at Archaeological site of Harappa were selected to see its contribution in decreasing salinity and increasing soil fertility. Soil samples were taken under the canopy of *S. persica* plants and were analyzed for different physical and chemical properties. The values of EC (mScm⁻¹) of the soil under *S. persica* trees at site I and II were ~ 11 and 6 folds less as compared to their original soils while the SAR values were half of their values in 2007. After four years of establishment of *S. persica* there was 51 folds increase in N, 6 folds in P and ~ 20 folds increase in K of the top soil of site I. At site II there was 64 folds increase in N, 6 in P and 47 folds increase in K concentration over a period of four years. There was significant difference (p<0.05) in plant heights (cm) and crown covers (cm²) between two sites as better growth was achieved at low salinity site (II). This shows that *S. persica* did not only reduced the salinity but also increased the fertility by adding litter and can effectively be used for the reclamation of saline-sodic soils. **Key words:** Saline-Sodic soils, Rehabilitation, Sodium adsorption ratio, *Salvadora persica.*

INTRODUCTION

Pakistan has 6.3 million hectares of salt-affected land and out of this 1.89 million hectare is saline, pervious saline-sodic is 1.85 million hectares, 1.02 million hectare is impervious saline-sodic and 0.028 million hectares is sodic in nature (Alam & Khan, 2000). Biological method i.e. planting the soil with halophytes could be used where the salts are taken up by halophytes and removed from soil. Worldwide more than 1500 plant species with high levels of salt tolerance have been identified for this purpose (Qureshi & Barrett-Lennard, 1998). Biological reclamation plays important role in the improvement of physical and chemical characteristics of the soil (Minhas et al., 1997; Oba et al., 2001). Biological methods take longer time (several years) to achieve results than chemical amendments. However they have the advantage that they require low initial investments, enhance the biological activity of the soil, add much needed organic matter and improve the supply of nutrients (Qureshi & Barrett-Lennard, 1998).

*Salvadora persica* L. is widely used for bioreclamation of saline sodic soils (Ramoliya, et al. 2004). It has the ability to survive from sand dunes to heavy soils and from non-saline to highly saline soil where the accumulations of salts prevent the growth of other crops (Zodape & Indusekhar, 1997).

It has economic as well as medicinal value because from its seeds oil can be produced that has application in soap and detergent industry.

At Harappa in 2001 the rehabilitation of natural thorn forest community at 36 acres of bare salt affected land was started by Government College University Lahore in collaboration with Archaeology Department and WWF Pakistan (Sharif & Khan, 2006). Thorn forest community is the natural vegetation of Harappa and all of its species are salt tolerant (Sharif & Khan 2009) but the salinity of the site is very high and the plants show slow growth and symptoms of salt injury. For the amelioration of salinity and sodicity on this land two sites were selected in 2007 and were planted with 100 saplings of *S. persica*. Both the sites were highly saline sodic with ECₑ (dSm⁻¹) values of 132 and 66 and ESP values of 71 and 64 for site I and site II, respectively. Ditches of 60 cm X 60 cm were dug and saplings were provided with Non-saline soil + Gypsum + Farmyard manure at the time of plantation. Instead of high salinity and sodicity at both sites transplants showed good growth and high percentage survival (98%) after one year (Sharif & Butt, 2010). The current study is focused on checking the growth and establishment of these *S. persica* plants and also to find out its effects on soil properties after four years.

The objectives of this study were:
1. To check the growth and establishment of *S. persica* plants transferred on saline- sodic soil after four years.

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2. To find out their contribution in reducing salinity, sodicity and in increasing the fertility of soil.

MATERIALS AND METHODS

Site selection and soil analyses

Three samples each from top soil samples (0-15 cm) and subsoil (15-60 cm) were collected from each site. Soil texture, EC(1:1), pH, CO$_3$-, HCO$_3$-, Ca$^{2+}$, Mg$^{2+}$, SO$_4^{2-}$, Cl, Na$^+$, K$^+$, N, available P, organic matter, SAR and ECP were determined as described by Ryan, et al. (2001).

Monitoring of plant growth

Measurements regarding plant height (cm), number of branches, crown cover (cm$^2$) and girth (cm) were made. Heights were measured with the help of measuring tape. Observations regarding the relative amount of chlorophyll were also made from 15 randomly selected plants from each site using the chlorophyll meter (Sp- 502 Konica Minolta). Girths of all branches arising upto 6 cm from soil level were noted. Crown cover (cm$^2$) of each plant on two sites was also measured with the help of measuring tape by taking two diameters of each plant that were more or less perpendicular to each other. Crown cover was calculated by using following formula

\[ CC = \frac{(D_1 + D_2)}{4} \times \pi \]  
(Mueller-Dombois & Ellenberg, 1974)

Statistical Analyses

Recorded data from different parameters were statistically analyzed by using SPSS version 17. Descriptive analysis was carried out to find the mean values and standard error of means. Significant difference of chemical properties of top and sub soil of each block and between the two blocks was determined by Independent sample t-test.

