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Effect of various concentrations of Zinc on Peroxidase activity of *Catla catla*

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**ABSTRACT**

Antioxidant enzymes are the first line of defense against reactive oxygen species (ROS) and other free radicals. When the rate of ROS exceeds the capacity of antioxidant enzymes, non-detoxified radicals begin to attack the bio-molecules. Therefore, present research work was planned to study the effect of zinc on the peroxidase activity in the liver and kidney of *Catla catla*. To check the concentration based effect, *C. catla* were exposed, separately, to 96-hr LC50 of zinc and its sub-lethal concentrations viz. 2/3rd, 1/4th and 1/5th of LC50 for 30 days at constant laboratory conditions. After 30-day, the fish were sacrificed and their liver and kidney were analyzed for peroxidase enzyme activity. Change in activity of peroxidase enzyme in the fish exposed to sub-lethal concentrations was compared with the control. Dose dependent increase in the activities of peroxidase enzyme was observed in the tissues of fish as compared to the control group. Peroxidase activity in the liver of fish from all the treatments was significantly (p<0.05) higher than that of kidney. The results of these studies in fish tissues may prove that peroxidase activity can be used as a sensitive bio-indicator of the antioxidant defense system.

**Key Words:** *Catla catla*, Sub-lethal, Zinc, Antioxidant Enzyme

**INTRODUCTION**

The fluctuating nature of the environment and human activities are continuously adding pollutants to the water bodies causing deleterious effects on the aquatic organisms (Javed, 2005). Metals are distinctive among various pollutants due to their non-biodegradable nature which helps them to get accumulated in the organs of aquatic organisms and become lethal (Sobha *et al.*, 2007). Metal contamination in aquatic environment is considered to be unsafe not only to the inhabitant of aquatic organisms like fish but also to human which are the ultimate consumers in the food chain (Ambreen *et al.*, 2015; Perera *et al.*, 2015). Among heavy metals, zinc is an essential metal which is used as cofactor in my enzymatic reactions. Although, it is essential for the normal fish growth and bio-mineralization (Clegg *et al.*, 2005) but accumulation of zinc can stimulate the production of reactive oxygen species (ROS) in the fish that can oxidize the proteins, DNA and lipids (Oteiza *et al.*, 2000). Zinc may also act as an antioxidant being an essential component of Cu/Zn-superoxide dismutase (Dondero *et al.*, 2005).

Production of reactive oxygen species (ROS) is an inevitable phenomenon in all aerobically respiring organisms (Nishida, 2011). Reactive oxygen species viz. superoxide anion, hydrogen peroxide and hydroxyl radicals are produced naturally by mitochondrial respiration and other cellular processes that lead to the oxidation of proteins, lipids and nucleic acids (Singh *et al.*, 2006). To minimize the hazardous impacts of ROS on biomolecules, there existed an antioxidant defense system in all aerobically respiring organisms (Geoffroy *et al.*, 2004). This system comprises of the enzymes like peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and glutathione peroxidase and low molecular weight antioxidants, like metallothionein, ascorbic acid and vitamin E (Tripathi *et al.*, 2006). Antioxidant enzymes may have variable activity in the liver and kidney of the fish. Liver is the house of redox reactions and bio-transformations that produce maximum amount of free metal ions (Basha & Rani, 2003). Research on oxidative stress in the fish focuses on different toxicological aspects, like effects of different heavy metals on antioxidant enzymes activities, the intensity of lipid per-oxidation as well as the induction of bio-transformational processes and other biomarkers of oxidative damage (Morales *et al.*, 2004). Antioxidants are extremely important biomarkers and act as strong indicators in the field of aquatic toxicology (Livingstone, 2003). Peroxidase is

**Author's Contribution:** M.A., Conduct experiment and collect data; M.J., Provide financial assistance and facilities for this experiment; F.A., Data analyses was done by this author, also corresponding author; F.L., Paper write up was done by this member
antioxidant enzyme that converts highly reactive H₂O₂ into non-toxicant forms, water and oxygen (Hansen et al., 2006). Peroxiadase has been reported in the elimination of ROS produced in pathological and physiological processes and plays a significant role in the stimulation of inflammation, apoptosis and signal transductions (Imai & Nakagawa, 2003). Fish liver and kidney are considered as vital organs, involved in metabolism, bio-transformation and excretion of contaminants (Figuiredo-Fernandes et al., 2006). C. catla, commonly called Thaila, an Indian Major Carp which is preferred due to its higher growth potential, consumer preference and its compatibility with other major carps in poly-culture system (FAO, 2005). These fish species are being highly affected with increasing pollution level in the freshwater bodies of Pakistan. To conserve them, it is essential to check the effects of metallic ions toxicity on these important edible fish species. Therefore, this research work was conducted to assess the concentration based effect of zinc on peroxidase activity in the liver and kidney of C. catla.

**MATERIALS AND METHODS**

This research work was conducted in the laboratories of Fisheries Research Farms, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan. One year old Catla catla were procured from the ponds and brought to the laboratory for acclimatization in cemented tanks. Fish were fed with pelleted feed (30%DP and 3.00Kcalg⁻¹ DE) twice daily. After acclimation period, C. catla of similar weights and lengths, were selected for these experiments. Pure chloride compound of zinc (ZnCl₂) was dissolved in 1000ml de-ionized water for the preparation of metal stock solution. Four fish groups (n=10) were transferred to the glass aquaria of 50L water capacity to check the effect of Zn on peroxidase activity in the selected tissues viz. liver and kidney of C. catla. To check the concentration/dose based enzyme activity, these fish groups were exposed for 96-hr to LC₅₀ of zinc (25.88±1.28mgL⁻¹) as determined by Abdullah & Javed (2006) and to 2/3rd, 1/4th and 1/5th LC₅₀ values for 96 hrs, separately, for 30 days. Each test was conducted with three replications for each concentration along with a control group. After 30-day exposure period, fish were sacrificed and their tissues viz. liver and kidney were isolated and preserved at 4°C for the estimation of enzyme assay.

**Enzyme Assay:** Red blood cells were removed from the liver and kidney, by rinsing these organs with phosphate buffer of pH 6.5 (0.2M) and homogenized in cold buffer (1:4W/V) by using a blender. After homogenization, the organ homogenate was centrifuged for 15 minutes at 10,000rpm at 4°C. After centrifugation process, clear supernatant was preserved at 4°C for enzyme assay while residue was discarded. For the determination of peroxidase activity, the samples were subjected to enzyme assay by following the method described by Civello et al. (1995). Activity of peroxidase was assessed by measuring the conversion of guaiacol to tetraguaiacol spectrophotometrically at 470nm.

**Preparation of 0.2M phosphate buffer (pH 6.5):** The 4g NaH₂PO₄ and 1g Na₃HPO₄ were taken in a flask and dissolved by adding distilled water and volume was raised up to 200ml and pH was adjusted at 6.5.

**Preparation of buffer substrate solution:** Guaiacol (750µl) was added to phosphate buffer (47ml) and mixed well on vortex agitator. After agitation, H₂O₂ (0.3ml) was added to buffer solution. Reaction mixture contained buffer substrate solution (3 ml), enzyme extract (0.06ml) and blank (phosphate buffer). A cuvette containing 3ml of blank solution was placed in the spectrophotometer and set it to zero at wavelength of 470nm. Then a cuvette containing buffered substrate solution was placed in the spectrophotometer and the reaction was initiated by adding 0.06ml of enzyme extract. After 3 minutes of reaction time, absorbance was observed and activity of enzyme was calculated by using the following formula:

\[
\text{Activity (Unit/mL)} = \frac{\Delta A/3}{26.60 \times 60 / 3000}
\]

**Statistical Analyses:** Factorial experiments, with three replications for each test concentration, were performed to find out statistical differences among various treatments of zinc under study. The means were compared by using Least Square Design (LSD). Possible relationships among different parameters were determined by Correlation and Regression analyses.

**RESULTS**

The peroxidase activity in the liver and kidney of zinc stressed fish was analyzed after 30-day metal exposure and represented in Table I.
Table I: Peroxidase activity (UmL⁻¹) in liver and kidney of *Catla catla* after sub-lethal exposure to zinc

<table>
<thead>
<tr>
<th>Organs</th>
<th>Sub-lethal Levels</th>
<th>Control</th>
<th>Overall Means±SD</th>
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<tr>
<td></td>
<td>96-hr LC₅₀</td>
<td>2/3rd LC₅₀</td>
<td>1/4th LC₅₀</td>
</tr>
<tr>
<td>Liver</td>
<td>0.550±0.007 a</td>
<td>0.451±0.006 b</td>
<td>0.381±0.006 c</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.147±0.002 a</td>
<td>0.085±0.002 b</td>
<td>0.060±0.002 c</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>0.348±0.004</td>
<td>0.268±0.004</td>
<td>0.220±0.004</td>
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</table>

Increased activities of peroxidase were observed in the liver and kidney of the fish exposed to various concentrations of zinc as compared to the control. Significantly higher peroxidase activities in the liver and kidney of fish were observed at 96-hr LC₅₀ exposure as compared to other treatments. Statistically significant differences at p<0.05 existed among all the treatments and organs. The overall means exhibit that the peroxidase activity increased with an increase in metal exposure concentrations that followed the order: 96-hr LC₅₀ > 2/3rd > 1/4th > 1/5th > control. Significantly higher enzyme activity was measured in the liver of fish (Fig., 1) exposed to 96-hr LC₅₀ (0.550±0.007U mL⁻¹) while it was lower in the liver of control fish group as 0.060±0.003U mL⁻¹. However, in the kidney of *C. catla*, the enzyme peroxidase activity under 96-hr LC₅₀, 2/3rd, 1/4th and 1/5th of LC₅₀ exposures were recorded as 0.147±0.002, 0.085±0.002, 0.060±0.002 and 0.040±0.001U mL⁻¹, respectively while the enzyme activity in control fish was observed as 0.022±0.003U mL⁻¹ (Fig., 2). The overall means computed for organs indicated that the peroxidase activity was more pronounced in the liver of *C. catla* as compared to the kidney (Fig., 3).

**DISCUSSION**

During the normal cell metabolism, endogenous cellular process causes the production of free...
radicals. However, the over production of reactive oxygen species (ROS) can cause changes in the cell redox status and alternation of gene expression, oxidation of lipids and proteins (Cao et al., 2010). Antioxidant defense system has been evolved in the aerobic organisms to protect them against toxicity of heavy metals and other substances that generate oxidative stress (George et al., 2004). Peroxidase is well known antioxidant enzyme present in mitochondrial matrix and cell which catalyzes the oxidation of glutathione-S-transferase into glutathione by converting \( \text{H}_2\text{O}_2 \) into the water and oxygen (Aruljothi & Sampillai, 2014). Antioxidant enzymes are sensitive biomarkers, and are considered as significant diagnostic tools for testing water for the presence of toxicants in the aquatic environment (Geoffroy et al., 2004).

During present study, increased activities of peroxidase were observed in the liver and kidney of the fish exposed to various concentrations of zinc as compared to the control. Significantly higher peroxidase activities in the liver and kidney of fish were observed at 96-hr \( \text{LC}_{50} \) exposure as compared to other treatments. The overall means exhibit that the peroxidase activity increased with an increase in metal exposure concentrations that followed the order: 96-hr \( \text{LC}_{50} > 2/3^\text{rd} > 1/4^\text{th} > 1/5^\text{th} > \text{control} \). Liver and kidney plays important role in the excretion and detoxification of heavy metals ingested in the body (Marijic & Raspor, 2006). Effect of contaminants and toxicity of heavy metals in the aquatic ecosystem can be assessed by measuring the physiological and biochemical parameters in the kidney and liver of the fish (Barhoumi et al., 2012). Farombi et al. (2007) also observed increased activity of peroxidase in zinc stressed African catfish, \( \text{Calarias gariepinus} \). During present study, liver exhibited significantly (\( p<0.05 \)) higher peroxidase activity than that of kidney. In agreement with this study, increased peroxidase enzyme activity in liver, following zinc exposure has been reported in \( \text{Labeo rohita} \) by Palaniappan et al. (2009). Increase in hepatic peroxidase activity of the fish exposed to zinc was interpreted to reflect hepatocytes damage due to toxicant (Devi & Gupta, 2014). Banni et al. (2011) reported that zinc can induce oxidative stress in the liver of zebra fish, \( \text{Brachydanio rerio} \). Alkaladi et al. (2014) reported that Zn can induce oxidative stress in the liver and kidney of Nile tilapia, \( \text{Oreochromis niloticus} \) and may cause damage to the cell membrane and mitochondria through over production of ROS. The present results are also in accordance with the findings of Saliu & Bawa-Allah (2012) who also observed higher activity of peroxidase (1.120±0.62UmL\(^{-1}\)) in the liver of zinc chloride stressed \( \text{Clarias gariepinus} \) as compared to the control (0.950±0.43UmL\(^{-1}\)) fish. However, Saddick et al. (2015) concluded from their research as peroxidase activity decreased in the liver of \( \text{Oreochromis niloticus} \) after exposure to higher concentration of zinc. Hao and Chen (2012) reported decreased activity of peroxidase in the liver of carp after exposure to higher concentration of zinc. Liver, a primary organ for various metabolic processes, may act as major target organ for zinc toxicity as zinc oxide induce ROS triggered mitochondrial mediated apoptosis, thus increasing antioxidant activity in response to it (Sharma et al., 2012). Change in the activity of peroxidase in kidney may be attributed to the fact that kidney is one of the major organs for detoxification and elimination of metallic toxicants (Gupta & Srivastava, 2006).

In conclusion the evaluation of metal’s toxicity in freshwater organisms is one of the imperative areas of research and there is an emergent concern on the development of new techniques for detecting toxic effects of metals in aquatic organisms, especially fish. Oxidative biomarkers are useful in assessing the health of aquatic life. Therefore, this experiment was conducted on fish to see the effect of zinc by using oxidative stress biomarker (peroxidase) in the liver and kidney of fish. The acquired information would further help in making the strategies for treating zinc polluted water bodies and making the water safe for the survival of fish species.

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Role of Non-Specific Esterases in the Incidence of Breast Cancer in Different Age Groups

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INTRODUCTION

Breast cancer is the most common types of cancer in females which cause 14 % deaths worldwide (Bayoumi et al., 2012). Occurrence of breast cancer is increasing and Pakistan showed highest prevalence (Naveed et al., 2014) i.e., almost one out of nine women is suffering from this disease (Sohail & Alam, 2007). The oxidative stress is a key risk factor for breast cancer (Brown & Bicknell, 2001) and reactive oxygen species (ROS) are main cause of oxidative stress in cancerous cells (Brown & Bicknell, 2001; Hanahan & Weinberg, 2011). ROS damage the DNA, proteins and lipids and this damage is a major cause of the mutations that cause the initiation and progression of tumors (Wu et al., 2004).

Esterases are enzymes that serve as a protective, metabolizing and clearing function for foreign substances (Mates & Sanchez-Jimenez, 2000). The esterases can be used as a reliable biomarker of physiological stress (Konduru, 2012) because the activity of various esterases and antioxidant defense system decreases during oxidative stress that is caused by ROS (Shreya et al., 2012). The goal of present study was to find relationship between stress enzymes (non-specific esterases) and incidence of breast cancer in different age groups individuals. The association of some risk factors (family history and marital status) on the occurrence of breast cancer was also studied.

MATERIALS AND METHODS

Population studied

The blood samples were collected in EDTA coated vials, from Niazi laboratory, Sahara laboratory and Rehman laboratory of Sargodha city, Punjab, Pakistan considering ethics and norms. Blood was collected from 200 individuals, half of them were patients of breast cancer (n=100) while half were age matched healthy individuals (n=100) and plasma was seperated by centrifuging fresh blood at 1300rpm for 5 minutes to estimate levels of alpha and beta esterases in cancer patients. A questioner was developed to record the data about risk factors of breast cancer patients such as age, stage of disease, family history, blood group, height and weight, was collected.

Biochemical estimation of non-specific esterases

For estimation of esterases, o-napthyl acetate and β-napthyl acetate was used as substrate (Baker et al., 1998). Initially, 0.1M substrate solution (20µl) and 100mM Phosphate buffer (150µl) was added to 20µl plasma and incubated at 37°C for 30 minutes. Further mixture of 1% FBB salt and 5% sodium dodecyl sulphate (SDS) (100µl) was added and absorption was recorded at the wavelength of 620nm (α-esterases) and 545nm (β-esterases). Reference mixture contained Phosphate buffer instead of plasma. Standard curve of alpha (α) naphthol and beta (β) naphthol were used for conversion of absorption

Key Words: Esterases, breast cancer, risk factors, diagnosis
values into the mM of product formed/min/mg of protein.

**Statistical analysis**

A nonparametric test was used for comparison of enzyme levels in breast cancer patients and healthy individuals. Difference was considered significance, if the p-value was < 0.05.

**RESULTS AND DISCUSSION**

The level of alpha and β-esterase was significantly higher in healthy individuals than breast cancer patients \((U=1684.5; \text{P-Value} =0.0103);\) (Fig. 1). While activity of α-esterases in healthy individuals was also high than breast cancer patients but statistically non-significant \((U = 1069; \text{P} =0.2085);\) (Fig. 2). Lund-Pero et al. (1994) reported significant reduction of alpha esterase activity when malignant colon tissue from the cancer patients was compared with normal colon tissue from healthy individuals. According to Vora et al. (2012), there is increased oxidative stress in D-galactose stressed mice that possibly caused a significant decrease of activity of non-specific esterase.

Many risk factors are associated with breast cancer. In this study the main focus was on family history and marital status. The data showed that 78% breast cancer patients were married and 22% were unmarried. Furthermore most of the breast cancer patients had family history of cancer (Table 4). Previously, researchers have revealed that family history and marital status are implicated with breast cancer (Aizer et al., 2013; Fakri et al. 2006; Naveed et al., 2014;). There was positive association of family history and incidence of breast cancer. Married females are at high risk as compared with unmarried ones. The Gilani et al. (2006) investigated that consanguineous marriages doubled the chance of breast cancer as compared with the risk of being married out of family. Hussain et al. (2013) did not find any association between family history and breast cancer in females. Bulotiene et al. (2008) have reported that marital status is associated with breast cancer mainly due to increase in the stress conditions. Our results are in contrary to the Aizer et al. (2013), who found that unmarried females have higher risk of metastatic cancer as compared with married ones. However, according to Fakri et al. (2006), marital status and breast cancer are not correlated.

The association between different age groups and incidence of breast cancer was also investigated in the present study. The results of present study showed that the age of most of the patients was around 40-50 (Fig. 3). The present results are in accordance with Naveed et al. (2014).