RESULTS

Soil Analyses

The mean values of pH, EC (1:1) and SAR for top soil for Site I were 8.43, 12.4 mS/cm and 113 respectively. While organic matter was 0.40%, N 0.05% and Phosphorous 28.3 mg/kg for top soil (Table. 1). The mean values of pH, EC (1:1) and SAR for top soil of Site II were 8.41, 11.8 mS/cm and 108, respectively. While value for organic matter was 0.46%, N 0.06% and P 29 mg/kg. There was a non-significant difference between the above mentioned parameters at both sites (p < 0.05).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± SE</th>
<th>T</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Site I</td>
<td>8.4367±0.5548</td>
<td>0.389</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>8.4100±0.4041</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC(1:1)(mS/cm)</td>
<td>Site I</td>
<td>12.4333±2.17414</td>
<td>0.273</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>11.633±1.48846</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAR</td>
<td>Site I</td>
<td>11367±2.76908</td>
<td>0.186</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>108±1.28582</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OM (%)</td>
<td>Site I</td>
<td>0.40±0.1220</td>
<td>0.707</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>0.46±0.03118</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td>Site I</td>
<td>0.05±0.49337</td>
<td>0.215</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>0.06±0.56496</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Available P(mg/kg)</td>
<td>Site I</td>
<td>28.333±2.64575</td>
<td>0.168</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Site II</td>
<td>29.300±2.96273</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1: Comparison of mean values of N%, OM%, pH, EC and SAR of top and subsoil of site I
*Significant difference at $p<0.05$ according to independent sample $t$-test.

Fig. 2: Comparison of mean values of N%, OM%, pH, EC and SAR of top and subsoil of site II
*Significant difference at $p<0.05$ according to independent sample $t$-test.

Fig. 3: Comparison of mean values of Heights (cm), Girth (cm) and Chlorophyll of plants growing at site I with site II.
Growth parameters of *S. persica* plants such as height (cm), crown cover (cm$^2$), girth (cm) and relative amount of chlorophyll were noted in order to check the difference in growth of plants between two sites. Crown cover (cm$^2$) and heights (cm) of *S. persica* plants growing at site II were significantly greater ($p < 0.05$) than the plants growing at site I. The mean values of crown cover (cm$^2$) of plants at site I and site II were 32905 and 45900, heights (cm) 1.389 and 1.738, respectively. The girths (cm) of *S. persica* plants growing at site I was significantly greater ($p < 0.05$) than plants growing at site II. Whereas difference between the relative amount of chlorophyll of *S. persica* plants growing at site I and site II was non-significant (Fig; 3 & 4)

**DISCUSSION**

High salinity effects plant growth. Soil of Harappa was highly saline-sodic so in 2007 at the time of plantation of *S. persica*, amendment with non-saline soil (Bhal) was provided at a depth of 60 cm with values of $E_{ce}$ (dSm$^{-1}$), pH and SAR 1.05, 7.5 and 4.60, respectively (Sharif & Butt, 2010). Over a period of four years (2007-11) this non-saline soil gradually accumulated salts from adjacent areas through side seepage and surface runoff and became slightly saline-sodic. Although there is a slight increase in salinity of this initially non-saline soil but these values are far less as compared to the actual adjacent soil having mean values of $E_{ce}$ (mS/cm$^{-1}$), pH and SAR of top soil 132 and 66, 8.86 and 8.80, 206 and 143 for site I and II, respectively. The values of $E_{ce}$ of the replaced soil under *S. persica* trees at site I and II are respectively ~ 11 and 6 folds less as compared to their original soils while the SAR values are half of their values in 2007. *S. persica* plants improved the chemical characteristics of soil by providing shade and adding litter. The roots of the plants also improve the soil structure which in turn improves aeration of soil and water holding capacity. As a result established plants help the soil by creating microenvironment, which can facilitate the recruitment of other community members under their shade. Established *S. persica* trees can serve as a nurse crop making natural regeneration and establishment possible. In 2007 the values of N and P were 0.01%, and 4.60 mg/kg, respectively and in 2011 after four years of the establishment of *S. persica* trees there is 51 folds increase in N, 6 folds in P of the top soil of site I. While this increase in N, P of Bhal under *S. persica* trees is 64 and 6, respectively at site II. This increase in fertility is due to the accumulation of litter under the canopy of *S. persica* plants overtime. When litter accumulates in the soil nutrients like P and N are integrated into its
composition and permit the soil to act as a pool for these nutrients (Gaskell et al. 2007).

Soil chemical analyses revealed that site II is slightly better in terms of N, P values and has less salinity and sodicity than site I that is also reflected in their growth pattern i.e. Heights (cm) and crown covers (cm²) of S. persica plants growing at site II are significantly greater as compared to plants of site I. There was a non-significant difference between relative amount of chlorophyll of plants of both sites because of the common effects of salinity like leaf burning, chlorosis and necrosis that is not only due to the phytotoxic response but is also related to the osmotic stress (Ali et al., 2004). The results of current study showed that S. persica plants not only reduced the salinity and sodicity but also increased the fertility of the soil. So S. persica plants can be effectively used for the reclamation of salt affected lands.