According to Hussain et al. (2013) the peak age for breast cancer is 50-59 years and it decreases in older age. Similarly Khan et al. (2004) reported that the prevalence of cancer was higher in age group of greater than 60 years and explained that it is significantly different in different age groups. According to Naeem et al. (2008) the middle age group (30-59years) women have high risk of developing breast cancer in local setup in various regions of Pakistan as reported in the present study. So it may be concluded that the above risk factors like family history and marital status and age are important in the pathogenesis of breast cancer. The association of the risk factors with frequency of breast cancer needs further study with large population size and in different ethnic groups. On the other hand the level of non-specific esterases like alpha and beta esterases have major role in the diagnosis of breast cancer.

![Fig. 1: Activity of β esterases (mM/min/mg) in control group and breast cancer patients.](image1)

![Fig. 2: Activity of α-esterases (mM/min/mg) in control and breast cancer patients.](image2)
Table I: Different factors associated with incidence of breast cancer.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total number of individuals 92</th>
</tr>
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<tbody>
<tr>
<td>Marital Status</td>
<td>Total</td>
</tr>
<tr>
<td>Married</td>
<td>72</td>
</tr>
<tr>
<td>Unmarried</td>
<td>20</td>
</tr>
<tr>
<td>Family History</td>
<td>No</td>
</tr>
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Fig. 3: Prevalence of breast cancer in different age groups.

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Development and Optimization of Molecular Technique for Diagnosis of Citrus Canker in Citrus Cultivars

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ABSTRACT
The conventional method for the detection of Xanthomonas axonopodis has been based on biochemical tests. A rapid and sensitive method for identification and detection of Xanthomonas axonopodis is required for management of citrus canker. PCR-based diagnostic test is appropriate for monitoring pathogen in a very short time compared to laborious, non-specific and expensive protocols. ELISA, Nested PCR has been used for many years in different countries. Citrus orchards in Sargodha region were surveyed and leaf samples showing typical symptoms of citrus canker were collected. Infected section of leaf was taken for isolation of bacteria. Lesions were cut into parts and streak bacteria by the help of inoculation loop grown in nutrient medium. Pure bacterial culture of Xanthomonas axonopodis were used for detection by standard PCR. Xanthomonas axonopodis was diagnosed by amplification of 16S rDNA. A fragment of ~1.4 kb was amplified and cloned for sequencing Out of three markers used (K1F-CIT/ K2R-CIT, K2F-CIT/ K2R-CIT, K3F-CUT/ K3R-CIY) K3F-CUT/ K3R-CIY gave best results repeatedly. So this primer pair can be used for identification/diagnosis of Xanthomonas axopodis. Bacterial culture used as template in PCR and colony PCR gave better results as compared with extracted DNA from infected leaf.

Key Words: Xanthomonas axonopodis citri, Citrus canker, Molecular diagnostics

INTRODUCTION
Xanthomonas axonopodis is the most devastating bacterium causing citrus canker. Citrus bacterial canker occurs in citrus producing countries which are in the region of tropical and sub-tropical areas. Strains of pthA, pthB, and pthC susceptible to citrus canker show abnormal increase of cells in normal host range (Garbiel et al., 2000). Canker affected plants show necrotic lesion that develops on leaves, fruits, and twigs. Surface of leaves becomes ruptured, shows blisters appearance due to hyperplasia which converts it into spongy pustules. Citrus canker has astringent contagiousness on grapefruit, pineapple, limes and lemons and their hybrids. Monetary citrus cultivars are susceptible that must be removed when diseases are exposed (Verniere, Hartung, Civerolo, & Pruvost, 1998). Citrus canker is prevalent and has a challenging effect on reservoirs eradication

Xanthomonas axonopodis citri, Xanthomonas axonopodis compestris and Xanthomonas axonopodis aurantifolii are gram-negative, rod like bacteria one flagellum. Size of flagellum is 1.5-2.0x0.5-0.75mm. Xanthomonas axonopodis have aerobic respiration and show obligate growth, yellow colonies are formed on culture medium. XAC have polysaccharide slime which helps them to form mucoid colonies; can grow in range of 25c-30c (Chand & Pal, 1982; Goto & Takahashi, 2000)

Economically, citrus fruit is considered very important. Chemical composition of essential oil extracted from citrus by the help of hydro distillation reveals six compounds i.e. limonene 80.51%, terpinene 6.80%, cymene 4.02%, β myrcene1.59%, and pinene 1.20% (Jin & Suk, 2009). Pakistan has attained 12th position in citrus production all over the world (Silva, 2013). In an area of 199 thousand hectares of citrus, Pakistan is producing 1832 thousand tons citrus is produced in Pakistan and the share of province Punjab is 95.10% and that of district Sargodha (Punjab) is 49.3% of total production in Pakistan (MINFAL, 2011-12).

Author’s Contribution: A.M.K. & A.A.K., designed experimental protocol; J.N. & M.M., did experimental work; H. M.T. & J. I., did editing and proof reading
Due to attack of *Xanthomonas axonopodis* corky lesion on leaf and fruit, vein chlorosis, premature fruit falling, hyperplasia, sunken centre, and elevated margins are formed (Schubert, 2001). In January 2006, USDA has revealed that eradication of citrus canker is not possible. While in Florida one billion canker eradication projects were applied in ten years (Gottwald & Irey, 2007). Citrus canker is established as epidemic in Argentina, Oman, USA, Australia, Asia, Brazil, Saudi Arabia, China, Reunion Island (Schubert & Miller, 2000).

In recent years demand of citrus fruit is increasing, but *Xanthomonas axonopodis citri* is damaging crops at large scale. Sweet fruits are more susceptible for canker attack. Eradication of crop helps to control incidence of citrus canker (Ribeirao & Machado, 2011).

Citrus canker is an alarming threat to citrus industry of Pakistan. *Xanthomonas axonopodis citri* (XAC) is the causative agent of citrus canker, prevalent in Subtropical citrus-growing areas. The disease can remarkably affect a wide range of citrus cultivars by defoliation, dieback, reduce fruit quality and pre-mature fruit drop and consequently is subjected to national eradication programs and international regulations.

**MATERIALS AND METHODS**

Marsh early (*Citrus paradisi* marsh), washington naval (*Citrus sinensis*), mid sweet (*Citrus maxima*), pineapple (*Ananas comosus*), kinnow (*Citrus reticulate* tarocco), pera rio (*Citrus paradisi* Rio Red), grapefruit (*Citrus paradisi*), kaghzi lemon (*Citrus limon*), and tarocco-N (*Citrus sinensis* tarocco-N) citrus cultivars were used in this study.

**Bacteria isolation and colonies formation:**

Bacteria were isolated from infected lesion of leaf and fruit after sterilization with 70% ethanol. Bacteria were oozeed out after lesion crushing. Oozed bacterial colonies were cloned on nutrient agar plate (0.5% peptone, 0.3% yeast extract, and 0.5% NaCl, 1.5% agar in 1 liter (L) water) by sterilizing loop. Bacterial colonies were cultured in nutrient medium (0.5% peptone, 0.3% yeast extract, 0.5% NaCl in 1 liter (L) water).

**Bacterial genomic DNA isolation:**

Bacterial genomic DNA was isolated from culture medium by the help TE lysis buffer (100mM Tris pH 8.0; 2% SDS), Proteinase K, 5M NaCl (292 gm of NaCl +700ml H2O), 24:1 chloroform: isoamyl alcohol, 0.5X TBE buffer(108gm Tris base+55gm Boric acid+40ml 0.5 EDTA: pH 8).

**PCR based amplification:**

PCR-based technique used for amplification of *Xanthomonas axonopodis* 16S rDNA, 23S rDNA region with the help of universal primers. Amplification was conducted in volumes of 25 µl in Peq STAR 96X universal gradient (PEQ Lab). Primers concentration and other condition were optimized to amplify DNA.

**PCR reaction mixture:**

PCR reaction mixture included MgCl2: 2 µl, DNTPs: 0.5µl (invitrogen Corp, San Diego-C, USA), Taq buffer: 2.5 µl (Invitrogen Corp), Taq DNA polymerase: 0.2 µl (Invitrogen Corp), Culture medium: 3 µl, Forward primer: 1.5 µl (16S rDNA) K3F, Reverse primer: 1.5 µl (16S rDNA) K3R, dd H2O (13.8 µl)

- Initial denaturation: 94 °C for 1 minute followed by:
  - Denaturation: 94 °C for 1 minute.
  - Annealing: 50 °C for 2 minutes.
  - Extension: 72 °C for 3 minutes.
- Final extension: 72 °C10-20 minutes.
- Store: 4 °C (Above mentioned range of PCR keep constant while annealing temperature changed according to samples)

**Confirmation of amplification:**

Amplification of PCR product was confirmed by loading 3 µl of PCR product in 0.8% agarose gel prepared in 0.5X TBE buffer. Gel documenting system visualized amplification of 16S rDNA and about ~1.4 Kb fragment size was amplified.

**Purification and ligation of PCR product:**

PCR amplified product was purified through phenol chloroform extraction. Purified PCR product was ligated into PTZ57/RT cloning vector (Fermentas). Ligation mixture (insert, PTZ57/RT vector, MgCl2, ddH2O, ligase buffer) added into competent cell (prepared from protocol of Cohen et al 1992).

**Screening of clones:**

Isolated DNA was digested with appropriate enzymes using appropriate buffers to confirm clones either single or double digestion.

**RESULTS**

**Bacterial DNA isolation:**

Bacterial DNA was isolated from bacterial colonies on agar plate. Isolated genomic DNA was used for PCR amplification.

**PCR amplification of 16S rDNA:**

DNA was diluted for suitable PCR amplification. ~1.4 kb bacterial genome was amplified. Initially Three strategies, like using extracted DNA from infected leaf, colony PCR and PCR using bacterial culture was used. Better and
consistent results were obtained while using 3µl of bacterial culture as a template and by colony PCR method as compared to using isolated DNA from infected leaf. Three pairs of universal primers were used for amplification of 16S rDNA viz K1F-CIT/ K1R-CIT, K2F-CIT/ K2R-CIT, K3F-CUT/ K3R-CIY (Table 1). Out of three primers checked, K3F-CUT/ K3R-CIY gave better results (Fig., 1, 2).

Further, PCR conditions were optimized for diagnosis of Xanthomonas axonopodis citri. Annealing temperature of marsh early (Citrus paradisi marsh), washington naval (Citrus sinensis), mid sweet (Citrus maxima), pineapple (Ananas comosus), kinnow (Citrus reticulate) was 50.5°C for 2 minutes while for tarocco (Citrus aurantium), pera rio (Citrus paradisi Rio Red), grapefruit (Citrus paradisi), kaghzi lemon (Citrus limon), and tarocco-N (Citrus sinensis tarocco-N) had 50.2 °C annealing temperature for 2 minutes (Table I).

**Cloning:**
PCR amplified product was cloned in PTZ57/RT vector. Cloning product was screened by digestion with appropriate enzymes. ~1.4 kb DNA was confirmed on 0.8% agarose gel.

**Table I: Optimization of primer for amplification**

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K1F-CIT</strong></td>
<td>GCATTTFATGACGCCATGAC</td>
<td>KIR-CIT TCCCTGATGCCTGGAGGATA</td>
</tr>
<tr>
<td><strong>K2F-CIT</strong></td>
<td>CTTCAACTCAACGCGGAC</td>
<td>K2R-CIT CATCGCGCGCTTGTTGGAG</td>
</tr>
<tr>
<td><strong>K3F-CUT</strong></td>
<td>AGAGTTGATCCTGGCTCAG</td>
<td>K3R-CIY ACGGCTACCTTGTTACGACTT</td>
</tr>
</tbody>
</table>

**Table II: Optimized temperature for amplification of DNA.**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Samples Name</th>
<th>Temperature(°C)</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Citrus paradisi marsh</td>
<td>50.5 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>2</td>
<td>Citrus sinensis</td>
<td>50.5 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>3</td>
<td>Citrus maxima</td>
<td>50.5 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>4</td>
<td>Citrus aurantium tarroco</td>
<td>50.2 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>5</td>
<td>Citrus paradisi rio red</td>
<td>50.2 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>6</td>
<td>Citrus aurantifolia swingle</td>
<td>50.2 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>7</td>
<td>Citrus sinensis tarroco-N</td>
<td>50.2 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>8</td>
<td>Citrus paradise</td>
<td>50.2 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>9</td>
<td>Ananas comosus</td>
<td>50.5 °C</td>
<td>~1.4Kb</td>
</tr>
<tr>
<td>10</td>
<td>Citrus reticulate</td>
<td>50.5 °C</td>
<td>~1.4Kb</td>
</tr>
</tbody>
</table>
Fig. 1: PCR amplification from cultured bacteria

Lane 1 shows *Citrus paradisi marsh* amplified DNA, Lane 2 shows *Citrus sinensis* amplified DNA, Lane 3 shows *Citrus maxima* amplified DNA, Lane 4 shows *Citrus aurantium* tarroco amplified DNA, Lane 5 shows *Citrus paradisi rio red* amplified DNA, Lane 6 shows *Citrus aurantifolia swingle* amplified DNA, Lane M shows ladder (1000kb), Lane 7 shows *Citrus sinensis tarroco-N* amplified DNA, Lane 8 shows *Citrus paradisi* amplified DNA, Lane 9 shows *Ananas comosus* amplified DNA, Lane 10 shows *Citrus reticulate* amplified DNA.

Fig. 2: Colony PCR amplification

Lane 1 shows *Citrus paradisi marsh* amplified DNA, Lane 2 shows *Citrus sinensis* amplified DNA, Lane 3 shows *Citrus aurantium* amplified DNA, Lane M shows ladder (1kb), Lane 4 *Citrus paradisi* amplified DNA, lane 5 negative controls, and Lane 6 shows *Citrus aurantifolia swingle* amplified DNA.

DISCUSSION

Henson & French, (1993) used bio-assay method for recognition of *Xanthomonas axonopodis* strains. Different pathogenicity tests were performed but the results were not accurate while in current study it was observed that standard PCR provided accurate results for recognition of *Xanthomonas axonopodis citri*. Cubero & Graham, (2002) used extracted DNA from bacterial colonies for PCR amplification while in recent study purified colonies suspension was directly used for PCR amplification.
and their clones were prepared. (Najafipour, 2014) used infected stem for identification of *Xanthomonas axonopodis* citri antecedently leaf was used for bacterial detection.

Ten citrus cultivars’ were used for diagnosis of *Xanthomonas axonopodis* by PCR. All cultivars have different genetic makeup but they have receptors on conserved region. These receptors provide a site for attack of *Xanthomonas axonopodis*. Diagnosis of *Xanthomonas axonopodis* on varying cultivars reduces ambiguity about *Xanthomonas axonopodis* attack. It is the first ever study of using PCR method to diagnose citrus canker on molecular level from our local varieties in Pakistan and their clone’s preparation.

**CONCLUSION**

*Xanthomonas axonopodis* attacks citrus cultivars on specific conserved region. Standard PCR provide accurate result for identification of *Xanthomonas axonopodis*.

**REFERENCES**


Seroprevalence of *Toxoplasma gondii* among Pregnant Women in Lahore, Pakistan

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**ABSTRACT**

*Toxoplasma gondii* a neglected protozoan parasite commonly infects humans worldwide. One third of human population has become victim of *T. gondii* and its infection in pregnant women has serious consequences on women as well as on fetus health. Present study was conducted to assess the seroprevalence of *Toxoplasma gondii* among pregnant women of Lahore, Pakistan. 239 blood samples of pregnant women were collected from Lady Walingdon hospital, Lahore along with other details on associated risk factors of infection. From these samples, 86 were randomly selected to be evaluated for the presence of IgG immunoglobulin by using enzyme-linked immunosorbent assay (ELISA) method. The overall seroprevalence of *T. gondii* in pregnant women was found 22% being IgG positive. The infection rate of *T. gondii* was observed higher (29%) in older age group (29-39 years) as compared to (18%) in younger age group (18-28 years). More seropositive women were found in 3rd trimester (26%) as compared to 1st (22%) and 2nd (15%) trimester of pregnancy. The highest seroprevalence of *T. gondii* was found in women having weight from 40-50kg and lowest rate (9%) was observed in women with 73-83kg weight. Miscarriages were observed in 31% seropositive women. Moreover, the toxoplasmosis rate was higher in socially poor and uneducated women. The seroprevalence of toxoplasmosis increases as age of mother increases due to accumulated exposure to *T. gondii* during the lifetime. The risk of infection increases in third trimester of pregnancy in women having low body weight.

**Key Words:** *Toxoplasma gondii*, pregnant women, congenital Toxoplasmosis, ELISA; seropositive.

**INTRODUCTION**

*Toxoplasma gondii* (*T. gondii*) is an obligate intracellular protozoan parasite commonly infecting humans worldwide (Fox *et al*. 2009). A careful estimate shows that *T. gondii* infects one third of human population globally (Kijlstra & Jongert, 2008). It causes toxoplasmosis that is characterized by clinical syndromes. Its definitive hosts are spread all over the world. The infection rate of *T. gondii* is increasing in human population and it has emerged as health threatening parasite all over the world (Flegr *et al*., 2014). *T. gondii* was first discovered in 1908 by two working groups in North Africa and Brazil (Black & Boothroyd, 2000) and has been placed in phylum Apicomplexa (Kessler *et al*., 2008).

There are major three modes of parasite transmission in humans. Firstly, ingestion of infected uncooked or inadequately cooked meat or uncooked foods that have come in contact with infected meat (Dubey, 1994). Secondly, parasitic transmission can occur from cat excreta containing oocysts to humans either from soil or from litter box. Third, infection can also spread from mother through placenta to its unborn fetus (Jones *et al*., 2003).

Toxoplasmosis is an international health problem. It is not only restricted to under developed countries but even the developed countries have substantial prevalence of *T. gondii*. In Belgium and France, the infection rate of congenital infection is 2–3 cases per 1000 live births. This infection rate is quite higher than the US reports, where seroprevalence is as low as 1 in 10,000 to 1 in 1000 live births (Dubey & Beattie, 1988; Guerina *et al*., 1994). Rate of *T. gondii* infection has fallen in many countries like United Kingdom and France over the past few decades (Montoya & Liesenfeld, 2004; Remington, 2006). United States has lowest seroprevalence of *T. gondii* and may show further

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decrease of seroprevalence in general population (Smith et al., 1996). But Central America and South America show higher seroprevalence of \textit{T. gondii}, approximately more than 60% (Remington et al., 1970; Remington, 2006). In Colombia, toxoplasmosis is considered a public health problem but still of little concern (Gómez et al., 1997). In Indonesia, it has been worked out that the seroprevalence of \textit{T. gondii} in humans is 58.8% and in pigs is 2.3% only (Tuda et al., 2017).

It is reported that pregnant woman becomes victim of \textit{T. gondii} at large scale due to environmental and demographic conditions. When a woman acquires infection during first or second trimester, then the new born will show severe symptoms of congenital toxoplasmosis (Dunn et al., 1999; Montoya & Remingtons, 2000). The infection of \textit{T. gondii} in unborn babies occurs through placenta. The parasitic infection in fetus leads to serious consequences like jaundice, abortion, brain calcification, microcephaly, hydrocephalus, mental retardation, blindness, and fetal death (Havelaar et al., 2007; Nissapatorn, 2009; Mwambe et al., 2013). There are some effective and efficient methods for diagnosis of \textit{T. gondii}. The common practice of parasite detection is the serological testing of Toxoplasma specific antibodies (IgG and IgM) in serum of patients (Alvarado-Esquivel, 2006; Binnicker, 2010; Mwambe et al., 2013; Cong et al., 2015).

In rural and urban areas, people keep cats and other pets which cause environmental contamination with oocysts leading to human infection (Shahzad et al., 2006). No substantial data is available regarding seroprevalence of \textit{T. gondii} in pregnant women in Punjab. It is, therefore, valuable to evaluate the blood serum of pregnant women for \textit{T. gondii} specific antibodies. The objective of the present study is to find the prevalence of \textit{Toxoplasma gondii} in pregnant women of Lahore, Pakistan and to collect information on associated risk factors to prevent toxoplasmosis.

\section*{MATERIALS AND METHODS}

Blood samples of 239 pregnant women were collected from the Lady Willingdon Hospital Lahore, Pakistan. Detailed information regarding risk factors like owning pets, educational level and socioeconomic conditions was gathered from these women through a designed questionnaire. All healthy pregnant women were included whereas the women with any kind of pathological infection such as HIV +ve, HCV +ve, HBC +ve, Anemia +ve and thalassemia +ve etc. were excluded from this study.

\section*{RESULTS}

Out of 239 collected blood samples, 86 samples were randomly selected for serological detection of toxoplasma-specific immunoglobulin, IgG.

ELISA immunoassay technique was employed to analyze the \textit{T. gondii} in blood samples. For diagnosis of toxoplasmosis, a commercially available kit “Toxoplasmosis Latex”, manufactured by Antec Diagnostic Products was used. The test was performed according to the procedures described by the kit manufacturer. Cut off value of every sample was determined and thus value index of every sample was calculated (Ahmad & Tasawar, 2016).

The ELISA test results and data obtained through questionnaires were analyzed by one way ANOVA using SPSS version 13.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Parameters} & \textbf{Total women included} & \textbf{IgG positive cases} & \textbf{Seroprevalence} \\
\hline
\textbf{Age (years)} & 86 & 19 & 22 \\
18-28 & 55 & 10 & 18 \\
29-39 & 31 & 9 & 29 \\
\hline
\end{tabular}
\caption{Overall seroprevalence of \textit{T. gondii} in pregnant women}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Parameters} & \textbf{Total women included} & \textbf{IgG positive cases} & \textbf{Seroprevalence} \\
\hline
\textbf{Trimester} & & & 22 \\
1\textsuperscript{st} & 18 & 4 & 22 \\
2\textsuperscript{nd} & 26 & 4 & 15 \\
3\textsuperscript{rd} & 42 & 11 & 26 \\
\hline
\end{tabular}
\caption{Seroprevalence of \textit{T. gondii} in women with respect to the stage of pregnancy}
\end{table}
The blood pressure is considered another determinant of *T. gondii* infection in pregnant women. A substantial no. of pregnant women (24%) had low blood pressure whereas 66% IgG positive women had high blood pressure and only 14% of women had normal blood pressure. Further the selected samples were divided into four groups with respect to weight. The highest seroprevalence of *T. gondii* (50%) was found in women having weight from 40-50kg and lowest rate (9%) was observed in women with 73-83kg of weight (Table IV). Further, out of 19 IgG positive women, 31% pregnant women had premature miscarriages of their last baby and some of them experienced miscarriages more than once.

**Table III: Relationship between Seroprevalence of *T. gondii* and Blood pressure**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total women included</th>
<th>IgG positive cases</th>
<th>Seroprevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mm/Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>34</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>High</td>
<td>3</td>
<td>2</td>
<td>66</td>
</tr>
<tr>
<td>Low</td>
<td>49</td>
<td>12</td>
<td>24</td>
</tr>
</tbody>
</table>

**Table IV: Relationship between Seroprevalence of *T. gondii* and body weight**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total women included</th>
<th>IgG positive cases</th>
<th>Seroprevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40-50</td>
<td>22</td>
<td>11</td>
<td>50</td>
</tr>
<tr>
<td>51-61</td>
<td>22</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>62-72</td>
<td>21</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>73-83</td>
<td>21</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

**DISCUSSION**

*T. gondii* is a common parasite is that transferred in humans by pets owning, blood transfusion and unhygienic environmental conditions. In Pakistan many people are unaware of the need of proper vaccination for their pets. This reality is considered the main cause of spreading infection among the owners. On the other hand, owning a pet does not mean the transfer of *T.gondii* but direct contact with cat feces and improper hand wash after contact causes transfer of parasite (Nissapatorn *et al*., 2011). Our findings could not relate to significant association of pet keeping with infection as most of the infected women did not keep pets at home.

The findings of existing study showed that the overall prevalence of *T. gondii* in pregnant women was 22% being positive for IgG which was even higher (29%) in older age group. The prevalence in our study is quite higher than the seroprevalence found in pregnant women of other countries such as Zambia, (5.87%), (Frimpong *et al*., 2011), South Africa, 6.4% (Mwambe *et al*., 2013) and Korea 3.7% (Aqeely *et al*., 2014). There are studies which showed relatively high rate of prevalence of *T.gondii* in other countries, for instance 18.7% in Mozambique (Sitoe *et al*., 2010), 30.9% in Tanzania (Nissapatorn *et al*., 2011) and 92.5% in Ghana (Ayi *et al*., 2009). Globally, the prevalence of toxoplasmosis is 24.1% in Saudi Arabia, (Aqeely *et al*., 2014), 28.3% in Southern Thailand (Sroka, 2010) and 17.3% in London (Kistiah, 2011). The seroprevalence of *T. gondii* in the present study was quite low as compared to that in Brazil, (68.37%) but comparable to that in Mexico (6.1–27.9%) and USA (17.5–29.2%) (Jones, 2001; Galvan-Ramirez *et al*., 2012; Silva *et al*., 2015).

There is a strong association between the risk of infection and the age of pregnant. It has been reported that risk of infection increases significantly with the increase of age (Nissapatorn *et al*., 2011; Singh *et al*., 2014). Our findings are in agreement to the study conducted on 955 pregnant women and results revealed that the prevalence of infection increased implicitly with increasing age (Rosso *et al*., 2008). It appears that as the age increases the risk of infection increases because time of exposure to toxoplasmosis increases as the one gets older (Ertug *et al*., 2005). There are some studies which revealed that age was not significantly associated with the infection (Ayi *et al*., 2009).

The transmission of *T.gondii* also occurs by blood transfusion. Different studies were conducted in Iran to find out the possibility of *T. gondii* in individuals who donate their blood to patients. The results showed that 102 out of total 270 samples were positive (37.8%) for IgG and none were IgM-positive (Tappeh *et al*., 2017). ELISA method is the most appropriate method for the detection of toxoplasmosis even after two weeks of infection. The presence of IgG and IgM antibodies in the serum indicates the incidence of infection. Some studies have reported the presence of IgM antibodies but in others studies consistent with our results did not find any IgM in serum (Mwambe *et al*., 2013; Sakae *et al*., 2013).
Our studies demonstrated that there was no significant association between gestational stage and the incidence of toxoplasmosis. However, the present results showed that the rate of toxoplasmosis was higher in third trimester as compared to first and second trimester of pregnancy. These findings are in agreement with the studies reported elsewhere (Ayi et al., 2009). Premature miscarriages were seen in toxoplasma IgG positive women in our study. Some pregnant women had with their previous baby died after birth while others experienced premature death of the fetus. Our data is in line with the study conducted in Norway, where 35940 women were investigated for toxoplasmosis and the results revealed that T.gondii infected women had miscarriage at the end of first trimester (Jenum et al., 1998).

There is valid association between parasite infection and socioeconomic status of the pregnant women. In our study, most of the seropositive pregnant women belonged to backward rural areas of Lahore where educational rate was very low (especially in women) due to poverty and low living standards. The findings of our study are consistent with the results of Frimpong et al. (2017), Andiappan et al., (2014) and Jones et al. (2009) who revealed that the prevalence of T.gondii was high in pregnant women with low socio-economic status as compared to the women with high socio-economic status. Similarly, in Bangladesh, the incidence of T.gondii was more common in poor people as compared to people belonging to upper socioeconomic status (Ashrafunnessa et al., 1998). This high prevalence of infection in low socio-economic group is due to unhygienic practices, poor living standards and low education.

ACKNOWLEDGMENTS

The authors are indebted to staff members of pathology department of the Lady Wilingdon Hospital, Lahore for their cooperation during sample collection.

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women and vertical transmission of Toxoplasma gondii in patients from basic units of health from Gurupi, Tocantins, Brazil, from 2012 to 2014. *Plos One.*, 10(11): e0141700.


Fossil molars of *Pachyportax* (Boselaphini, Bovidae) from Middle Siwalik Subgroup of Pakistan

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**INTRODUCTION**

The Siwaliks are Neogene deposits found in Pakistan, India, Nepal and Bhutan. It is divided into three subgroups namely Lower Siwaliks, Middle Siwaliks and Upper Siwaliks. The Middle Siwalik fauna is richly recorded formation known as Dhok Pathan Formation of Pakistani Siwaliks. The fossiliferous localities of the Siwalik Group are known since the 17th century (Lydekker, 1876, 1878, 1884; Pilgrim, 1926, 1937, 1939; Hooijer, 1950; Akhtar, 1992; Khan *et al.*, 2009, 2012, 2014, 2015; Babar *et al.*, 2016). *Pachyportax latidens* was reported for the very first time in Middle Siwaliks at Late Miocene ages. The said species has very short stratigraphic range from Late Miocene to Early Pliocene of the Middle Siwaliks of Pakistan. The species disappeared in Upper Siwaliks during Pleistocene ages, so it has a very short interval geologically (Pilgrim, 1939; Gentry, 1999 Bibi, 2007; Khan *et al.*, 2009).

The newly recovered material from Dhok Pathan Formation of the Siwaliks confirms the existence of the *Pachyportax latidens*, a large sized bovid species, in the Siwaliks. The isolated molars have been recovered from two villages (Hasnot and Padhri) of district Jhelum, Pakistan (Fig.1). The stratigraphy and biochronology of the Potwar plateau of Pakistan was provided by Barry *et al.* (2002). “The material from the Late Miocene of the Siwaliks can be assigned to *Pachyportax latidens*, which is common at the Late Miocene-Early Pliocene sites in the subcontinent Siwaliks.

**METHODOLOGY**

The newly discovered material has been deposited in Fossil Display and Research Centre, Department of Zoology, University of the Punjab, Lahore, Pakistan. The aim of the article is to describe additional material of the Late Miocene large sized Siwalik bovid and it will be an addition to the existing knowledge about these genera.

**SYSTEMATIC PALAEONTOLOGY**

“Family Bovidae Gray, 1821”
“Tribe Boselaphini Simpson, 1945”
“Genus *Pachyportax* Pilgrim, 1937”

*Pachyportax latidens* (Lydekker) Pilgrim, 1937*

**New material:** “PUPC 16/17, an isolated highly damaged upper premolar; PUPC 16/13, an isolated partially broken left M2; PUPC 16/15, an isolated left M2; PUPC 16/14, an isolated partial left M3.”

**DESCRIPTION**

PUPC 16/17 is a damaged upper premolar (Fig. 2). The protocone is present whereas the paracone is highly damaged. The metacone and hypocone are missing. The molars are quadrates (Fig. 2). The protocone is extended lingually. The
‘preprotocrista’ is smaller than the ‘postprotocrista’. The dentinal eyelet is present occlusally in the center of the molars. The enamel is rugose. The mesostyle is more developed than the parastyle and metastyle. “The lower third molar is partially preserved (Fig. 2). The major conids: protoconid, metaconid, hypoconid and entoconid are well developed. The ectostylid is transversally extended. The prefossettid and postfossettid are deep.”

**Fig., 1:** “Map of Potwar Plateau showing studied localities (encircled) in northern Pakistan.”

**Fig., 2:** *Pachyportax latidens*: 1. PUPC 16/17, P; 2. PUPC 16/13, M2; 3. PUPC 16/15, M2; 4. PUPC 16/14, m3. a = occlusal, b = lingual, c = buccal. Scale bar 10mm.
Table I: Comparative measurements of the cheek teeth of *Pachyportax latidens* in millimeters. *The studied specimens. Referred data are taken from Akhtar (1992), Khan et al. (2008, 2009).

<table>
<thead>
<tr>
<th>Inventory No.</th>
<th>Nature</th>
<th>Length</th>
<th>Width</th>
<th>W/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUPC 16/17*</td>
<td>P</td>
<td>ca.20.14</td>
<td>ca.18.08</td>
<td>0.89</td>
</tr>
<tr>
<td>PUPC 16/16*</td>
<td>M</td>
<td>ca.17.87</td>
<td>ca.23.83</td>
<td>1.33</td>
</tr>
<tr>
<td>PUPC 16/15*</td>
<td>M2</td>
<td>22.30</td>
<td>23.41</td>
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</tr>
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<td>21.45</td>
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<td>28.08</td>
<td>17.05</td>
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<td>m3</td>
<td>37.2</td>
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<tr>
<td>PUPC 86/7</td>
<td>m3</td>
<td>33.0</td>
<td>14.0</td>
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</table>

**COMPARISON AND DISCUSSION**

The most prominent features of the upper molars are large sized and quadrate shape with transversely extended median basal pillars, relatively heavy styles and strong ribs. The systematic study of the specimens reflects all the morphometrical features of the genus *Pachyportax* and the species *P. latidens* (Khan et al., 2008 a,b 2009). Genus *Selenoportax* has constricted crown neck as compared to *Pachyportax*. It is already noted that *Pachyportax* adapted habitats like other living bosalpines, *Boselaphus tragocamelus* (Scott, 1985; Khan et al., 2009). The development of folds and strong pillars with rounded and wearing cusps suited for open habitat and diets available in these habitats. In diet, much tougher items are indicated by more complex and advanced morphological characters of teeth in the group (Bibi & Gülec, 2008). In fact, the Hasnot and Padhri regions indicate patchy drier habitats with dense forests and wet lands.

**CONCLUSIONS**

The new molars of *Pachyportax latidens* recovered from Hasnot and Padhri Villages of District Jhelum of Middle Siwalik Subgroup of Pakistan. The recovery of molars updates the palaeontological record of *Pachyportax latidens* and confirms the existence of species in the Siwalik Late Miocene – Early Pliocene.

**REFERENCES**


Species Diversity and Community Assemblage of Planktonic Rotifers in Pipnakha Pond, Gujranwala, Pakistan

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ABSTRACT

Species diversity and community assemblage of planktonic rotifers were determined with respect to seasonal variations in a pond of Pipnakha village, Gujranwala. Sampling was executed on monthly basis at three sites of the pond from October, 2011 to September, 2012. Altogether, 74 rotifers belonging to 24 genera and 13 families were identified. Highest (128.7±40 ind/ml) population density of rotifers was observed in June while lowest (64.9±22 ind/ml) population density was seen in January. Brachionus havanaensis was found to be the dominant species with the highest mean population density (40±11.9 ind/ml) while Philodina roseola was a least dominant with lowest population density (3±1.2 ind/ml). ANOVA displayed a significant difference in population density of rotifers among months. Rotifers exhibited positive correlations with temperature, total hardness pH, TDS, electrical conductivity and turbidity however, DO and transparency indicated negative correlations. Shannon-Weaver index stretched from 0.936 to 3.802 and exhibited great diversity. Values of species evenness extended from 0.932 to 0.970 that showed even distribution.

Key Words: Species diversity, rotifers, seasonal fluctuations, abiotic parameters

INTRODUCTION

Freshwater zooplanktons are classified into Rotifers, Cladocerans and Copepods. The members of the phylum Rotifera are distributed into three classes i.e. Bdelloidea, Monogononta and Seisonidea and represented by about 2200 described species (Ejaz et al., 2016). About 95% rotifer fauna is found in freshwater bodies and remaining 5% in marine (Miller & Harley, 2007). Rotifers live in lotic waters (rivers, streams, canals) and lentic water bodies (ponds, floodplains, lakes) (Lansac-Toha et al., 2009). These are important members of the littoral and limnetic un-segmented, pseudocoelomate, bilaterally symmetrical invertebrates (Wallace & Snell, 2010; Segers, 2007; Sulehria et al., 2013).

Some rotifer species dwell boundary covering aquatic and terrestrial settings i.e., they colonize film of water covering lichens, mushrooms, mosses and liverworts. This type of habitat is called limnoterrestrial. They are also found in soil, rainy puddles, tree holes, pitcher plants, gutters and on aquatic larvae of insects, freshwater crustaceans and also in sewage treatment plants (Wallace et al., 2015). There are also some epiphytic rotifers (Sulehria et al., 2012).

A small number of rotifer species is very particular in their feeding habits, but most are opportunistic and ingest numerous types of food such as algae, bacteria and ciliates although some are detritivorous (Sulehria & Malik, 2013).

Larvae of most planktivorous fish, due to high protein content use rotifers as food for rapid growth (Clarke et al., 2013). Several large animals fed on rotifers such as tadpoles (Barua, 1988) and small rotifers are eaten by bryozoans and copepods and they also fall prey to bigger rotifers (Wallace et al., 2006).

Dispersal of rotifers is affected by ecological barriers instead of geographical obstructions (Pejler, 1995). Abundance of predators, food resources, temperature and contestants are the main issues that upset the community structure of rotifers (Ekhande et al., 2013). As compared to other zooplankton rotifers react more sharply and quickly to the deviations in aquatic environment. The study
of rotifers is vital to assess the features of a pond (Kumar et al., 2011).

In this study we identified the rotifer species of pond from study area and recorded their seasonal dynamics. We also explored and studied the correlation of abiotic factors with the rotifer fauna.

MATERIALS AND METHODS

Study area

In the centre of the Pipnakha village, a pond is located on the western side, at the distance of 14 km from Gujranwala city. This pond is approximately 234 ft long and 150 ft wide. In the pond three sites (PS1, PS2 and PS3) were selected for sampling which were further divided into three sub-sites.