REFERENCES


Anti-fungal activity of different parts of Ethno-medicinally important plant

*Butea monosperma* (Lamk.) Taub.

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**ABSTRACT**

The antifungal activity of extracts of different parts of *Butea monosperma* (Lamk.) Taub. in polar solvents such as methanol and water and nonpolar solvents like Petroleum ether and chloroform were evaluated against four species of fungi viz. *Aspergillus oryzae, Candida utilis, Humicola linuginosa* and *Sporotrichum thermophile*. Methanolic extract of leaves extracted by Soxhlet method showed maximum antifungal activity in form of zone of inhibition i.e. 21mm among all the extracts which was comparable to the zone of inhibition produced by standard drug (Gentamycin). The MIC values of the extracts against microorganisms tested were ranging from 6 mg/ml to 2 mg/ml. The ranges of MIC values of Petroleum ether extracts were 2 mg/ml to 6mg/ml; chloroform extract from 5 mg/ml to 6mg/ml; aqueous extract from 3 mg/ml to 5mg/ml and methanolic extract prepared by Soxhlet method were recorded from 2 mg/ml to 5 mg/ml.

**Key words:** *Butea monosperma*, Medicinal plant, Antifungal activity, Plant extracts.

**INTRODUCTION**

Medicinal plants are part of human medicine since the beginning of civilization. Plants make the backbone of traditional herbal medicine (Nayak et al., 2011). Plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world’s inhabitants relying mainly on traditional medicines for their primary health care (Owolabi et al., 2007). World Health Organization reported that medicinal plants are the best source to obtain a variety of drugs (Nascimento et al., 2000). Therefore, such plants should be investigated to better understand their properties, safety and efficacy.

The success story of chemotherapy lies in the continuous search for new drugs to counter the challenge posed by resistant strains of microorganisms. The investigation of certain indigenous plants for their antimicrobial properties may yield useful results. Many studies indicate that some plants have substances such as peptides, unsaturated long chain aldehydes, alkaloids, essential oils, phenolics, as well as different compounds soluble in organic solvents like ethanol, chloroform, methanol and butanol. These plants have emerged as plants with compounds possessing significant therapeutic potential against human pathogens, including bacteria, fungi or virus (El astal et al., 2005). The plant used in this study is *Butea monosperma* (Lamk.) Taub. is ethno-medicinally important and used locally to cure different diseases. The present was conducted to evaluate the antifungal activity of different parts of *B. monosperma* by different extraction methods.

**MATERIALS AND METHODS**

**Plant material**

Fresh parts of *Butea monosperma* (Lamk.) Taub. was obtained from the forests of Shakargarh, District Narowal, Punjab.

**Preparation of plant extracts**

Collected plant materials were air dried and grind into fine powder. The crude extracts were obtained by using standard techniques of maceration method and Soxhlet method. The filtrates were then evaporated by using rotary evaporator at 55 °C. Dried extracts were stored in refrigerator at 4 °C for further use.

**Antifungal activity**

The test microorganisms used in this study include four fungi species *Aspergillus oryzae, Candida utilis, Humicola linuginosa* and *Sporotrichum thermophile*. The fungi were cultured on the Potato Dextrose Agar medium. The hole plate method was used for the determination of zone of inhibition. MIC was carried out according to Murray et al., 1999 by Broth dilution assay.

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Statistical analysis
All data were represented as a mean of three independent measurements. Means were compared by Student's T test and differences were considered to be significant when p< 0.05.

RESULTS

Antifungal activity of crude extracts of leaves
The results in Table 1 indicated that petroleum ether extract of leaves of B. monosperma by maceration method had inhibitory effects against most of the tested organisms except S. thermophile while chloroform extract inhibited the growth of C. utilis. However the methanol extracts showed inhibition against all the fungi used and the aqueous extracts of leaves obtained by soxhlet method had inhibitory action against A. oryzae, C. utilis and S. thermophile, but no effect against H. linuginosa. The methanol extract taken by soxhlet method showed inhibitory action against all the microorganisms tested. The inhibitory ability of methanol extract was more pronounced against S. thermophile.

Antifungal activity of crude extract of bark
The petroleum ether and chloroform extracts of bark taken by maceration method showed no inhibitory effect on the growth of most of the fungi tested (Table 1). Methanolic extract exhibited action on A. oryzae, C. utilis and S. thermophile. The aqueous extract obtained by soxhlet method of bark had inhibitory action on K. pneumonia, P. aeruginosa, B. subtilis and C. utilis and methanolic extract exhibited activity against most of the tested organisms except K. pneumonia, S. aureus and H. linuginosa.