Fig., 1: Map of a pond in Pipnakha village. Physico-chemical analysis of water

For physico-chemical analysis, water sampling was done for one year (October, 2011 to September, 2012). HCl (2.5%) solution was used for soaking the bottles before sampling and then washed by using distilled water. Just before sampling the bottles were rinsed with pond water. Samples were collected from below the surface (20-25 cm) of the pond.

Temperature (°C), electrical conductivity (µS/cm), total dissolved solids (mg/l), dissolved oxygen (mg/l), turbidity (NTU) and pH were noted at the spot by using their respective metres such as thermometer (HANNA HI-8053), conductivity meter (YSI-Eco Sense EC300), TDS meter (YSI-Eco Sense EC 300), D.O meter (YSI-Eco Sense DO 200), turbidity meter (HANNA HI-93703) and pH meter (YSI-Eco Sense pH 100). A Secchi disc was used to measure transparency (cm). Total hardness (mg/l) was noted by using the technique given in APHA (2005).

Rotifer Collection

A net of 37µm mesh size was used for rotifer sampling on monthly basis. This net was towed for 2 to 3 minutes horizontally, so that 40 to 50 L of water could pass through it. From each site three samples of rotifer were taken and mixed to make a composite sample. Calculation of sampled volume was done after Perry (2003). Rotifers were conserved by putting a few drops of 4 to 5% formaldehyde solution (Koste, 1978). To study live rotifers an additional rotifer sample was taken from each site.

Rotifer Counting and Identification

Rotifer species were recognized by observing their morphology, behavior, shape and size (Ward & Whipple, 1959; Pennak, 1978; Segers, 2007). By using a Sedgewick-Rafter cell and inverted Olympus microscope, rotifers were counted at 60-100x (APHA, 2005) and studied after staining with 1% neutral red (vital stain).

Diversity Indices

Diversity indices (Simpson and Shannon-Weaver) were applied to figure out the rotifer diversity. Diversity indices, Species evenness (SE) and Species richness were exhibited by the method used by Sulehria et al. (2013); Ejaz et al. (2016).

Data analyses

Pearson’s correlation was applied to anticipate the relationship between physico-chemical limits and rotifer species. To find the difference among rotifer density of various months ANOVA was applied. Software MINITAB 2013 was used for ANOVA and Pearson’s correlation. MS Excel 2010 was used to draw abundance curve and graphs.

RESULTS AND DISCUSSION

Physico-chemical limitations of water influence the diversity and community assemblage of rotifers either negatively or positively (Chittapun et al., 2007; Sulehria et al., 2012). Highest water temperature (37.46±0.23) was seen in June and lowest (9.67±0.19) in the month of January (Fig. 2). Results of this study, showed a positive correlation of rotifers with temperature (Table I). Density of rotifers was observed highest (128.7±40 ind/ml) in June while lowest (64.9±22 ind/ml) in January (Fig. 3). Results indicated that the rate of rotifer population growth enhanced with the rise in temperature. Related findings were also reported in various studies by Baloch et al., 2008; Schöll & Kiss, 2008; Ejaz et al., 2015. Increase in
temperature proportionately increases the rate of growth of rotifer population.

pH is the total of proton activities and ideal pH for rotifer growth ranges from 6.5 to 8.5 (Neschuk et al., 2002). The maximum pH (8.9±0.01) was seen during the month of June while minimum pH (6.59±0.06) was noted during January (Fig. 2). During this study, Pearson correlations reflected positive relationship between rotifers and pH (Table I). Dai et al. (2014) and Ejaz et al. (2016) reported similar findings. Contrary results were obtained by Sulehria and Malik (2012) in some earlier studies. Increase in pH in hot months may be due to increased quantity of nitrates, phosphates and CaCO₃.

Dissolved oxygen (DO) is critical for aquatic life. Highest DO (10±0.01) was seen in the month of January and lowest (6.5±0.05) in June (Fig. 2). During hot months this drop in DO might be due to falling solubility of oxygen and increasing decomposition. The correlations between rotifers and dissolved oxygen reflected negative influence (Table I). These conclusions agreed with the findings of Saler & Sen (2002), Sulehria et al. (2013) and Shumka (2014). However, these results were totally diverse from earlier studies conducted by Malik & Sulehria (2004); Sulehria et al. (2009b). Five genera i.e., Testudinella Notholca, Lepadella, Synchaeta and Lecane, revealed high density and diversity with rise in dissolved oxygen, which might be due to cold temperature rather than high level of DO.

Electrical conductivity was highest (891.33±0.88) in August and lowest (468.67±0.88) in January (Fig. 2). Highest conductivity in hot months might be due to lower solubility, high temperature and decomposition of organic matter. Rotifer density and diversity reflected positive correlations with electrical conductivity (Table I). Similar effects had also been reported in other studies in Pakistan by Sulehria & Malik (2012, 2013) and Ejaz et al. (2015, 2016).

Total Dissolved Solids (TDS) increased in the pond with natural means and also agricultural run-off and urban wastes. TDS are infiltrable solids and show direct relationship with EC. Samal (2001) found that EC would increase with rise in TDS. Total dissolved solids were recorded maximum (579.36±0.64) in August and minimum (304.64±0.63) in January (Fig., 2). Total dissolved solids (TDS) revealed positive correlations with rotifers (Table I). Similar findings were also reported by Mustapha (2009) and Hussain et al. (2014). Increase in TDS might be due to natural resources, urban and agricultural run-off, industrial and sewage wastes.

The maximum total hardness (314.65±0.12) was calculated in June and minimum (242.73±0.13) in January (Fig., 2). Pearson correlations showed positive relationship between rotifers and total hardness (Table I). Increased hardness might be the result of more detergents, organic substances, chlorides and other pollutants. Similar results were also noted by Malik & Sulehria (2004) and Ejaz et al. (2016). Transparency extended from 10.33±0.20 and 20.13±0.19 and reflected negative correlations with the rotifer population. Turbidity reflected positive correlation with rotifer species (Fig., 2).

During this work, 74 rotifer species belonging to 24 genera and 13 families were identified (Table II). June reflected the maximum (128.7±40 ind/ml) mean density of rotifers and minimum was seen (64.9±22 ind/ml) in January. Philodina roseola showed lowest (3±1.2 ind/ml) density and highest (40±11.9 ind/ml) mean population density was shown by Brachionus havanaensis making it a dominant species (Fig. 3). Month of June revealed the highest (52 species) diversity of rotifers and lowest (26 species) diversity was observed in January (Fig., 4).

From 24 genera, Brachionus was the highest (8.37%) contributor and Pleosoma was the lowest (1.56%) one. Percentage composition of other rotifer genera was 6.71% (Keratella), 5.57% (Lecane), 5.34% (Lepadella), 5.31% (Filinia), 5.3% (Testudinella), 5.15% (Trichocerca), 5% (Rotaria), 4.99% (Polyarthra), 4.74% (Synchaeta), 4.65% (Notommata), 4.65% (Cephalodella), 4.5% (Notholca), 4.38% (Philodina), 3.71% (Colurella), 3.59% (Hexarthra), 3.3% (Collotheca), 2.72% (Macrochaetus), 2.47% (Monomamma), 2.39% (Kellcottia), 1.98% (Trichoria), 1.81% (Anuraeopsis) and 1.81% (Dicroanophorus) (Fig., 6). Similar findings were also reported by Ejaz et al. (2016).

ANOVA reflected a significant difference (P<0.05) in rotifer density during the study period (Table III).

Values of Shannon Weaver index ranged from 3.036 (lowest) in January to 3.802 (highest) in June. Minimum Simpson index of dominance (0.025) was observed in June and highest (0.053) in the month of January. Values of Simpson index of diversity exhibited minimum (0.947) in January and maximum (0.975) in June. Increase in temperature enhanced the rate of photosynthesis, and detritus which might be the cause of the high density and diversity of rotifers due to enhanced growth. Lowest species richness (2.256) of rotifers was observed in January and highest (6.998) in the month of March. In the month of January, minimum (0.932) value of
species evenness was calculated and maximum (0.970) in March (Fig., 5).
In species abundance curve, Brachionus havanaensis showed the maximum abundance (40) and minimum (3) by Philodina roseola. All the remaining species ranged between these two borders (Fig., 7).

Table I: Correlations (Pearson) between Rotifers and physico-chemical parameters.

<table>
<thead>
<tr>
<th>Rotifers</th>
<th>Temp</th>
<th>pH</th>
<th>DO</th>
<th>EC</th>
<th>TDS</th>
<th>TH</th>
<th>Trans</th>
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</tr>
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<td></td>
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<tr>
<td>TDS</td>
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<td>0.739</td>
<td>0.073</td>
<td>-0.424</td>
<td>1.000</td>
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<td>0.913</td>
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<td>TH</td>
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<td>0.726</td>
<td>0.176</td>
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<td>0.913</td>
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<tr>
<td>Trans</td>
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<td>-0.882</td>
<td>-0.516</td>
<td>0.805</td>
<td>-0.688</td>
<td>-0.688</td>
<td>-0.681</td>
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<tr>
<td>Turb</td>
<td>0.800</td>
<td>0.848</td>
<td>0.437</td>
<td>-0.794</td>
<td>0.683</td>
<td>0.683</td>
<td>0.695</td>
</tr>
</tbody>
</table>

Temp= Temperature, DO= Dissolved oxygen, EC= Electrical conductivity, TDS= Total dissolved solids, TH= Total hardness, Trans= Transparency, Turb= Turbidity

Table II: List of rotifer species identified from Pipnakha Pond.

<table>
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<th>S. No</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
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</thead>
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<tr>
<td>1</td>
<td>Brachionidae</td>
<td>Anuraeopsis</td>
<td>Anuraeopsis fissa Gosse, 1851</td>
</tr>
<tr>
<td>2</td>
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<td>Brachionus</td>
<td>Brachionus angularis Gosse, 1851</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Brachionus</td>
<td>Brachionus budapestiensis Daday, 1885</td>
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<tr>
<td>4</td>
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<td>Brachionus</td>
<td>Brachionus calyciflorus Pallas, 1766</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Brachionus</td>
<td>Brachionus diversicornis (Daday, 1883)</td>
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<tr>
<td>6</td>
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<td>Brachionus</td>
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</tr>
<tr>
<td>7</td>
<td></td>
<td>Brachionus</td>
<td>Brachionus havanaensis Rousselet, 1911</td>
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<tr>
<td>8</td>
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<td>Keratella tropica (Apstein, 1907)</td>
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<td>Keratella cochlearis (Gosse, 1851)</td>
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<tr>
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<td>Lepadella tripeta (Ehrenberg, 1832)</td>
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<td>Cephalodella catellina (Müller, 1786)</td>
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<td>Cephalodella sterea (Gosse, 1887)</td>
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</tr>
<tr>
<td>52</td>
<td>Rotaria rotatoria (Pallas, 1766)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>Rotaria citrina (Ehrenberg, 1838)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>Rotaria neptunia (Ehrenberg, 1830)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>Ploesoma lenticulare Herrick, 1885</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>Polyarthra vulgaris Carlin, 1943</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>Polyarthra dolichoptera Idelson, 1925</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>Polyarthra euryptera Wierzejski, 1891</td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>Polyarthra trigla Ehrenberg, 1834</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>Synchaeta oblonga Ehrenberg, 1832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>Synchaeta pectinata Ehrenberg, 1832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>Testudinella patina (Hermann, 1783)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>Testudinella emarginula (Stenroos, 1898)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>Testudinella triglata Smirnov, 1931</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Testudinella parva (Ternetz, 1892)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>Trichotria tectatis Ehrenberg, 1830</td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>Trichocerca porcellus (Gosse, 1851)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>Trichocerca capucina (Wierzejski &amp; Zacharias, 1893)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>Trichocerca cavia (Gosse, 1886)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>Trichocerca cylindrica (Imhof, 1891)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>Trichocerca bicristata (Gosse, 1887)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>Trichocerca flagellata Hauer, 1937</td>
<td></td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>Trichocerca longiseta (Schrank, 1802)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>Trichocerca similis (Wierzejski, 1893)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Total** 13 24 74
Table III: Analysis of Variance of Rotifers (P<0.05).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>1</td>
<td>40674</td>
<td>40674</td>
<td>134.70</td>
<td>&gt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>22</td>
<td>6643</td>
<td>302</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>47318</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DF= Degree of freedom, SS= Sum of square, MS= Mean of square, F= f-Distribution, P= Probability, Significance level= 0.05

Temp= Temperature, D.O= Dissolved oxygen, E.Cond= Electrical conductivity, TDS= Total dissolved solids, T.Hard= Total Hardness, Trans= Transparency

Fig. 2: Variations of different physico-chemical parameters in Pipnakha Pond

Fig. 3: Density of rotifers isolated from Pipnakha Pond.
Fig. 4: Diversity of rotifers isolated from Pipnakha Pond.

Fig. 5: Variations of diversity indices of rotifers isolated from Pipnakha Pond.

H (Shannon-weaver diversity index), D (Simpson index of dominance), 1-D (Simpson index of diversity), SR (Species richness), SE (Species evenness)
Fig., 6: Percentage representation of rotifer genera isolated from Pipnakha Pond

Fig., 7: Species abundance curve of rotifers isolated from Pipnakha Pond

CONCLUSION

Analysis of planktonic rotifers of Pipnakha Pond is the first work. Our knowledge regarding rotifer fauna of Gujranwala is still not thorough. This research work is an input to the existing knowledge of distribution of rotifers and desires further research work to obtain a consistent representation of planktonic rotifer fauna of lentic water bodies and ecology of this group.

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Microbial Diversity Associated With Rice Seeds

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ABSTRACT

During present investigation, seed-born micro-flora of five rice (Oryza sativa L.) varieties viz. Basmati-198, Basmati-802, Basmati Pak, Basmati Shaheen and Basmati-370 were studied using agar plate and blotter paper method. Highest percentage of myco-flora (17%) was found to be associated with seeds of Basmati Pak that was considerably more than myco-flora associated with all other varieties of rice. The highest percentage of bacterial isolates (16%) was recorded with Basmati Shaheen variety. Total of six fungal and three bacterial species namely Fusarium sp., Alternaria alternata, Pyricularia sp., Dreschlera sp., Penicilium sp., Curvularia sp., Acetobacterium, Deniobacter and Micrococcus were isolated from different rice varieties.

Key Words: Rice, seed-borne micro-flora, blotter paper, diversity

INTRODUCTION

Oryza sativa (rice) is a member of the grass family (Arnold et al., 1987). As a cereals crop, it is generally consumed as food for most of the world’s population, especially in Asia (Neue, 1993). Cereals manufacturing in Pakistan keeps very important position in farming and the national economic system (Abid et al., 2014). Pakistan is the world’s 11th biggest manufacturer of rice, after China, India, Indonesia, Bangladesh, Vietnam, Thailand and Burma. Each year, it produces an average of 6 million tones and together with the rest of the South Asia, the country is responsible for providing 30% of the paddy grain output (FAO, 2006). Many fungi, ranging from pathogens to non-pathogens of rice, have been recorded as seed borne on rice (Aluko et al., 2004). The rice crop has suffered from various types of diseases, majority of them are known to be caused by bacteria e.g. Bacterial blight (Xanthomonas oryzae) Bacterial leaf streak (Xanthomonas oryzae) Foot rot (Erwinia chrysanthemi) Grain rot (Burkholderia glumae) Sheath brown rot (Pseudomonas tuscovaginae) (Dean et al., 2005). The current study was conducted to recognize the fungal and bacterial micro-flora related to different varieties of rice seeds.

MATERIAL AND METHODS

Sample Collection

Seeds of following rice varieties namely Basmati-198, Basmati-802, Basmati Pak, Basmati Shaheen, Basmati-370 were collected from Rice Research Institute of Kala Shaha Kaku, Pakistan. One thousand seed of each variety was selected randomly. Physical characteristics of all the seed varieties were measured by means of seed length, seed width, seed thickness, seed length width ratio and weight.

Selection of Seeds

Seeds from each rice samples were randomly selected and tested for different physical traits (seed length, seed width, seed thickness, length/width, and 1000 seed weight) and germination tests.
Measurement of Physical Characteristics of Local Germplasm lines

Physical characteristic of 5 local varieties of rice were measured. For this purpose, 1000 seed weight for each sample was measured on a digital weighing balance, and three readings were taken for each sample. Seed length, seed width and seed thickness of these local rice varieties was measured with the help of a digital Vernier Calliper. For each sample of rice, these measurements were taken in three replicates; in each replicate 5 seeds were measured. The seeds were randomly selected from the seed samples.

Selective isolation procedures and media

Rice seeds were air dried for 48 hours at room temperature and then thoroughly washed to remove surface debris completely. After drying, the seed samples were divided into two groups i.e. unsterilized and sterilized. The seed samples were subjected to surface sterilization procedure: 2-3 min wash in 1% NaOCl, followed by a 5 min wash in sterile water. After being thoroughly dried under sterile conditions, the seeds were then pretreated by one of the following methods for isolation purposes.

Method 1

Seeds samples from both groups were aseptically placed on selective medium i.e. MEA (Malt Extract Agar) and LBA (Laura Bertani Agar) for fungal and bacterial isolation. The inoculated plates were incubated at 25±2 ºC for 5-7 days and at 37 ºC for 24 hours respectively.

Method 2

Occurrence of seed borne micro-flora on seeds was determined by Modified Blotter paper method. Ten seeds of each cultivar were selected. Ten surface sterilized and unsterilized seeds of each cultivar were spaced on Blotter paper in plastic petri dishes. These plastic petri dishes were incubated at -20 ºC for 8 days for breaking seed dormancy. The infested seeds were transferred to MEA and LBA medium plates. The inoculated plates were incubated at 25±2 ºC for 5-7 days and at 37 ºC for 24 hours respectively. The isolated colonies were subsequently sub cultured in order to obtain pure cultures. The percentage frequency of various fungal and bacterial varieties was calculated as follows:

\[
\text{Frequency of occurrence (\%)} = \frac{\text{No. of seeds on which a microbial species occurs}}{\text{Total No. of seeds}} \times 100
\]

Identification of Fungal Isolates

The isolated pure fungal colonies were subjected to macro and microscopic identification.

i. Colonial morphology

Pure and fresh fungal isolates were identified using cultural and morphological features such as; colony growth pattern, color, texture and growth elevation. From the incubated plates different fungal isolates with different coloration included; (a) White (b) Brown (c) Grey black, which signified the %age occurrence of different fungal colonies (Emory, 2007; Mirza et al., 1979).

ii. Cellular morphology

A small piece of mycelia from representative culture was placed on the glass slide containing lecto-phenol cotton blue stain for colored cultures and trypan blue for white culture colonies using a sterile inoculating needle. The morphological characteristics and appearance of various fungal isolates from rice seeds were confirmed and authenticated with the help of Identification manual (Emory, 2007; Mirza et al., 1979).

Biochemical Test and Identification of Bacterial Isolates

For bacterial isolation the surface sterilized sample was cut in to small portions and placed on LBA (Luria Bertani Agar) media plates under aseptic conditions. Inoculated plates were incubated at 37 ºC for 24hours. Different bacterial isolates were observed from inoculated plates, which signified the %age occurrence of various bacterial isolates. Bacterial isolates were re-cultured in order to obtain pure bacterial isolates. Identification of bacteria was carried out following the standardized procedure starting with the colony and cell morphology followed by Gram staining and finally testing the metabolic activities of unknown strain. Identification of bacterial species was done by recording colony morphological features (Beishir, 1991) (color, shape, size, texture, margins and odor etc.) and cell microscopic characters (color, cell wall, contents,
shape, arrangement, material, Gram stain, spore stain, capsular material and motility). The pure colonies were differentiated by biochemical tests (Holt et al., 1997; Benson, 1996).

**Statistical analysis:**

All the collected data was evaluated by Analysis of variance followed by Duncan's Multiple Range Test to separate the treatment means at P≤0.05 (Steel et al., 1997).

**RESULTS AND DISCUSSION**

In present study various physical parameters of different rice varieties were studied. Results exhibited the Basmati-198 (10.36mm) had the healthiest looking grain with husk among fine and coarse rice varieties used for study; since it had longest grain, equal in seed weight and thickness with Basmati-Shaheen (2.93mm and 2.92mm). Among fine grain, Basmati-198 had highest 1000 seed weight (30.2g) whereas Basmati-370 had the least weight of 17.2g (Table I & II). Analysis of variance indicated different effects of genotypes in almost all seed morphological traits at various levels of significance (P<0.01 and 0.05). All the genotypes showed significant differences among all the traits studied. Highly significant variations associated with high genetic diversity among the genotypes in all the desirable traits studied are helpful for further screening and selection process. Variance analysis also indicated polymorphism of the genotypes with respect to their various morphological traits (Ashfaq et al., 2013). These will also be providing the basic information to the scientific community, researcher and farmers community (Li et al., 2008). Pearson correlation analysis showed significant positive, significant negative and non-significant correlation in some seed morphological traits. Most of the traits showed positive association with each other and considering important morphological tool for the selection of high yielding diverse genotypes (Muthuramu et al., 2010). 1000 grain weight showed highly significant correlation with seed length, seed thickness and seed length width ratio (r= 0.6767**, r= 0.4617**, r= 0.44145**) and significant association with seed width (r = 0.4699*). On the other hand, seed length width ratio showed positive significant association with seed width and seed thickness (r= 0.4615*, r= 0.4692*) and seed width associated width seed length (r= 0.7135*) (Simple correlation of all these seed morphological and root shoot traits are shown in the table II. Various fungal and bacterial species were isolated from different rice varieties. The morphological data is shown in tables III & IV. Highest percentage of myco-flora (17%) was found to be associated with seeds of Basmati-Pak that was considerably more than myco-flora associated with all other seed types. While highest percentage of bacterial isolates (16%) was recorded with Basmati-Shaheen variety. There was 14%, 13%, 12% and 10% bacterial isolates associated with the seeds of Basmati-370, Basmati-198 and Basmati- 386, respectively. In figure 1, Fusarium sp., Alternaria alternata, Pyricularia sp., Dreschslera sp., Penicillium sp. and Curvularia sp. were recorded from various rice varieties with varying percentage incidence. Early research has recorded Alternaria alternata, Aspergillus niger, Curvularia lunata, Curvularia oryzae, Pyricularia oryzae, Drchslera oryzae, Fusarium miniliforme and Fusarium oxyssporum from different rice varieties (Khan et al., 2000; Wahid et al., 2001; Javaid et al., 2002; Ibiam et al., 2006; Nguefack et al., 2008). Greatest percentage of Alternaria alternata (16%) was found in seeds of Bas-Pak that was significantly greater than its incidence of all other seed varieties. There was 7%, 9%, 4% and 2% incidence of this fungal species in seeds of Basmati-370, Basmati-386, Basmati- 198 and Basmati- Shaheen, respectively (Figure 2). Distinction in incidence of Alternaria alternata among various varieties was insignificant. In the same way, determined percentage of Fusarium sp. was documented in seeds of Basmati- 386 (6%) followed by 4.5% in seeds of each of Basmati- 198 and Basmati-370, and 2.5% in seeds of Basmati-Pak and Basmati-Shaheen. Maximum percentage of occurrence of Curvularia sp. (3%) was found in Basmati-Pak and Basmati-198 followed by Basmati-386 (2.5%), Basmati-370 (2%) and Basmati-Shaheen (1.5%). Percentage occurrence of Pyricularia sp., Dreschslera sp. and Penicillium sp. was recorded 2.0, 1.0, 2.5, 0.5, 1.0%, respectively in Basmati- 198 and Basmati-370, and Basmati- 386, Basmati-Pak and Basmati-Shaheen. Three bacterial species Acetobacterium, Deniobacter and Micrococcus were isolated from the various rice varieties under investigation (Ashfaq et al., 2015). Highest
percentage of occurrence of *Acetobacterium* (9%) was recorded in seeds of Basmati-Shaheen followed by 0%, 2% 3.5%, and 4% in Basmati-Pak, Basmati-370, Basmati-386 and Basmati-198, respectively. On the other hand highest percentages of *Deniobacter* and *Micrococcus* (5%, 3%) were found in seeds of Basmati- 370 and Basmati-Shaheen, respectively Fig., 3.

CONCLUSION

Overall, Basmati-198, Basmati-370 and Basmati-Shaheen showed fruitful results that could be used for the screening, assessment and improvement of rice crop for producing healthy rice for nourishing more people. On the other hand, the information obtained from this study may be helpful for breeders, scientists and farmers community for starting a new research program for producing disease free rice in the rice World by utilizing and exploiting the potential rice lines.

ACKNOWLEDGEMENT

We are highly thankful to the University of the Punjab, Lahore for providing funds to complete this research work in time.

Table I: Analysis of variance of seed morphological traits of various rice genotypes

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>SL</th>
<th>SW</th>
<th>ST</th>
<th>SL/SW</th>
<th>1000 SW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td>4</td>
<td>2.05425</td>
<td>0.04096</td>
<td>0.12585</td>
<td>0.00131</td>
<td>0.007</td>
</tr>
<tr>
<td>Replications</td>
<td>3</td>
<td>2.31589</td>
<td>0.18038</td>
<td>0.26212</td>
<td>0.90938</td>
<td>228.652</td>
</tr>
<tr>
<td>Errors</td>
<td>12</td>
<td>1.95567</td>
<td>0.04814</td>
<td>0.08686</td>
<td>0.02793</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Level of significance p<0.05=* and p<0.01=**
SL= seed length, SW= seed width, ST= seed thickness, L/W=length/width ratio and 1000 grain weight

Table II: Pearson’s Correlation among different seed morphological traits of rice

<table>
<thead>
<tr>
<th>Traits</th>
<th>SL(mm)</th>
<th>SW(mm)</th>
<th>ST(mm)</th>
<th>SL/SW(mm)</th>
<th>1000SW(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW</td>
<td>0.7135</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST</td>
<td>0.6829</td>
<td>0.9397</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL/SW</td>
<td>0.8287</td>
<td>0.4615</td>
<td>0.4692</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>1000 SW</td>
<td>0.6767</td>
<td>0.4699</td>
<td>0.4617</td>
<td>0.4145</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Level of significance p<0.05=* and p<0.01=**
SL= seed length, SW= seed width, ST= seed thickness, L/W=length/width ratio and 1000 grain weight
### Table III: Biochemical characteristics of bacterial isolates encountered

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Colony characters</th>
<th>Gram stain</th>
<th>Spore</th>
<th>Motility</th>
<th>Catalase</th>
<th>Citrate Utilization</th>
<th>Urease</th>
<th>Indole</th>
<th>Glucos e</th>
<th>Methyl red</th>
<th>Hydrogen Sulfide</th>
<th>Nitrate reduction</th>
<th>Probable identity of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Raised smooth spherical entire creamy opaque</td>
<td>+ Rods</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Acetobacterium sp.</td>
</tr>
<tr>
<td>2</td>
<td>Raised smooth Spherical entire Translucent</td>
<td>+ Coci</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Denibacter sp.</td>
</tr>
<tr>
<td>3</td>
<td>Flat smooth spherical entire creamy opaque</td>
<td>+ Coci</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Micrococcus sp.</td>
</tr>
</tbody>
</table>

### Table IV: Microbiological characterization of fungi isolates encountered

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Colony morphology</th>
<th>Mycelium</th>
<th>Conidial color and shape</th>
<th>No. of septa</th>
<th>Conidial size</th>
<th>Identity of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Greenish brown-black, smooth edge, sporulation on surface. Reverse; black</td>
<td>Branch chained, having 4-6 conidia</td>
<td>Brown, ovoid, ellipsoid</td>
<td>Transverse septa; 4-7 Longisepta; 0-2</td>
<td>20-32 x 6-12µm</td>
<td>Alternaria alternata</td>
</tr>
<tr>
<td>2</td>
<td>White, cottony Reverse; off white</td>
<td>Aerial mycelia, hyaline to colorless</td>
<td>Curved blunt apical, pediculate basal part</td>
<td>Microconidia: 0-1 septate, blunt</td>
<td>Macroconidia: 3-5 septate</td>
<td>25-30x 48 µm</td>
</tr>
<tr>
<td>3</td>
<td>Blackish brown, velvety Reverse; black</td>
<td>Immersed, dark brown</td>
<td>Ellipsoidal, round at ends, pale brown</td>
<td>4-5 pseudo-septa</td>
<td>13-30 x 6-10 µm</td>
<td>Drechlera sp.</td>
</tr>
<tr>
<td>4</td>
<td>Effuse, greyish brown</td>
<td>Pale brown, un-branched,</td>
<td>Solitary, pyriform, olivaceous brown</td>
<td>1-3</td>
<td>17-25 x 6-9 µm</td>
<td>Pyricularia sp.</td>
</tr>
<tr>
<td>5</td>
<td>Black, velvety Reverse; black</td>
<td>Dark brown, thick walled</td>
<td>Cylindrical, sub-ellipsoidal</td>
<td>4- celled Central 2 cells darker</td>
<td>20-28 x 6-58 µm</td>
<td>Curvularia sp.</td>
</tr>
<tr>
<td>6</td>
<td>Green grey Reverse; off white</td>
<td>Hyaline, erect, pennisetated, verticillate phialides</td>
<td>Pale green, sub-globose</td>
<td>No</td>
<td>2-3 µm</td>
<td>Penicillum sp.</td>
</tr>
</tbody>
</table>
Fig. 1: Percentage of micro-flora associated with different rice varieties

Fig. 2: Percentage occurrence of fungal species in five commonly cultivated rice varieties.
Fig. 3: Percentage occurrence of bacterial species in five commonly cultivated rice varieties.

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Extracts from unripe and ripened peels of Citrus limon reveal variation in composition of bioactive compounds and exhibit antibacterial activity in relation to different extraction solvents

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INTRODUCTION

Citrus limon is commonly available in Pakistan and generally known as limo. It has considerable economic value for its peels essential oil and is documented to be the source of many bioactive compounds such as minerals, vitamin C, flavonoids, phenols, limonoids, folic acid (Deyhim et al., 2006). Lemon peels bioactive compounds have been used for their antioxidant, germicidal and anticarcinogenic activities (Guenther, 1948; Mukhopadhyay, 2000).

Presently, world over attention is focused to extract valued compounds from natural sources so as to explore their commercial uses in cosmetics, medicines and food protection. C. lemon fruit peels, are a possible source of numerous bioactive compounds such as flavonoids, tannins, and specifically limonoids which are infrequent to other plants. These components have significant biological activities including antioxidant, antimicrobial, anti-inflammatory and anti-cancer (Sikora et al., 2008).

Extraction is the chief significant step in the recovery and purification of active constituents from plant materials (Delfanian et al., 2015). Solvent extraction technique is a traditional method for extraction and is more regularly used for the separation of bioactive compounds. In this process, extraction yield of bioactive compounds is reliant on conditions of extraction and the solvent polarity etc.

Extract yield is dependent on method of extraction and nature of solvent used (Goli et al., 2004). The extraction process must allow the complete extraction of required compounds and should avoid chemical modification of compound of interest (Zuo et al., 2002). Various solvent systems are used for extraction of phenolic compounds from plant extracts (Chavan et al., 2001).

Some recent studies have shown that C. limon is a rich source of flavonoids, phenolic compounds and essential oil, hence it would be interesting to evaluate different solvent extracts to maximally obtain the valuable bioactive compounds found in it. In this scenario, this particular project was initiated to obtain and analyze the different chemical constituents obtained from unripe and repined peels of lemon

ABSTRACT

The goal of present study was to appraise the bioactivity of compounds using TFC (total flavonoid content), DPPH scavenging activity, reducing power assay and ABTS (radical scavenging activity) of different solvent extracts, from Citrus limon (unripe and ripened) peels. In unripe peels the total flavonoid contents, DPPH radical scavenging activity, ABTS radical scavenging activity and reducing power ranged from 18.23-57.56 mg/g CE (catechin equivalent), 21.69-65.82%, 38.96-84.83% and 0.01-1.8 mg /mL, respectively. For ripened lemon peels all these indicators ranged from 10.53-47.88 mg/g CE, 37.99-81.40%, 42.96-95.93% and 0.07-1.47 mg /mL respectively. Lemon peel extracts exhibited good antimicrobial activity against Escherichia coli, Bacillus subtilis, Staphylococcus aureus and Salmonella typhimurium. Overall ripened lemon peel extracts showed higher antioxidant activity than unripe peels. The data presented in present study is an important factor to select ripened lemon peels as high potential values for nutraceutical, pharmaceutical and cosmetic industries.

Key Words: Citrus limon, Extraction solvent system, Antioxidant activity, Antimicrobial activity.
by applying various organic solvents and their dilution. To the best of our knowledge no such comparative study has yet been reported on C. limon. The commonly used solvent extraction technique is utilized to separate antioxidant compounds from peels. This report describes the antioxidant properties of different extracts from unripe and ripened lemon peel by using in-vitro antioxidant assays.

MATERIALS AND METHODS

Plant material

Unripe (110 days of fruiting) and ripened (150 days of fruiting) C. limon peels were harvested at two different time intervals from local farms of district Sargodha. Lemons were peeled manually and dried at ambient conditions.

Chemicals and reagents

Analytical grade chemicals of Sigma-Aldrich Chemical Corporation, Germany including Trichloroacetic acid (TCA), Ascorbic acid, Catechin, Methanol, Butylated hydroxyl tolune (BHT), Distilled water, Ferric chloride, Acetone Potassium ferricyanide, Sodium carbonate, Sodium dihydrogen phosphate, Sodium nitrite, Sodium hydroxide, Trichloroacetic acid (TCA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS, MnO2 Nitrium hydrogen phosphate, n-Hexane, Broth, nutrient agar, Folin-Ciocalteu reagent were used in this work.

Preparation of extracts and determination of antioxidant activity

Extraction

Dried sample of unripe and ripened lemon peels were ground into fine powder in a grinder. Pulverized sample of both unripe and ripened peels (20g) were individually mixed with 200mL of extraction solvents;100 % methanol (pure methanol), 100% ethyl acetate (pure ethyl acetate), 100% chloroform (pure chloroform), 70 % methanol:methanol:water,70:30 v/v) 50% methanol:water, 50:50 v/v), 70% ethyl acetate (ethyl acetate:water,70:30 v/v), 50% ethyl acetate (ethyl acetate:water, 50:50 v/v), 70% chloroform (chloroform:water, 70:30 v/v) 50% chloroform (chloroform:water,50:50 v/v) in conical flasks. Extraction was carried out in an orbital shaker (Optima OS-752) for 27 hours. Each extract was filtered and solvents were evaporated using rotary evaporator (HB Heidolph digital laborota 4001 efficient) under reduced pressure. The concentrated crude extracts of unripe and ripened lemon peels were stored under refrigeration for further analysis.

Determination of TFCs

The total flavonoid contents (TFCs) were calculated by a method stated by (Zhishen et al., 1999). For TFC, 1mL crude extract aqueous solution (10mg/1mL) was taken and filled up to 5mL with distilled water in 10 mL volumetric flask. Then (0.3 mL of NaNO2 1:20) was added in each mixture and incubated for 5 minutes at room temperature. After 5 minute incubation, 0.3 mL of AlCl3 (1:10) was added in sample and again kept at room temperature for 6 minutes and then a 2mL of NaOH(1M) was added and filled up to 10 mL using distilled water. Absorbance was measured at 510 nm against blank (having all chemicals in equal amount without extract). The results were reported as CE (Catechin Equivalent) mg/g of DW (Dry Weight). Absorbance was measured three times for each sample and then average mean reading was obtained. Each test was performed in triplicates.

Assessment of reducing power of crude extracts

The reducing power of all extracts was determined by using method described by (Jayaparakasha et al., 2008) with slight modifications. Different concentration of extracts (2-10) mg were made in a mixture (1:1) phoshoric acid buffer (0.2 M, pH 6.6) and Potassium ferricyanide (1%) (K3Fe(CN)6). These were mixed and placed for 20 min at 50°C in water bath and then chilled rapidly with ice. Then added 1 mL 10% trichloroacetic acid to each concentration and allowed to react for 10 minutes in dark. Then 1 mL of distilled water and 0.8 mL of 0.1% ferric chloride (FeCl3) was added and incubated in dark for further 10 min. Absorbance of reaction mixtures was taken at 700 nm. Absorbance value relates positively with reduction power. The reduction ability tests were run in triplicate.

Estimation of ABTS radical scavenging activity

The ABTS radical scavenging activity was determined by using the procedure reported by (Proestos et al., 2013). First ABTS was dissolved in distilled water and made 7mM solution. MnO2 solution of 2.45 mM was prepared. Both solution were mixed in 1:1 ratio and kept it in the dark for 24 hours. 1ml of ABTS was added to 25ml of aqueous methanol. A volume of 20μL (diluted 1:10) of aqueous plant extract was added to two mL of ABTS++ radical cation solution, and the mixture was kept at a standard temperature of 30 °C. The absorbance was taken at 734 nm directly. The results were measured as % inhibition of ABTS++ radical cation by dried sample. The
following formula was used to calculate percentage radical scavenging activity.

\[ I\% = \frac{A_0 - A}{A} \times 100 \]

Where \( I \) = ABTS⁺ inhibition (%), \( A_0 \) = absorbance of control and \( A \) = absorbance of a tested sample.