Antifungal activity of crude extracts of flowers
Petroleum ether extract of flowers obtained by maceration method inhibited the growth of A. oryzae and C. utilis and chloroform extract of the flower exhibited antifungal activity against A. oryzae and S. thermophile. The methanolic extract showed inhibitory action against most of the organisms tested except H. linuginosa (Table 1). Aqueous extract of flowers taken by soxhlet method showed antifungal activity against A. oryzae, C. utilis and S. thermophile.

Antifungal activity of crude extracts of seeds
Petroleum ether extract obtained by maceration method showed antifungal activity against C. utilis, H. linuginosa and S. thermophile but chloroform extract retarded the growth of S. thermophile. Methanolic extract of seeds exhibited the antifungal activity against all the fungi tested (Table 1). Aqueous and methanolic extracts of flowers taken by Soxhlet method showed antifungal activity against all the test microorganisms.

Antifungal activity of standard disc
The Gentamycin 40 µg inhibited the growth of all fungi tested and produced 25mm zone of inhibition against A. oryzae.

Minimum Inhibitory Concentration (MIC)
MIC of crude extracts of leaves
5 mgl, respectively (Table 3). But the chloroform extra/m ct showed MIC value for C. utilis was 5 mg/ml. While the MIC value of methanolic extract against The MIC value of petroleum ether extract obtained by maceration method of leaves against A. oryzae, C. utilis and H. linuginosa were 3 mg/ml, 2 mg/ml and A. oryzae, C. utilis, S. thermophile and H. linuginosa were 4 mg/ml, 4 mg/ml, 3 mg/ml and 5 mg/ml, respectively.

The MIC value of aqueous extract obtained by Soxhlet method of leaves against C. utilis and S. thermophile was 5 mg/ml and for A. oryzae was 3 mg/ml and the MIC value of methanolic extract against A. oryzae and H. linuginosa was 5 mg/ml, for C. utilis was 4 mg/ml and for S. thermophile was 2 mg/ml.

MIC vale of crude extracts of bark
The methanolic extract taken by maceration method of bark showed 5 mg/ml, 4 mg/ml and 5 mg/ml MIC values for A. oryzae, C. utilis and S. thermophile, respectively (Table 3). The MIC value of the aqueous extract obtained by Soxhlet method of bark against C. utilis was 3 mg/ml and the MIC value of methanolic extract of bark against A. oryzae and C. utilis was 3 mg/ml while for S. thermophile was 4 mg/ml.

MIC value of crude extracts of flowers
The petroleum ether extract taken by maceration method of flowers showed 5 mg/ml MIC value for A. oryzae and C. utilis and the MIC value of chloroform against A. oryzae and C. utilis was 5 mg/ml. The MIC value of methanol extract against A. oryzae, C. utilis, and S. thermophile was 3 mg/ml (Table 3).

The aqueous extract of flowers obtained by Soxhlet method showed 4 mg/ml value against A. oryzae and S. thermophile while 3 mg/ml for C. utilis.
and the MIC value of methanol extract against A. oryzae and S. thermophile was 3 mg/ml while for C. utilis was 4 mg/ml.

**MIC value of crude extract of seeds**

The petroleum ether extract of seeds taken by maceration method showed 6 mg/ml MIC value against H. linuginosa and 5 mg/ml for S. thermophile and MIC value of chloroform extract against S. thermophile was 6 mg/ml. The methanol extract showed 5 mg/ml MIC value against A. oryzae and C. utilis while 4 mg/ml for H. linuginosa and 3 mg/ml for S. thermophile (Table 3).

The aqueous extract of seeds obtained by Soxhlet method showed 3 mg/ml MIC value against H. linuginosa, 5 mg/ml for A. oryzae and S. thermophile and the MIC value of methanol extract A. oryzae and H. linuginosa was 4 mg/ml, for C. utilis was 5 mg/ml and S. thermophile was 3 mg/ml.

**DISCUSSION**

Antifungal properties of medicinal plants are being increasingly reported from different parts of the world. The world health organization estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world’s population. In the present work, the extracts obtained from different parts of *Butea monosperma* shows antifungal activity against most of the tested fungal strains. The results were compared with standard antibiotic drugs (Table 2). The well plate method was preferred to be used in this study since it was found to be better than the disc diffusion method (Essawi & Scour, 2000).

The extracts obtained by Soxhlet method showed more antifungal activity against the tested microorganisms than the extracts obtained by maceration method. Therefore the Soxhlet method of extraction is more reliable as compared to the maceration method. The extracts taken by Soxhlet method of different parts of *B. monosperma* also showed more antibacterial activity than maceration method (Ahmad & Khan, 2012).

The antifungal activity of these extracts elucidated the possible presence of biologically active compounds such as flavonoids in different parts of *B. monosperma*. Flavonoids were implicated by Barnabas & Nagarajan (1988) to be responsible for the antimicrobial activity of some medicinal plants.

The methanolic extract of flowers exhibited slightly better inhibitory action against most of the microorganisms tested than the methanolic extracts of the other parts. On the other hand, the aqueous extracts of leaves and flowers showed better potential of growth inhibition of the microorganisms tested.

The MIC values of extracts were effective as that of the MIC of the standard discs evaluated by Dulger & Gonuz (2004). Thus, the extracts of all parts of plants were active against microorganisms tested although the activity was lower. The activity of plant extracts against fungi may be indicative of the presence of antifungal compounds or simple general metabolic toxins in the plant materials. Furthermore, it may help to discover new chemical classes of antimicrobial agents for the maintenance of human health.

**REFERENCES**


Table 1: Mean Zone of Inhibition produced by crude extracts of parts of *B. monosperma*

<table>
<thead>
<tr>
<th>Plant Part</th>
<th>Fungi tested</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>By Maceration method</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Petroleum ether extract</td>
</tr>
<tr>
<td>Leaves</td>
<td>A. oryzae</td>
<td>16±1.154</td>
</tr>
<tr>
<td></td>
<td>C. utilis</td>
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</tr>
<tr>
<td></td>
<td>H. linuginosa</td>
<td>10±0.577</td>
</tr>
<tr>
<td></td>
<td>S. thermophile</td>
<td>--</td>
</tr>
<tr>
<td>Bark</td>
<td>A. oryzae</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>C. utilis</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>H. linuginosa</td>
<td>--</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>S. thermophile</td>
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</tr>
<tr>
<td>Seeds</td>
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<td></td>
<td>S. thermophile</td>
<td>12±1.154</td>
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</table>

All the results are mean of three parallel replicates. ± indicates the standard error.

Table 2: Antifungal Activity of standard disc

<table>
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<tr>
<th>Fungal species</th>
<th>Zone of Inhibition of Gentamycin (mm)</th>
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<td>S. thermophile</td>
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All the results are mean of three parallel replicates. ± indicates the standard error.

Table 3: The MIC value in mg/ml of crude extracts of parts of *B. monosperma*.

<table>
<thead>
<tr>
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<th>Fungi tested</th>
<th>MIC (mg/ml)</th>
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<tr>
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<td>Petroleum ether extract</td>
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<tr>
<td></td>
<td>C. utilis</td>
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<td></td>
<td>C. utilis</td>
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<tr>
<td></td>
<td>H. linuginosa</td>
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<td></td>
<td>S. thermophile</td>
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</tr>
<tr>
<td>Flower</td>
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<tr>
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<td></td>
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<tr>
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</tr>
<tr>
<td></td>
<td>C. utilis</td>
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<tr>
<td></td>
<td>H. linuginosa</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>S. thermophile</td>
<td>5</td>
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</table>
Monthly variations in physicochemical parameters of a flood plain reservoir on River Ravi near Balloki Headworks (Pakistan)

ALTAF HUSSAIN*, ABDUL QAYYUM KHAN SULEHRIA, MUHAMMAD EJAZ AND ASMA MAQBOOL

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ABSTRACT

Monthly variations of different physicochemical parameters of water of a flood plain reservoir on River Ravi near Balloki Headworks were studied. Atmospheric temperature ranged from 16.62 to 40.32 (°C). Water temperature ranged from 14.15 to 33.89 (°C). pH ranged from 6.82 to 8.53, dissolved oxygen from 5.00 to 9.46 (mg/l), electrical conductivity from 232.76 to 330 (µS/cm), total dissolved solids from 148.96 to 211.20 (mg/l), turbidity from 4.62 to 63.70 (NTU), visibility from 30.48 to 150 (cm), total hardness from 120 to 160 (mg/l), total alkalinity from 100 to 119 (mg/l) and chlorides ranged from 20.0 to 34.93 (mg/l).

Key words: Physicochemical parameters, flood plain, River Ravi, monthly variations, Balloki Headworks.

INTRODUCTION

Rivers and reservoirs play a major role in agricultural, fishery and electricity production along with the use of water for drinking purposes. Several factors which determine the water quality of a reservoir includes seasonal climatic changes (Chapman, 1996; Barik et al., 2010), seasonal precipitation, wind action, geologic origin of the catchment basin and pattern of hydrological cycle prevalent in the dam (Tundisi & Straskraba, 1999). Several limnological parameters such as conductivity, total dissolved solids, phytoplankton and reservoir morphometry have been used in estimating potential fish yields from reservoirs. Several physicochemical or biological factors, in suitable range, help in increased activities and growth for aquatic animals. On the other hand, some factors exert stress and adversely affect growth and reproduction of different animals (Iwama et al., 2000). Studies on water quality mostly centre on fish production and aquatic biotic integrity (Boyd, 1982; Abohweyere, 1990; King, 1998). Therefore, protection of water quality is very important issue so it should be kept within acceptable range (Quyang et al. 2006).

Limnology covers the biological, chemical, physical, geological, and other attributes of inland waters including rivers, streams, wetlands, lakes, ponds and springs pools etc. It is an interdisciplinary science which deals with the detailed field as well as laboratory studies to understand the structural and functional aspects and suggest solution to all the problems associated with the freshwater environment (Adoni et al., 1985).