**Determination of DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity**

Antioxidant potential taken as DPPH radical scavenging capacity assay was calculated using method of Clarke et al., 2013. Briefly 20μL of plant extract solution (4mg/mL) was taken and then added to 180 μL of DPPH solution (40 μg/mL). The mixture was incubated for 15 min in the dark and measured spectrophotometrically at 540 nm by UV-Vis spectrophotometer and the results were expressed as % inhibition of dried sample. The % inhibition was calculated as

\[ I\% = \frac{A_0 - A}{A} \times 100 \]

\( I \) = (% inhibition of DPPH), \( A_0 \) = (absorbance of control sample) (t = 0 h) and \( A \) = (absorbance of a tested sample at the end of the reaction) (t = 15 minutes). Results were calculated as % inhibition of DPPH radical scavenging activity; maximum values of DPPH scavenging are associated with stronger antioxidant activity.

**Evaluation of antimicrobial activity**

**Antimicrobial activity of lemon peel extracts**

Antimicrobial activity of all extracts was calculated using method described by Afzal et al., 2014. Bacterial strains used were *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*. Briefly, 100mL inoculum of each bacterial strain was poured into the nutrient agar. Then filter paper discs of 6mm were placed on medium and each disc was loaded with 100 μL of sample extract followed by incubation at 37 °C for 24 hours. Inhibition zones were developed by extracts showing antimicrobial activity.

**Statistical analysis**

Two way ANOVA was employed through the software SPSS 16.0 and evinced the whole data as mean ± standard deviation. Significant differences for mean values were determined and p>0.05 revealed non-significant difference.

**RESULTS AND DISCUSSION**

**Yield of crude extracts**

The extraction yield for antioxidant constituents from lemon with methanol (100, 70 and 50 %), ethyl acetate (100, 70 and 50 %) and chloroform (100, 70 and 50 %) has been presented in Table I. The extract yield of different unripe lemon peel extract varied over a range of 5.29 – 28.12 % (g / 100g). Maximum yield (28.12 %) was obtained with 70 % methanol whereas least yield (5.29%) with 100 percent chloroform. So, it can be considered that there is significant difference (p < 0.05) in relation to the different solvents used for extraction of antioxidant components from lemon. The present results showed 17.48 % extract yield of mature lemon peels with 100 % methanol which was in exact agreement with the percentage yield reported by Sekar et al., (2013) for methanolic extracts of mature lemon peels. Sultana et al., (2014) reported 16.62% extraction yield of clove with 70 % methanol but that percentage yield was lower than that observed in our present study of unripe and ripened lemon peels which was 28.12 and 20.54 % respectively. The present results also showed higher extraction yield with methanol as compared to chloroform which was comparable to percentage yield of methanol and chloroform extract of thyme reported by (Hossain et al., 2013). Sultana et al. (2007) reported 11.3% extract yield of corncob with ethyl acetate which was greater than present results of 100% ethyl acetate of unripe and ripened which were 8.13, 7.34 %, respectively.

Present results showed higher percentage yield of ripened lemon peels than unripe lemon peels which was in agreement with those reported by Gull et al., 2012 for unripe and fully ripe guava fruit. Kumar et al., (2011) reported the lemon peels extract yield of 18% using ethyl acetate as solvent which was higher than our results of using 100% ethyl acetate but lower than 50 % ethyl acetate yield (20.20%).
Table I: Extract yields (g/100g) from different peel extracts of *Citrus limon*.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Unripe peels</th>
<th>Ripened peels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100%</td>
<td>70%</td>
</tr>
<tr>
<td>MeOH</td>
<td>19.28±0.36***</td>
<td>28.12±0.13***</td>
</tr>
<tr>
<td>EtOAc</td>
<td>8.13±0.11**</td>
<td>7.11±0.12**</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>5.29±0.27***</td>
<td>7.04±0.06***</td>
</tr>
</tbody>
</table>

- Values represent mean ± standard deviation (n=3) of three separate samples of extracts individually analyzed.
- Stars show significance, p<0.001=***, p<0.01=**, p<0.5=*, ns=non-significant.

**Total flavonoid contents**

Total flavonoid contents from different unripe lemon peel extracts (Table II) obtained by using different solvents such as methanol, ethyl acetate and chloroform at different concentration (100%, 70% and 50%) ranged from 18.23 to 54.76 CE (mg/g) of dry weight (DW). Details are given in Table II. The lowest value of ripened lemon peel was obtained for 100% chloroform extract of ripened lemon peel (10.53 CE mg/g), while highest value was obtained for 100% methanol extract of unripe lemon peel (47.88 CE mg/g).

Eghdami et al. (2013) reported higher flavonoid contents of methanolic extracts of thyme which is in good agreement with our trend of methanolic extracts of unripe and ripened lemon peel extracts of present study. Present work also showed higher total flavonoid contents with methanolic extracts followed by chloroform and ethyl acetate which are also supported by the results of Hossain et al., 2013 for thyme extracts using methanol, chloroform and ethyl acetate as solvents.

Results of present study showed higher flavonoid contents in unripe lemon peels than ripened one, which agree with results obtained by Gull et al., (2012) for different stages of ripening of guava fruit but are not in agreement with results reported by Mahmood et al., (2013) that cherry fruits contain higher flavonoid contents at ripening stage than at un ripened stage.

The higher concentrations of total flavonoid compounds of present study in younger un-ripe lemon fruit as compared to those in fully-ripe lemon fruits can be explained by the fact during ripening, at various stages different phenolic acid compounds might be condensed to form complex phenolic acids such as lignin and tannins etc (Ben-Ahmed et al., 2009). So, due to conversion of phenolic acids to complex phenolic compounds during maturity, ripened fruits possess lower concentrations of flavonoids than un-ripe fruits. Differences in total flavonoid contents of lemon peels at two different stages of maturity could also be explained by a report that stated that phenolic presence was influenced by species genetic makeup, growing conditions, soil circumstances and nutrients availability at harvesting stages (Jaffery et al., 2003).
Table II: Total flavonoid contents (CE mg / g (DW) of different extracts from *Citrus limon*.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Unripe peels</th>
<th>Ripened peels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100%</td>
<td>70%</td>
</tr>
<tr>
<td>MeOH</td>
<td>54.76±0.68***</td>
<td>48.03±0.05***</td>
</tr>
<tr>
<td>EtOAc</td>
<td>23.16±0.28***</td>
<td>40.06±0.11***</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>18.23±0.40***</td>
<td>34.77±0.69***</td>
</tr>
</tbody>
</table>

- Values represent mean ± standard deviation (n=3) of three separate samples of extracts individually analyzed
- Stars show significance (p<0.001=***, p<0.01=**, p<0.5=*, ns=non-significant)

DPPH radical scavenging activity

The % DPPH radical scavenging activity was carried out for unripe lemon peels extracts of different solvents: methanol, ethyl acetate and chloroform at varying concentrations (100,70 and 50 %) ranged from 21.69 to 65.82 % (Table III). The lowest value of unripe lemon peel was obtained for 50% chloroform (21.69 %) while highest value was obtained for 100 % methanol extract of unripe lemon peel (65.82 %). Likewise % DPPH radical scavenging activity for ripe lemon peels was determined by using various solvents: methanol, ethyl acetate and chloroform in varying concentrations (100, 70 and 50 %) ranged from 37.99 to 81.40 %. The lowest value obtained for ripened lemon peels was for 100% methanol (37.99 %) and highest for 100 % ethyl acetate (81.40 %). Details are given in Table III. Substantial rise in reducing potential was observed in relation to the ripening/maturity of fruit. The present results showed significant rise in DPPH scavenging activity at progression of maturity which is also supported by the previous study on cherry fruits at varying stages of ripening (Mahmood et al., 2013). Our trend of increasing DPPH radical scavenging activity was not in agreement to the results shown by other authors for various ripening stages of guava orange and lemon juice (Gull et al., 2012; Omoba et al., 2015; Kumari et al., 2014).

Table III: DPPH radical scavenging activity of different extracts from *Citrus limon*.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Unripe peels</th>
<th>Ripened peels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100%</td>
<td>70%</td>
</tr>
<tr>
<td>MeOH</td>
<td>65.82±0.75</td>
<td>50.46±0.50</td>
</tr>
<tr>
<td>EtOAc</td>
<td>44.97±0.95</td>
<td>43.98±0.97</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>32.50±1.32</td>
<td>60.49±0.50</td>
</tr>
</tbody>
</table>

- Values represent mean ± standard deviation (n=3) of three separate samples of extracts individually analyzed
- Stars show significance (p<0.001=***, p<0.01=**, p<0.5=*, ns=non-significant)

ABTS radical scavenging activity

The ABTS radical scavenging action detected for unripe lemon peels using 100, 70 and 50 % of Methanol, ethyl acetate and chloroform (100, 70 and 50 %) ranged from 38.96 to 84.43 % (Table IV). The lowest value of unripe lemon peel was obtained for 100% ethyl acetate (38.96 %) whereas maximum value was obtained for 100 % methanol extract (84.43%). Likewise ABTS radical scavenging activity for ripened lemon peels using various solvents: methanol, ethyl acetate and
chloroform at varying concentrations (100, 70 and 50 %) ranged from 43.31 to 96.81 %. The lowest value obtained (Table IV) for ripened lemon peels were for 70% ethyl acetate (43.31 %) and highest for 50% chloroform (88.53 %). Our present findings showed that ripened lemon peels possessed higher ABTS radical scavenging activity than unripe which was in good agreement to the work done by (Omoba et al., 2015) for unripe and ripened orange peels.

Table IV: ABTS radical scavenging activity of different extracts from *Citrus limon*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Unripe peels</th>
<th>Ripened peels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100%</td>
<td>70%</td>
</tr>
<tr>
<td>MeOH</td>
<td>84.43±0.38***</td>
<td>50.33±0.57***</td>
</tr>
<tr>
<td>EtOAc</td>
<td>38.96±0.95</td>
<td>41.84±0.77</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>75.33±0.57</td>
<td>60.38±0.56</td>
</tr>
</tbody>
</table>

- Values represent mean ± standard deviation (n=3) of three separate extracts of extracts individually analyzed.
- Stars in above table shows significance (p<0.001=***, p<0.01=**, p<0.5=*, ns=non-significant).

Reducing power assay

The lowest and highest reduction potential of unripe and ripened lemon peel extracts measured at concentration range of 2-10 mg/mL was in the range of 0.009-1.62 and 0.075-1.47, respectively (Fig., 1A and 1B). Methanolic, ethyl acetate and chloroform extracts of unripe lemon peels showed highest reductive potential of 0.853, 1.65 and 1.77 at 10 mg/mL, respectively. Similarly the ripened lemon peel extracts of methanol, ethyl acetate and chloroform showed highest reductive potential of 0.67, 1.21 and 1.47 at 10 mg/mL, respectively. Relation between the reduction potential and concentration was linearly increasing as shown in graph which was in good agreement to previous report (Manzoor et al., 2013). Antimicrobial activity by disc diffusion method

Lemon is considered an important medicinal plant cultivated for alkaloids which shows antibacterial and anticancer activities (Pandey et al., 2011). Antimicrobial activity of different extracts of lemon peels was determined using disc diffusion method. Ciprofloxacin was used as a standard in present study.

Zones of inhibition of different unripe and ripened methanolic (100%, 70% and 50%) lemon peel extracts against different bacterial strains were found to be in following ranges: 5.50 – 28.83mm against *Bacillus subtilis*, 3.63 – 21.70mm against *Staphylococcus aureus*, 1.00-18.60mm against *Salmonella typhimurium* and 3.00 – 22.00mm against *Escherichia coli* (Table V (A)). Zones of inhibition of different unripe and ripened ethyl acetate (100%, 70% and 50%) lemon peel extracts against different bacterial strains were found to be in following ranges: 2.23 – 18.27 mm against *B. subtilus*, 5.43 – 11.33mm against *S. aureus*, 10.06-23.53mm against *Salmonella typhimurium* and 5.18 – 16.23mm against *E.coli* (Table V (B)).

Zones of inhibition of different unripe and ripened chloroform (100%, 70% and 50%) lemon peel extracts against different bacterial strains were found to be in following ranges: 10.20 – 30.43 mm against *B. subtilus*, 10.30 – 31.33mm against *S. aureus*, 5.1-30.13mm against *S. typhimurium* and 0 – 10.11mm against *E. coli* (Table V (C)).

Present study of methanolic extracts of unripe lemon peel extracts showed good antimicrobial activity against *E. coli* and *S. aureus* which is greater than that reported by Pandey et al., (2011) for methanolic and ethyl acetate extracts of lemon peels against various strains of bacteria. While ethyl acetate extracts activity of lemon peels against *Escherichia coli* and *Staphylococcus aureus* observed in present study was lower than that reported by Pandey et al., (2011) for lemon peel extracts. Kumar et al., (2011) reported the antimicrobial activity of ethyl acetate extracts of lemon peels against all four strains: *Bacillus subtilus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* which was higher than our present research findings.
Fig. 1A: Reducing power assay of Unripe *Citrus limon* peel

Fig. 1B: Reducing power assay of Ripened Citrus Limon peels
Table V (A): Antimicrobial activity of methanolic extracts of *Citrus limon*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvents</th>
<th>Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Bacillus subtilis</em> ZOI(mm)</td>
</tr>
<tr>
<td>Unripe peels</td>
<td>100% MeOH</td>
<td>10.39±0.53***</td>
</tr>
<tr>
<td></td>
<td>70% MeOH</td>
<td>28.83±0.76***</td>
</tr>
<tr>
<td></td>
<td>50% MeOH</td>
<td>5.50±0.50***</td>
</tr>
<tr>
<td>Ripened Peels</td>
<td>100% MeOH</td>
<td>15.63±0.55***</td>
</tr>
<tr>
<td></td>
<td>70% MeOH</td>
<td>10.33±0.57***</td>
</tr>
<tr>
<td></td>
<td>50% MeOH</td>
<td>20.33±0.57***</td>
</tr>
<tr>
<td>Drug</td>
<td>Ciprofloxacin</td>
<td>40.00±0.01***</td>
</tr>
</tbody>
</table>

- Values represent mean ± standard deviation (n=3) of three separate extracts of extracts individually analyzed.
- Stars show significance (p<0.001=***, p<0.01=**, p<0.5=*, ns=non-significant).

Table V (B): Antimicrobial activity of ethyl acetate extracts of *Citrus limon*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvents</th>
<th>Bacterial stain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Bacillus subtilis</em> ZOI(mm)</td>
</tr>
<tr>
<td>Unripe peels</td>
<td>100% EtOAc</td>
<td>22.3±0.20***</td>
</tr>
<tr>
<td></td>
<td>70% EtOAc</td>
<td>10.63±0.55***</td>
</tr>
<tr>
<td></td>
<td>50% EtOAc</td>
<td>5.07±0.06***</td>
</tr>
<tr>
<td>Ripened Peels</td>
<td>100% EtOAc</td>
<td>12.33±0.57***</td>
</tr>
<tr>
<td></td>
<td>70% EtOAc</td>
<td>18.27±0.25***</td>
</tr>
<tr>
<td></td>
<td>50% EtOAc</td>
<td>23.3±0.32***</td>
</tr>
<tr>
<td>Drug</td>
<td>Ciprofloxacin</td>
<td>48.00±0.00***</td>
</tr>
</tbody>
</table>

- Values represent mean ± standard deviation (n=3) of three separate extracts of extracts individually analyzed.
- Stars show significance (p<0.001=***, p<0.01=**, p<0.5=*, ns=non-significant).
Table V (C): Antimicrobial activity of chloroform extracts of *Citrus limon*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvents</th>
<th>Bacillus subtilis ZOI(mm)</th>
<th>Staphylococcus aureus ZOI(mm)</th>
<th>Salmonella typhimurium ZOI(mm)</th>
<th>Escherichia coli ZOI(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unripe peels</td>
<td>100% CHCl₃</td>
<td>20.10±0.10***</td>
<td>2.03±0.05***</td>
<td>21.13±0.15***</td>
<td>3.10±0.10***</td>
</tr>
<tr>
<td></td>
<td>70% CHCl₃</td>
<td>22.00±1.00</td>
<td>10.30±0.30</td>
<td>20.44±0.50</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>50% CHCl₃</td>
<td>30.43±0.15***</td>
<td>31.33±0.57***</td>
<td>5.1±0.17***</td>
<td>10.10±0.17***</td>
</tr>
<tr>
<td>Ripened Peels</td>
<td>100% CHCl₃</td>
<td>20.38±0.54***</td>
<td>15.23±0.40***</td>
<td>21.50±0.50***</td>
<td>2.00±0.10***</td>
</tr>
<tr>
<td></td>
<td>70% CHCl₃</td>
<td>10.20±0.26</td>
<td>20.33±0.57</td>
<td>20.18±0.31</td>
<td>5.18±0.15</td>
</tr>
<tr>
<td></td>
<td>50% CHCl₃</td>
<td>14.83±5.92***</td>
<td>15.13±0.15***</td>
<td>30.13±0.15***</td>
<td>10.11±0.10***</td>
</tr>
<tr>
<td>Drug</td>
<td>Ciprofloxacin</td>
<td>48.00±0.00***</td>
<td>40.08±0.10***</td>
<td>40.03±0.57***</td>
<td>40.01±0.56***</td>
</tr>
</tbody>
</table>

- Values represent mean ± standard deviation (n=3) of three separate extracts of extracts individually analyzed.
- Stars show significance (\(p<0.001=***\), \(p<0.01=**\), \(p<0.5=*\), ns=non-significant)

**CONCLUSION**

Current research project provides a comprehensive study on antioxidant potential of various extracts of unripe and ripened lemon peels cultivated in Pakistan. It has been concluded from results that antioxidant activity of *Citrus limon* (unripe and ripened) peels was greatly affected during ripening stages and nature of solvents that were employed to obtain bioactive compounds. Based on present findings it can be concluded that ripened lemon peels has high potential value for development and supply of highly valuable compounds. It can be said that assessment of antioxidants characteristics of *Citrus limon* peels needs selection of appropriate extraction solvent and multiple assays analysis. As such both the time of harvesting fruits and nature of extraction solvent has prominent effects on the antioxidant and phenolics compounds from citrus peels. Generally, the results showed that limon peel extracts were a potential source of natural antioxidants and exhibited potential for antibacterial activities.