Water quality assessment generally involves analysis of physico-chemical, biological and microbiological parameters and addresses abiotic and biotic status of the ecosystem (IAAB, 1998; Kulshrestha and Sharma, 2006; Mulani et al., 2009).

Recently, lot of work has been done on fresh water reservoirs and changing ecological behavior of reservoirs, ponds and dams (Mirza et al., 2013; Sulehria et al., 2012; Sulehria & Malik, 2012; Janjua et al., 2009; Sulehria et al., 2009a, 2009b; Malik & Sulehria, 2004:).

Temperature is one of the major factors affecting freshwater ecosystems, temporal and spatial distribution of organisms. Invertebrates are most sensitive to changes in temperature. In a shallow pond there could be a substantial increase in temperature over a diurnal period with cooling at night. The upper region of a lake will warm in the sun and, if wind turbulence is low, an underlying cool layer will be present. This thermal stratification is very important in determining other abiotic and biotic factors. Factors controlling the rate of photosynthesis and the amount of oxygen evolved include light, species and abundance of plant (Adeniji, 1991), temperature and turbulence (Welch, 1948; Arooye, 2007).

Dissolved oxygen concentration and the pH of water bodies are also important parameters which determine the spatial and temporal distribution of aquatic organisms particularly the fish fauna. Dissolved oxygen is required for respiration by most aquatic animals. Dissolved oxygen combined with other important elements such as Carbon, Sulphur, Nitrogen and Phosphorous to form carbonate, sulphate, nitrate and sulphate respectively which constitute the required compounds for aquatic organisms for survival.
Dissolved oxygen and pH affects directly or indirectly other water parameters such as transparency, viscosity, total dissolved solids and conductivity (Whitney, 1942). Photosynthesis by aquatic plants during the daylight removes carbon dioxide (CO₂) from the medium hence pH would increase. At night, respiratory processes of aquatic organisms release CO₂ into the medium and pH declines. Similarly warm waters develop increased pH levels due to conversion of CO₂ into organic carbon by photosynthesis and the rate may exceed the rate of the release of CO₂ from organic carbon by the process of respiration (King, 1970).

Anthropogenic impact such as urban, industrial and agricultural activities as well as natural processes (precipitation inputs, erosion, etc.) diminish the surface water quality lowering the use of water for drinking, agricultural and other purposes (Carpenter et al. 1998). The concentrations of toxic materials such as heavy metals, pesticides, and nutrients in excess not only affect human health but also cause various problems such as loss of oxygen, fish deaths and loss of biodiversity. It is, therefore, necessary that the water quality should be checked at regular intervals for increased density and diversity of aquatic organisms. Due to spatial and temporal fluctuations in water quality, a monitoring program providing a representative and reliable estimation of the quality of surface waters is necessary (Dixon & Chrisswell 1996). The assessment of the water quality can be performed by classification, modeling, and interpretation of the monitored data (Simeonov et al., 2003, Boyacýðlu, 2006).

The purpose of this study was to evaluate the seasonal variations of the water quality parameters, to determine temporal and spatial variations in water quality and to investigate the similarities or dissimilarities of water quality between the sampling sites.

MATERIALS AND METHODS

Study area

The floodplain understudy is situated on River Ravi near Balloki Headworks in District Kasur, Pakistan. It is 65 Km from Lahore lying at a Latitude: 31° 11' 25" North, and at Longitude: 73° 52' 40" East. The total area of the floodplain is about 8.6 Km. It has distinct tropical climate with a marked monsoonal effect with an average rainfall of 52.01mm, humidity 70.40% and average atmospheric temperature ranging from a minimum of 5°C in winter to a maximum of 50°C in summer. Water level varies in different months of the year, being highest in summer (July to September) and lowest in winter (October to April) every year.

Sampling

Monthly variations of physicochemical characteristics of water were studied from January to December, 2012. Samples were taken separately in one liter sample bottles for the evaluation of physicochemical parameters. Atmospheric and water temperature (°C), pH, dissolved Oxygen (mg/l), electrical conductivity (µS/cm), total dissolved solids (mg/l), turbidity (FTU) and transparency (cm) were measured on the spot. Temperature and DO were measured by DO meter (DO200 Ecosence), electrical conductivity and total dissolved solids were measured by conductivity meter (EC300 Ecosence), pH was measured by pH meter (PH100 Ecosence), turbidity was measured by turbidity meter (Hi 93703 HANNA). Transparency was measured by secchi disc plate of 20 cm in diameter, painted with alternate black and white quadrates. For the determination of total hardness (mg/l ), total alkalinity (mg/l ), chlorides (mg/l ) and free CO₂ , ppm , water was taken in IL sampling bottles and brought to the Laboratories at Govt. College University, Lahore, for further processing, employing methods described in APHA (2005) and Hach (2003).

Statistical analysis

XLSTAT 2013 for MS Excel 2007 was used in analyzing the data sets. Graphs were plotted with the help of MS Excel 2007.

RESULTS AND DISCUSSION

A summary statistics of the different water parameters recorded during the whole year are shown in Table 1. Air temperature was recorded maximum in April (40.32 °C) and minimum in January (16.62 °C). Water temperature was recorded maximum in July (33.89 °C) and minimum in December (14.15 °C). The present observation revealed that the annual air temperature cycle maintained a close parallel relationship with annual cycle of water temperature. Both showed similar trends having highest values in summer and lowest values in winter respectively. Both the temperatures started to increase from February, reached at peak during summer (April to June) and then dropped suddenly in August, mainly due to rain fall and mixing of incoming cold water of river with hot flood plain stagnant water. Differences between air and water temperatures were maximum during summer and minimum during winter. This increase and decrease in temperature corresponded to the
seasional and climatic variations in the region. This observation was also in agreement with the findings of Hidetoshi (2002), Kolo & Oladimeji (2004) and Caldwril (2003).

pH was maximum in July (8.53) and minimum in December (6.82). pH started increasing steadily from January to May. This increase in pH was due to increase in temperature until July and then dropped abruptly in August. The increase in pH in warm months may be due to the increase of CaCO$_3$ in stagnant waters and increased amount of nitrates, phosphates and ultimately eutrophication in summer. Kamble et al (2009) has also reported that HCO$_3$ ions formed during summer, due to reduced photosynthesis also increased pH. Sudden decrease in pH in August was due to stirring effect of incoming water in stagnant water of the flood plain. Silva & Ronald (1987), Araoye (2009) and Mustapha (2009) had also reported the similar findings.

DO is a very important indicator of a water body’s ability to support aquatic life. Aquatic organisms need dissolved oxygen for their survival. In present studies maximum concentration of dissolved oxygen was present from November (8.0 mg/l) to February (7.68 mg/l) being maximum in January ((9.46 mg/l), while minimum amount of Oxygen was observed in summer, being lowest in June (5.0 mg/l). Oxygen concentration started increasing from July during rainy season. It might be due to water agitation and influx of rain and flood water into the flood plain. Flood water having increased oxygen concentration, was responsible for improving oxygen concentration in water. Low quantity of oxygen in summer can be attributed to the decreasing solubility level of oxygen during increasing temperature in summer months, along with the increasing decomposition ratio in warm days. Similarly higher amount of oxygen during winter months may be due to increasing solubility of oxygen during winter along with the decrease in decomposition ratio in winter months. Similar observation was recorded by Janjua et al (2009) and Morrison et al (2001).

Electrical conductivity was highest in June (330 µS/cm) and lowest in January (232.76 µS/cm). Electrical conductivity increased with increase in temperature. In warm month’s evaporation in water bodies resulted in decrease in the total quantity of water, causing increase in electrical conductivity. In the present study maximum conductivity was recorded in June (300 µS/cm) and minimum in January (232.76 µS/cm). It increased from January to June and then decreased abruptly in July and August due to dilution effect caused by rain and flood water. Again increase in conductivity in September was due to increase in water temperature and again decrease from October to December was due to decrease in water temperature. This also agreed with the findings of Mirza et al (2013) and Kolo & Oladimeji (2004).

Total dissolved solids were maximum in June (211.2 mg/l) and minimum in January (148.97 mg/l). TDS values decreased in July and August, then increased in September, after which the values decreased up till January. From January the TDS values again started to increase till it reached to maximum in June. The TDS values followed the same trend as Electrical conductivity. It is seen that a linear relationship existed between TDS and EC. TDS can be calculated by multiplying the electrical conductivity with a specific factor (usually which ranged from 0.55 to 0.75). A similar trend of TDS was also observed by Mustapha, (2009) and Singh et al (2010). Samal (2001) had also concluded that Electrical conductivity would increase with increase in TDS values.

Turbidity was found highest in July (63.7 NTU) and low turbidity was observed from September to April, being minimum in February (6.42 NTU). High value of turbidity in July was due to the maximum agitation of water caused by rainfall. During rainstorm particles from surrounding land also washed into the river making the water a muddy brown colour, indicating higher turbidity. Similarly during high flows, water velocities were faster and water volumes were higher, which could more easily stir up and suspend materials from the stream bed, increasing turbidities.

Transparency values were recorded maximum in December (150 cm) and minimum in July (30.48 cm). Reduced transparency during rainy season (July) might be due to the erosion of soil by precipitation and the transport of silt particles through run off. On the other hand, higher transparency values during dry season were due to the absence of flood water, surface run off and setting effect of suspended solids. Lower readings of transparency indicated turbid or coloured water. Transparency and turbidity were inversely proportional to each other. Mirza et al (2013) and Khan & Chaudhury (1994) also made similar observations. Mustapha (2009) reported that low transparency in the wet season might be due to the washing of debris, organic matter and silt into flood plain through run off.