**REFERENCES**


Preliminary screening of some plants of Punjab, Pakistan for Phytochemicals

SYED ZULFIQAR ALI ABIDI, HAFIZ ABDULLAH SHAKIR, SYED SHAHID ALI, & JAVED IQBAL QAZI

Department of Zoology, University of the Punjab, Lahore

ABSTRACT

Plants produce different metabolites and some of them are used for antimicrobial, fungicidal, herbicidal, piscicidal and molluscicidal. The present study was aimed to find out metabolites of piscicidal potential. Total thirty plants were collected on the basis of their availability and abundance around the year from different locations of Punjab, Pakistan. The aqueous and alcoholic extract of fresh and dried parts of each plant were used for qualitative estimation of thirteen metabolites (alkaloids, carbohydrate, cardiac glycoside, flavonoid, phenol, phlobatannine, free amino acids, saponins, tannins, terpenoids, quinine, oxalic acid and steroids). Twenty one plants showed the presence of phytochemicals. The use of piscicidal property of these plants may improve the production of aquaculture by getting rid of unwanted fish species.

Key Words: Alcoholic extract, Aqueous extract, Phytochemicals, Metabolites, Plants

INTRODUCTION

Pakistan supports a very rich flora in alluvial plains of the Himalaya (Nasir & Ali, 1970-89; Shinwari, 2010). Punjab (one of the provinces of Pakistan) is the land of five rivers and has all the four seasons for the seed germination and growth of plants. Different plants / weeds grow in different seasons of the years. Resultantly, different types of plants/ weeds are available throughout the year (Qureshi et al., 2009; Siddiqui et al., 2009; Qureshi et al., 2010, 2011; Salehi et al., 2011; Hamed et al., 2015). Each plant has its own peculiar qualities due to its physical as well as its chemical characteristics. These plants synthesize different bioactive chemicals (phytochemicals) which being the secondary metabolites are not required directly for the plant itself (Ayoola, 2006, 2008; Dastgir & Hussain, 2013). These secondary metabolites (phenol, tannin, saponin etc.) are synthesized in all parts of the plants. However, their quality and quantity may be different in different parts of a plant depending upon age of the plant, climatic conditions, soil of the area and biological activity of the particular part of the plant (Hill, 1952; Fransworth, 1966; Ha et al., 2001; Hussain et al., 2011; Khan et al., 2011; Savithramma et al., 2011; Ugochukwu et al., 2013; Jayanth & Lalith, 2013 and 2014; Bharti & Bhushan, 2015; Zare et al., 2015).

The parts of the plants due to the presence of secondary metabolites are sometimes used directly for antibacterial, fungicidal, herbicidal, molluscicidal and piscicidal purposes but mostly metabolites (phytochemicals) are used after extraction and purification. The quality of metabolites can be further improved by adding/subtracting certain chemicals (Fansworth et al., 1966; Everiest, 1974, 1981; Das et al., 2010; Hussain et al., 2011; Kumar et al., 2014). Quality of each phytochemical (alkaloids, tannins, flavonoid, glycosides, terpenoids and phenolic compounds) depends upon the method and nature of the solvent used for their extraction (Harbone, 1973; Zahid et al., 2002; Zhang and Guo, 2005, 2006; Sharma & Kumar, 2008; Mirjalili et al., 2009; Das et al., 2010; Savithramma et al., 2011; Tiwari et al., 2011; Litha & Jayanthi, 2012; Sardhara & Gopal, 2013; Kumar et al., 2014; Mungenge et al., 2014; Mariappan et al., 2015; Sogbesan & Emmanuel, 2015; Pushpa et al., 2015; Greenshina & Murugan, 2016; Tasneem et al., 2016). The present study was aimed to find out the presence of different phytochemicals both in fresh and dried parts of locally available plants which may be used as antibacterial, fungicidal, herbicidal, molluscicidal and piscicidal agents.

MATERIALS AND METHODS

Collection and identification of plants

Thirty plants were collected from the different localities of Punjab, Pakistan (Table 1) and immediately transported to laboratory. The identification of plants were confirmed following the identification keys (Nasir & Ali, 1970-89; Shinwari, 2010).
Table I: Description of plants collected from different localities

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Scientific Name</th>
<th>Lab. code</th>
<th>Local name</th>
<th>Family</th>
<th>Order</th>
<th>Collection Area</th>
<th>Distribution, seasons and economic value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Achyranthus aspera</td>
<td>ZAA-089</td>
<td>-</td>
<td>Amaranthaceae</td>
<td>Caryophyliales</td>
<td>Islamabad</td>
<td>Locally available weed throughout Pakistan in winter with no economic value</td>
</tr>
<tr>
<td>2.</td>
<td>Anamirta cocculus (Seeds)</td>
<td>ZAA-102</td>
<td>Magar Mahi</td>
<td>Menispermaceae</td>
<td>Ranunculales</td>
<td>Local market</td>
<td>Imported from India. Seeds available in all seasons and have moderate economic value</td>
</tr>
<tr>
<td>3.</td>
<td>Calendula arvensis</td>
<td>ZAA-103</td>
<td>-</td>
<td>Asteraceae</td>
<td>Asterales</td>
<td>Islamabad</td>
<td>Locally available weed throughout Pakistan in winter with no economic value</td>
</tr>
<tr>
<td>4.</td>
<td>Calotropis procera</td>
<td>ZAA-104</td>
<td>Ak</td>
<td>Asciepiadaceae</td>
<td>Gentianales</td>
<td>Lahore, Faisalabad</td>
<td>Locally available weed throughout Pakistan in all seasons with no economic value</td>
</tr>
<tr>
<td>5.</td>
<td>Carthamus Oxyacantha</td>
<td>ZAA-105</td>
<td></td>
<td>Asteraceae</td>
<td>Asterales</td>
<td>Islamabad</td>
<td>Locally available weed in winter in hilly areas of Pakistan with no economic value</td>
</tr>
<tr>
<td>6.</td>
<td>Chenopodium ambrosioides</td>
<td>ZAA-106</td>
<td>Wild bathu</td>
<td>Amaranthaceae</td>
<td>Caryophyliales</td>
<td>Islamabad, Lahore</td>
<td>Locally available weed in winter throughout Pakistan with no economic value</td>
</tr>
<tr>
<td>7.</td>
<td>Cichorium intybus</td>
<td>ZAA-107</td>
<td></td>
<td>Asteraceae</td>
<td>Asterales</td>
<td>Islamabad</td>
<td>Locally available weed in winter only in hilly areas of Pakistan with no economic value</td>
</tr>
<tr>
<td>8.</td>
<td>Colocasia esculenta</td>
<td>ZAA-108</td>
<td>Elephant ear</td>
<td>Araceae</td>
<td>Alismatales</td>
<td>Faisalabad, Islamabad</td>
<td>Ornamental Plant Found throughout Pakistan in all seasons with moderate economic value</td>
</tr>
<tr>
<td>9.</td>
<td>Conyza canadensis</td>
<td>ZAA-109</td>
<td></td>
<td>Asteraceae</td>
<td>Asterales</td>
<td>Islamabad</td>
<td>Locally available weed in winter only in hilly areas of Pakistan with no economic value</td>
</tr>
<tr>
<td>10.</td>
<td>Coronopus didymus</td>
<td>ZAA-110</td>
<td></td>
<td>Brassicaceae</td>
<td>Brassicales</td>
<td>Islamabad</td>
<td>Locally available weed in winter only in hilly areas</td>
</tr>
<tr>
<td>No.</td>
<td>Species</td>
<td>ZAA</td>
<td>Family</td>
<td>Order</td>
<td>Location</td>
<td>Economic Value</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>--------------------------</td>
<td>-----</td>
<td>------------</td>
<td>-------</td>
<td>--------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Crozophore tinctora</td>
<td>ZAA-111</td>
<td>Euphorbiaceae</td>
<td>Malipighiales</td>
<td>Islamabad</td>
<td>Locally available weed in winter only in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Datura alba</td>
<td>ZAA-112</td>
<td>Solanaceae</td>
<td>Solanales</td>
<td>Lahore, Islamabad</td>
<td>Locally available weed in all seasons throughout Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Echinops echinatus</td>
<td>ZAA-113</td>
<td>Asteraceae</td>
<td>Asterales</td>
<td>Islamabad</td>
<td>Locally available weed in winter only in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Euphorbia heliscopia</td>
<td>ZAA-114</td>
<td>Euphorbiaceae</td>
<td>Malipighiales</td>
<td>Islamabad</td>
<td>Locally available weed in winter only in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>Lactuca dissecta</td>
<td>ZAA-115</td>
<td>Asteraceae</td>
<td>Asterales</td>
<td>Islamabad</td>
<td>Locally available weed in winter only in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>Lantana indica</td>
<td>ZAA-116</td>
<td>Verbenaceae</td>
<td>Lamiales</td>
<td>Lahore, Faisalabad</td>
<td>Locally available weed in all seasons throughout Pakistan</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>Malia azedarach</td>
<td>ZAA-117</td>
<td>Derhaik</td>
<td>Malvaceae</td>
<td>Sapindales</td>
<td>Lahore, Faisalabad</td>
<td>Locally available plant throughout Pakistan in all seasons with no economic value</td>
</tr>
<tr>
<td>18.</td>
<td>Malvestrum coromandeliaennum</td>
<td>ZAA-118</td>
<td>Malvaceae</td>
<td>Malvales</td>
<td>Islamabad</td>
<td>Locally available weed in winter in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>Nerium oleander</td>
<td>ZAA-119</td>
<td>Kanair</td>
<td>Apocynaceae</td>
<td>Gentianales</td>
<td>Faisalabad, Islamabad</td>
<td>Locally available ornamental plant almost everywhere in Pakistan in all seasons with moderate economic value</td>
</tr>
<tr>
<td>20.</td>
<td>Physalis peruviana</td>
<td>ZAA-120</td>
<td>Solanaceae</td>
<td>Solanales</td>
<td>Islamabad</td>
<td>Locally available weed in winter in</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Species Name</td>
<td>ZAA-Code</td>
<td>Family</td>
<td>Order</td>
<td>Location</td>
<td>Economic Value</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>---------------------------</td>
<td>----------</td>
<td>--------------</td>
<td>-------------</td>
<td>---------------------------------------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td><em>Ricinus communis</em></td>
<td>ZAA-121</td>
<td>Euphorbiaceae</td>
<td>Malpighiales</td>
<td>Lahore, Faisalabad</td>
<td>Locally available plant in all seasons throughout Pakistan with moderate economic value</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td><em>Rumex dentatus</em></td>
<td>ZAA-122</td>
<td>Polygonaceae</td>
<td>Caryophyliales</td>
<td>Islamabad</td>
<td>Locally available weed in winter in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td><em>Salvia moorcroftina</em></td>
<td>ZAA-131</td>
<td>Lamiaceae</td>
<td>Lamiales</td>
<td>Islamabad</td>
<td>Locally rarely available weed in winter in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td><em>Sapium sebifer</em></td>
<td>ZAA-134</td>
<td>Euphorbiaceae</td>
<td>Malpighiales</td>
<td>Lahore, Islamabad</td>
<td>Locally available ornamental plant in all seasons throughout Pakistan with moderate economic value</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td><em>Sassuria heteromalia</em></td>
<td>ZAA-154</td>
<td>Asteraceae</td>
<td>Asterales</td>
<td>Islamabad</td>
<td>Locally available weed in winter in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td><em>Silybum marianum</em></td>
<td>ZAA-161</td>
<td>Asteraceae</td>
<td>Asterales</td>
<td>Islamabad</td>
<td>Locally available weed in winter in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td><em>Trichodesma indicum</em></td>
<td>ZAA-162</td>
<td>Boranginaceae</td>
<td>Unplaced</td>
<td>Islamabad</td>
<td>Locally available weed in winter in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td><em>Trifolium repens</em></td>
<td>ZAA-166</td>
<td>Fabaceae</td>
<td>Fabales</td>
<td>Islamabad</td>
<td>Locally available weed in winter in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td><em>Verbena tenuisecta</em></td>
<td>ZAA-172</td>
<td>Verbenaceae</td>
<td>Lamiales</td>
<td>Islamabad</td>
<td>Locally available weed in winter in hilly areas of Pakistan with no economic value</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td><em>Withania somnifera</em></td>
<td>ZAA-178</td>
<td>Solanaceae</td>
<td>Solanales</td>
<td>Lahore, Faisalabad, Islamabad</td>
<td>Locally available weed in winter throughout Pakistan with no economic value</td>
<td></td>
</tr>
</tbody>
</table>
Preparation of Extracts

Different parts of every plant (roots, stem, leaves, and flowers) were divided into two portions. One portion was proceeded as fresh and second part of every plant was dried at room temperature and then grinded into fine powder by electric grinder (OSAKA, China) at speed of 500 rpm separately. The fine powder (50 g) of each portion was suspended in different extraction medium (water or ethanol) in a ratio of 1:2 w/v at room temperature for 24 h. The fluid was then filtered by using cheese cloth in reagent bottle and then Whatmann filter paper No. 1. The aqueous and alcoholic extracts of fresh and dried parts of each plant were kept at room temperature in air tight vials.

Phytochemical screening of aqueous and ethanolic extracts

Standard procedures as described by Ugochukwu et al. (2013), and Nandagoapalan et al. (2016) were used to evaluate the presence of different categories of phytochemicals in each plant extract. Following is a brief description of the standard procedures. All the experiments were performed in triplicate.

Total alkaloids (Wagner’s reagent)

Each extract (2 ml) was taken in a test tube and mixed with 3-5 drops of Wagner’s reagent (2g of iodine and 6g of KI in 100ml of water). The control was treated similarly after adding water instead of plant extract. Formation of reddish brown precipitate or coloration as compared to control was considered positive for the presence of alkaloids (Pershant et al., 2011).

Total carbohydrate (Molisch’reagent)

Two ml of an extract was mixed with two ml of water in a test tube labeled as sample. Whereas control contained 4ml water and then few drops of Molisch’s reagent (5% α-naphthol in absolute alcohol) were added in each test tube followed by addition of 2 ml of Conc. H₂SO₄ carefully. After 2-3 minutes, formation of red or dull violet color in comparison with control at the mixing point of two layers indicated the presence of carbohydrates (Pershant et al., 2011).

Flavonoids (Alkaline reagent test)

Each extract (2 ml) was mixed with 2-3 drops of 20 % NaOH solution. Two ml of water served as control. Formation of dense yellow color which became colorless by mixing with few drops of dilute HCl indicated the presence of flavonoid in the extract (Pershant et al., 2011).

Phenols (Ferric Chloride test)

Two ml of each extract was mixed with 3-4 drops of 5% ferric chloride solution. Dark blue color or black color formation in the test tube was indication for the presence of phenols in the extract (Harborne et al., 1973; Tyler & Herbalgram, 1994).

Salkowski’s test for terpenoids

Each extract (2 ml) was mixed with 1 ml of chloroform in test tube followed by the addition of 3-4 drops of conc. H₂SO₄. Formation of reddish brown precipitate was considered positive for the presence of terpenoids in the extract (Ayoola et al., 2008; Pershant et al., 2011).

Foam test for saponins

Two ml of extract was diluted with 6ml of water. Formation of relentless foam confirmed the presence of saponins in the extract (Harborne et al., 1973).

Braymer’s test for tannins

Two ml of each extract was mixed with the 1-2 drops of 10% ethanolic ferric chloride solution. Creation of blue to greenish color showed the presence of tannins in the extract (Harborne et al 1973).

Keller kalian’s test for cardiac glycosides

Five ml of each extract was mixed with 2ml of glacial acetic acid followed by the addition of 1-2 drops of 5% ferric chloride. Thereafter, 1 ml of Conc. H₂SO₄ was added in the bottom of test tube carefully. Formation of brown ring at the mixing point was considered positive for the presence of de-oxy-sugar; distinctiveness of cardenolides (Ayoola et al., 2008).

Test for quinones

One ml of each extract was mixed with few drops of conc. HCl. Formation of yellow precipitate indicated the presence of quinines in the extract (Pershant et al., 2011).

Test for oxalate

Few drops of glacial ethanolic acid were added in the 3ml of extract in a test tube. Formation of greenish black color was considered positive for the presence of oxalate in the extract (Pershant et al., 2011).

Test for phlobatannins (Precipitate test)

Two ml of each extract was boiled in a test tube with 1ml of 1% aqueous HCl. Formation of red precipitate was considered positive for the presence of phlobatannine in the extract (Pershant et al., 2011).

Ninhydrin test for amino acids and proteins

A few (2-3) drops of ninhydrin solution (made in 1% acetone) were added to 2 ml of an extract and the mixture boiled in water bath for 1-2 minutes. Formation of purple color was considered positive for the presence of amino acid and protein in the extracts (Pershant et al., 2011).

Liebermann-burchard test for steroids

One ml of each extract mixed with few drops of chloroform, acetic anhydride and Conc.
In the present study, the cardiac glycosides, phenol, saponins, tannin and sterol were present in aqueous extract of stem of white flowered *Narum oliender*. Both extracts of leaves of white flowered *N. oliender* showed the presence of alkaloids, carbohydrates, cardiac glycosides, phenol, saponins, tannin and sterol. Aqueous extract of leave from red flowered *N. oliender* plant showed presence of terpenoids in addition to the other chemicals present in white flowered *N. oliender*. Aqueous extract of white flowers *N. oliender* showed the presence of carbohydrate, phenol, saponins, tannin and steroids whereas ethanolic extract of white flowers *N. oliender* also contained

**RESULTS AND DISCUSSION**

In present study, 13 phytochemicals in different parts (fresh and dried) of thirty plants were studied after preparing aqueous as well as ethanolic extract. Twenty one plants showed positive results for the presence of phytochemicals. While 9 plants showed complete absence of these phytochemicals. When the %age distribution of the phytochemicals were worked out for the twenty one plants species investigated it was revealed that most of the phytochemicals were extracted in equal percentage from both fresh and dried forms except in case of alkaloids and terpenoids which were in fresh and dried leaves: 56 and 63, 56 and 59 %, respectively. Same was the position for terpenoids in fresh stem as 57% and in dried stem it was 62%. It was also observed that leaves were better part of plants to extract the phytochemicals (19 to 78%) as compared to stem (14 to 76%) and roots (14 to 71%). Similar finding was reported by other researchers (Offer & Uchenwoke, 2015; Zaman et al., 2016).

It was also observed that ethanol was better extractor than aqueous medium. The present study was in line with other findings (Yadav et al., 2014; Jardat et al., 2015). In aqueous medium, alkaloid, cardiac glycosides, phenol, terpenoid quinene, oxalic acid and steroid from leaves were extracted in 56, 59, 67, 56, 41, 22 and 63%, respectively. Whereas in ethanol their quantities were 74, 63, 81, 74, 52, 37 and 78%, respectively (Table II-III).