Total hardness and alkalinity are expressed as CaCO$_3$ which is not correct. Total hardness means the concentration of cations and alkalinity means the concentration of anions. Hardness is
Table 1: Summary statistics of Water parameters recorded from January 2012- December 2012

<table>
<thead>
<tr>
<th>Months</th>
<th>Air temp. (°C)</th>
<th>Water temp. (°C)</th>
<th>pH</th>
<th>DO. (ppm)</th>
<th>E.Cod. (µs/cm)</th>
<th>TDS (mg/L)</th>
<th>Turbidity (FTU)</th>
<th>Visibility (cm)</th>
<th>T. H (mg/l)</th>
<th>T.A. (mg/l)</th>
<th>Chlorides (mg/l)</th>
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<td>108.53</td>
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<td>7.6</td>
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<td>139</td>
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**Minimun**

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<tr>
<th>Months</th>
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<th>Water temp. (°C)</th>
<th>pH</th>
<th>DO. (ppm)</th>
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**Maximun**

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**Mean**

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<td>6.94</td>
<td>265.66</td>
<td>170.02</td>
<td>16.38</td>
<td>87.33</td>
<td>143.33</td>
<td>113.04</td>
<td>28.1125</td>
</tr>
</tbody>
</table>

**St. Er.**

<table>
<thead>
<tr>
<th>Months</th>
<th>Air temp. (°C)</th>
<th>Water temp. (°C)</th>
<th>pH</th>
<th>DO. (ppm)</th>
<th>E.Cod. (µs/cm)</th>
<th>TDS (mg/L)</th>
<th>Turbidity (FTU)</th>
<th>Visibility (cm)</th>
<th>T. H (mg/l)</th>
<th>T.A. (mg/l)</th>
<th>Chlorides (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>St. Er.</td>
<td>2.24</td>
<td>1.87</td>
<td>0.2</td>
<td>0.48</td>
<td>8.09</td>
<td>5.18</td>
<td>5.04</td>
<td>9.09</td>
<td>3.19</td>
<td>1.49</td>
<td>3.744722</td>
</tr>
</tbody>
</table>

**St.Dev.**

<table>
<thead>
<tr>
<th>Months</th>
<th>Air temp. (°C)</th>
<th>Water temp. (°C)</th>
<th>pH</th>
<th>DO. (ppm)</th>
<th>E.Cod. (µs/cm)</th>
<th>TDS (mg/L)</th>
<th>Turbidity (FTU)</th>
<th>Visibility (cm)</th>
<th>T. H (mg/l)</th>
<th>T.A. (mg/l)</th>
<th>Chlorides (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>St.Dev.</td>
<td>7.75</td>
<td>6.47</td>
<td>0.5</td>
<td>1.55</td>
<td>28.03</td>
<td>17.94</td>
<td>17.46</td>
<td>31.47</td>
<td>11.05</td>
<td>5.17</td>
<td>3.744722</td>
</tr>
</tbody>
</table>

T.H=Total hardness; T.A.=Total alkalinity

mainly concerned with the concentration of calcium and magnesium ions. Total hardness was found highest in June (160 mg/l) and lowest in August (120 mg/l). It showed positive correlation with temperature. As temperature increased total hardness also increased. Similarly with decrease in temperature hardness also decreased. The high values of hardness during summer can be attributed to increased rate of evaporation of water in a flood plain during summer. Sudden change in hardness in August is due to rain fall and addition of flood water in flood plain. Similar findings were observed by Ratushnyak et al (2006) and Park & Shin (2007) but different findings were observed by Mirza et al (2013).

Total alkalinity of water is due to presence of mineral salts in it. It is primarily caused by the presence of carbonate and bicarbonate ions. Total alkalinity also followed almost same pattern as Total hardness, being highest in June and lowest in August. High values in June were due to evolution of CO2 during decomposition of organic matter in summer and reduction of water in the flood plain. The lowest alkalinity in August was attributed to the dilution factor in August, due to rain fall and incoming flood water. Similar results were produced by Mirza et al (2013).

Chloride is one of the important parameter in water. Its concentrations vary widely, especially in pond waters, ranging from less than 1mg/l to more than 100 mg/l. The Chlorides is also one of the important indicators of Pollution. The value of chlorides was present from 20 to 35 mg/l, in present studies. Maximum values were recorded in the months from December to February whereas minimum value was recorded in August. High concentrations of chlorides might be due to the invasion of domestic and agricultural wastes. High domestic wastes are added to the river near Lahore, which ultimately are responsible for rise in chloride contents in the flood plain. Similar results were also reported by Ahmad (2004).

The physico-chemical characteristics of water analyzed during the study period revealed that due to anthropogenic activities, the water quality is deteriorating day by day. Therefore, there is an urgent need to properly manage wastes in the cities and control and monitor human activities in order to ensure minimized effects of these parameters on the River Ravi.
Fig. 1: Different physicochemical parameters of water i.e., Air temperature (°C), water temperature (°C), pH, dissolved oxygen (mg/l), electrical conductivity (µs/cm), total dissolved solids (mg/l), turbidity (NTU), visibility (cm), total hardness (mg/l), total alkalinity (mg/l) and chlorides (mg/l).

REFERENCES