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Aqueous Extract</th>
<th>Dried Extract</th>
<th>Fresh Extract</th>
<th>Aqueous Extract</th>
<th>Dried Extract</th>
<th>Fresh Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>56</td>
<td>33</td>
<td>36</td>
<td>47</td>
<td>53</td>
<td>50</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>78</td>
<td>78</td>
<td>76</td>
<td>71</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>Cardiac glycoside</td>
<td>59</td>
<td>56</td>
<td>71</td>
<td>71</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>41</td>
<td>41</td>
<td>33</td>
<td>43</td>
<td>43</td>
<td>25</td>
</tr>
<tr>
<td>Phenol</td>
<td>67</td>
<td>63</td>
<td>71</td>
<td>71</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Phosphatamine</td>
<td>19</td>
<td>19</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Amino acids</td>
<td>19</td>
<td>19</td>
<td>24</td>
<td>24</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Saponins</td>
<td>74</td>
<td>74</td>
<td>71</td>
<td>71</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td>Tannins</td>
<td>74</td>
<td>74</td>
<td>71</td>
<td>71</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>56</td>
<td>59</td>
<td>67</td>
<td>67</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Steroids</td>
<td>63</td>
<td>63</td>
<td>71</td>
<td>71</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

**Table II: Percent of presence of different phytochemicals in aqueous extracts of fresh (F) and dried (D) parts of plants**

**Table III: Percent of presence of different phytochemicals in alcoholic extracts of fresh (F) and dried (D) parts of plants**

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Alcoholic Extract</th>
<th>Aqueous Extract</th>
<th>Dried Extract</th>
<th>Fresh Extract</th>
<th>Aqueous Extract</th>
<th>Dried Extract</th>
<th>Fresh Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>74</td>
<td>74</td>
<td>33</td>
<td>33</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>78</td>
<td>78</td>
<td>81</td>
<td>81</td>
<td>71</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Cardiac glycoside</td>
<td>63</td>
<td>63</td>
<td>71</td>
<td>71</td>
<td>57</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Flavonoid</td>
<td>44</td>
<td>44</td>
<td>33</td>
<td>33</td>
<td>43</td>
<td>43</td>
<td></td>
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<tr>
<td>Phenol</td>
<td>81</td>
<td>81</td>
<td>81</td>
<td>81</td>
<td>79</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>Phosphatamine</td>
<td>19</td>
<td>19</td>
<td>24</td>
<td>24</td>
<td>43</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Amino acids</td>
<td>19</td>
<td>19</td>
<td>14</td>
<td>14</td>
<td>21</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Saponins</td>
<td>74</td>
<td>74</td>
<td>76</td>
<td>76</td>
<td>64</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Tannins</td>
<td>78</td>
<td>78</td>
<td>76</td>
<td>76</td>
<td>63</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
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In the present study, the cardiac glycosides, phenol, saponins, tannin and sterol were present in aqueous extract of stem of white flowered *Narum oliender*. Both extracts of leaves of white flowered *N. oliender* showed the presence of alkaloids, carbohydrates, cardiac glycosides, phenol, saponins, tannin and sterol. Aqueous extract of leave from red flowered *N. oliender* plant showed presence of terpenoids in addition to the other chemicals present in white flowered *N. oliender*. Aqueous extract of white flowers *N. oliender* showed the presence of carbohydrate, phenol, saponins, tannin and steroids whereas ethanolic extract of white flowers *N. oliender* also contained...
oxalic acid (Table IV). Aqueous extract of the seeds of *Melia azedarach* showed presence of alkaloids, carbohydrate, cardiac glycoside, amino acid, terpenoids, quinine, and sterol, whereas in ethanolic extract of *M. azedarach*, amino acids were absent. Both categories of the extracts of leaves of *M. azedarach* showed the presence of alkaloids, carbohydrate, phenol, tannin, terpenoids, quinine and oxalic acid. Aqueous extract of stem of *M. azedarach* showed the presence of carbohydrate, cardiac glycoside, and saponins, whereas ethanolic extract of stem of *M. azedarach* also expressed the presence of terpenoids in addition to the chemicals recognized in aqueous extract (Table IV.).

Both aqueous as well as ethanolic extracts of roots of *Salvia moorcroftina* showed the presence of alkaloid, carbohydrate, phenol, saponins, tannin, quinines and steroids, whereas the latter category of the extract of *S. moorcroftina* also showed the presence of terpenoids. Aqueous leave extract of *S. moorcroftina* showed the presence of alkaloid, phenol, phlobatannine, tannin, terpenoids, saponins, quinines, and steroids, whereas ethanolic extract of *S. moorcroftina* showed the presence of same chemicals except saponins. Both types of extracts of stem of *S. moorcroftina* showed the presence of alkaloids, phenol, phlobatannine, saponins, tannin, terpenoids, quinines and steroids. (Table IV). Aqueous extract of the leaves of *Datura alba* showed the presence of carbohydrate, cardiac glycosides, saponins, and tannin, whereas ethanolic extract of *D. alba* showed the presence of, phenol, terpenoids, quinines and sterol in addition to the chemicals recognized in aqueous extract of *D. alba*. Aqueous extract of root of *D. alba* showed the presence of carbohydrate, cardiac glycosides and saponins whereas ethanolic extract of *D. alba* showed the presence of tannin, terpenoids and sterol in addition to the chemicals recognized in aqueous extract of *D. alba*. However, cardiac glycosides were absent in ethanolic extract of the root. Aqueous extract of stem of *D. alba* showed the presence of carbohydrate, cardiac glycosides and saponins, whereas ethanolic extract of *D. alba* also showed the presence of quinines and steroids (Table IV).

Aqueous extract of the roots of *Withania somnifera* showed the presence of carbohydrate, amino acid, saponins and sterol whereas the ethanolic extract of roots of *W. somnifera* also showed the presence of phlobatannine and terpenoids. However, amino acids were absent in the ethanolic extract of *W. somnifera*. Aqueous extract of the dried stem of *W. somnifera* showed the presence of carbohydrate, saponins, tannin and steroids whereas ethanolic extract of *W. somnifera* also showed the presence of terpenoids. However, tannins were absent in ethanolic extract of *W. somnifera*. Both categories of the extracts of fresh stem *W. somnifera* showed the presence of carbohydrate, cardiac glycosides, saponins, tannin and steroids. Aqueous extract of both fresh and dried leaves of *W. somnifera* showed the presence of carbohydrate, saponins tannin and steroids whereas in aqueous dried leaves extract of *W. somnifera* cardiac glycosides and phenol were absent. Ethanolic extract of both fresh and dried leaves of *W. somnifera* showed the presence of carbohydrate, cardiac glycosides, phenol, saponins, tannin, terpenoids and steroids (Table IV). Aqueous extracts of fresh and dried leaves of *Cryzophore tinctora* showed the presence of phenol, saponins, tannin and steroids, whereas ethanolic extract also showed terpenoids. Aqueous extract of dried leaves of *C. tinctora* also showed the presence of quinine and absence of phenol. Both aqueous as well as ethanolic extracts of fresh and dried stem of *C. tinctora* showed the presence of phenol, saponins, tannin, quinine, terpenoids and steroids. Both aqueous as well as ethanolic extracts of fresh and dried roots of *C. tinctora* showed the presence of phenol, saponins, tannin, quinine, terpenoids and steroids (Table IV). Also showed the presence of terpenoids.

Both aqueous as well as ethanolic extracts of leaves, stem and roots of *Chenopodium ambrosioides* showed the presence of cardiac glycosides, saponins and steroids whereas ethanolic extract of stem and roots of *C. ambrosioides* also showed the presence of terpenoids (Table IV). Both aqueous as well as ethanolic extracts of the flowers and stems of *Carthamus oxyacantha* showed the presence of carbohydrate, cardiac glycosides, saponins, tannin, and terpenoids, whereas ethanolic extract of the flowers and stem of *C. oxyacantha* also showed alkaloids, phenol, and steroids. Both *Aqueous* as well as ethanolic extracts of roots of *C. oxyacantha* showed the presence of carbohydrate, cardiac glycosides, saponins, tannin, and terpenoids whereas ethanolic extract of roots of *C. oxyacantha* also showed alkaloids, phenol, and steroids (Table IV). Aqueous extracts of the stem of *Conyza canadensis* showed the presence of alkaloid and carbohydrate whereas the ethanolic extract of *C. canadensis* also showed the presence of saponins. Aqueous extract of the leaves of *C. canadensis* showed the presence of carbohydrate, phenol, saponins and tannin, whereas the ethanolic extract of the leaves of *C. canadensis* also showed the presence of alkaloids, terpenoids and quinines. Both categories of the extracts of the flowers of *C. canadensis* showed the presence of alkaloids,
carbohydrate, cardiac glycosides acid, flavonoid, phenol, tannin, terpenoids and quinines. Aqueous extract of the roots of *C. canadensis* showed the presence of carbohydrate phenol, amino acid saponins and tannins whereas the ethanolic extract of roots of *C. canadensis* also showed the presence of phenol, quinines and terpenoids (Table IV).

Aqueous extracts of the stem and leaves of *Trichodesma indicum* showed the presence of flavonoid, phenol and tannin whereas aqueous extract of leaves of *T. indicum* also showed the presence of terpenoids. Ethanolic extracts of the stem and leaves of *T. indicum* showed the presence of flavonoid and quinines, whereas ethanolic extract of the stem of *T. indicum* also showed phenol and tannin but the extract of leaves of *T. indicum* showed alkaloids. Both aqueous as well as ethanolic extract of the roots of *T. indicum* showed the presence of alkaloid, flavonoid, phenol, tannin, terpenoids and quinine (Table IV). Both aqueous as well as ethanolic extracts of the stem of *Lantana indica* showed the presence of carbohydrate, cardioglycosides, amino acid, saponins, terpenoids and sterol. Aqueous extract of the flowers and leaves of *L. indica* showed the presence of alkaloids, carbohydrate, cardioglycosides, phenol, saponins and sterol, whereas the ethanolic extracts of flowers and leaves of *L. indica* also showed the presence of flavonoid and tannin. Aqueous extract of the roots of *L. indica* showed the presence of carbohydrates, cardioglycoside, phenol, saponins, and steroids whereas the ethanolic extract of the roots of *L. indica* also showed the presence of alkaloids, amino acids, terpenoids and quinines (Table IV).

Aqueous extract of the stem of *Lactuca dissecta* showed the presence of carbohydrate, cardioglycosides, phenol, saponins, tannin and sterol, whereas the ethanolic extract of *L. dissecta* also showed the presence of terpenoids. Aqueous extract of the leaves of *L. dissecta* showed the presence of alkaloid, carbohydrate, flavonoid, phenol, saponins, tannin, quinines and sterol, whereas the ethanolic extract of *L. dissecta* also showed the presence of carbohydrate glycosides and terpenoids. Aqueous extract of the root of *L. dissecta* showed the presence of carbohydrate, cardioglycosides, flavonoid, and sterol, whereas sterol was absent in ethanolic extract of *L. dissecta* (Table IV). Both aqueous as well as ethanolic extracts of the stem *Achyranthus aspera* showed the presence of carbohydrate, phenol and sterol. Both aqueous as well as ethanolic extracts of thorns of *A. aspera* showed the presence of alkaloid, carbohydrate, cardioglycosides, flavonoid, phenol, saponins, tannin, terpenoids and sterol. Both aqueous as well as ethanolic extracts of the leaves of *A. aspera* showed the presence of phenol, saponins, tannin, terpenoids and sterol. Both aqueous as well as ethanolic extracts of the roots of *A. aspera* showed the presence of phenol, saponins, tannin, terpenoids and sterol (Table IV).

Both aqueous as well as ethanolic extracts of the stem of *Malvestrum coromandelianum* showed the presence of carbohydrate, phenol, saponins, tannin and terpenoids. Both aqueous as well as ethanolic extract of the root of *M. coromandelianum* showed the presence of alkaloids, carbohydrate and saponins (Table IV).

Both aqueous as well as ethanolic extracts of the leaves of *Silybum marianum* showed the presence of carbohydrate, phenol, saponins, tannin, terpenoids and steroids. Both aqueous as well as ethanolic extract of the roots of *S. marianum* showed the presence of saponins, quinines and steroids (Table IV). Aqueous extract of the leaves of *Coronopus didymus* showed the presence of carbohydrate, phenol, amino acid, saponins, and tannin, whereas ethanolic extract of *C. didymus* also showed the presence of terpenoids and steroid (Table IV). Aqueous extract of the leaves of *Calotropis procera* showed the presence of alkaloids, carbohydrate, cardioglycosides, flavonoid, phenol, amino acid, tannin, terpenoids, quinines and steroids, whereas ethanolic extract of *C. procera* also showed oxalic acid. Aqueous extract of the stem showed the presence of carbohydrate, cardioglycosides, phenol, amino acid and sterol, whereas ethanolic extract of *C. procera* also showed tannin. Aqueous extract of the fruits of *C. procera* showed the presence of alkaloids, carbohydrate, cardioglycosides, flavonoid, phenols, amino acid, tannin, terpenoids, quinine, oxalic acid, and sterols. Both categories of extracts of the latex showed the presence of alkaloids, carbohydrate, cardioglycosides, flavonoid, phenols, amino acid, tannin, terpenoids, quinine, oxalic acid, and sterols. Both categories of extracts of the roots of *C. procera* showed the presence of alkaloids, carbohydrate, cardioglycosides, flavonoid, phenols, amino acid, tannin, terpenoids, quinine, oxalic acid, and sterols (Table IV).

Both aqueous as well as ethanolic extracts of the leaves of *Colocasia esculenta* showed the presence of carbohydrate, cardioglycosides, flavonoid, phenol, tannin and sterol whereas ethanolic extract of *C. esculenta* also showed terpenoids, quinines and oxalic acid. Both categories of extracts of the stems of *C. esculenta* showed the presence of carbohydrate, cardiac
glycosides, flavonoid, phenol, tannin, terpenoids, quinines, oxalic acid and sterol (Table IV). Both aqueous as well as ethanolic extracts of the whole plant of *Echinops echinatus* showed the presence of carbohydrate, phenol amino acid and saponins, whereas the ethanolic extracts of *E. echinatus* also showed quinines and sterol (Table IV). Aqueous extract of the whole plant of *Sassuria heteromalia* showed the presence of alkaloids, carbohydrate, cardiac glycosides and saponins whereas ethanolic extract of *S. heteromalia* also showed sterol (Table IV). Aqueous extract of the leaves of *Sapium sebiferum* showed the presence of carbohydrates, cardiac glycosides, saponins and sterol whereas ethanolic extract of *S. sebiferum* also showed phenol, tannins, oxalic acid and alkaloids (Table IV). The present study agreed with reported results on similar plants for instance. Zahid et al. (2002) extracted flavonoid, glycosides from *S. moorcroftiana*. *E. helioscopia* had been reported to possess tannin, saponins, diterpenoids, and amino acids (Ha et al., 2001; Zhang & Guo, 2005, 2006; Zhang et al., 2005; 2006). *Achyranthus aspera* contains triterpenoid and saponins. Mirjalili et al., (2009) extracted steroidal lactones from *Withania somnifera*. Muller et al. (1968) declared the presence of amino acids in latex of *Euphorbia helioscopia*. Yamamura et al. (1989) reported extraction of diterpenes from *Euphorbia helioscopia*. *Sapium sabiferum* contains diterpene esters (Everist, 1981). Nature of the phytochemicals reported in the present study advocate for their fungicidal, bactericidal, piscicidal and molluscidic potential; as the phytochemicals have been demonstrated for such activities (Mungenge et al., 2014).

### Table IV: Phytochemicals detected in aqueous (A) and ethanolic (B), fresh/ dried parts of the plants

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**CONCLUSION**

It is evident from the present study that the selected plants have different metabolites which are known for their poisonous effects on the user/fishes. Some of them have cardiac glycosides, flavonoid, saponins, terpenoids etc, which can be easily extracted and used as natural piscidial...
agents. In many countries like India, Nigeria, Nepal, these chemicals are being used for the eradication of unwanted fishes from the ponds.

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REFERENCES


Bacterial Contamination of Drinking Water in Tehsil Oghi, Mansehra Pakistan

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ABSTRACT
To analyze the microbiological quality of drinking water from different sources (tap water, open wells, stream water, spring water and water supply networks such as municipal main storage tank) in rural communities of the Oghi; a total of 175 water samples were collected from the study area with 35 samples from each source. All the samples were analyzed for microbial contamination by pour plate method, membrane filtration technique and MacConkey agar for the isolation of Enterobacteriaceae. Different identification tests have been carried out by using various selective and differential media (MacConkey agar, Eosine & Methylene Blue agar) for isolation and identification of microorganisms. Water samples showed the presence of faecal contamination; especially coliform bacteria which is a risk for the health of local community. Salmonella spp. was detected in 7.42 % of water samples having range 1-20 CFU 100ml⁻¹, while E.coli was detected in 91.4% of water samples having colonial population between 1-40 CFU/100ml. According to this study, 98 % of the water sources of study area did not meet the safe limits for drinking water of the country as well as the WHO guidelines. Conclusively it was declared that water quality is unacceptable for human consumption. It is therefore recommended that the authorities should take every possible measure to provide safe and clean water to household especially drinking water. Filtration plants, proper chlorination and awareness program should be carried out for safe drinking water.

Key Words: Drinking water, Microbial contamination, waste management.

INTRODUCTION
Water being the basic requirement of people, is used for drinking, domestic consumption and in agriculture activities (Sulehria et al., 2013). Globally, contaminated drinking water is responsible for the death of 1.6 million toddlers per annum with majority in the rural areas (Hisam et al., 2014). According to World Health Organization (WHO) a large group of people in the developing world are devoid of clean and potable water (Sulehria et al., 2013). Also polluted water is one of the most important contributors to health issues in Pakistan (Hisam et al., 2014). Some of the responsible factors for microbial contamination are poor waste management, use of polluted water and poor level of awareness (Nabeela et al., 2014).

World Health Organization has claimed that 80 % of human health problems in the emerging nations are due to biological contamination of drinking water. Pakistan has been ranked at 80th position among 122 nations regarding poor potable water quality. According to the studies of Bhutta et al., (2002), drinking water in densely populated cities of Pakistan like Karachi, Lahore, Rawalpindi, Peshawar, Faisalabad, Qasur, Sialkot and Gujrat is polluted due to various human activities and cannot be recommended for human consumption. Among various sources of contamination, microbial contamination is of prime importance which is due to improper disposal of faecal matter. This bacterial contamination has been reported to be the alarming issue throughout the country including urban and rural areas (PCRWR, 2005). Most of the bacterial pathogens are transmitted through drinking water to human body and the presence of coliforms indicates faecal contamination in the water (Sulehria et al., 2013). A research conducted by Pakistan Council for Research in Water Resources (PCRWR), have analyzed the water quality of rural areas in 64 Tehsils of four provinces with the findings of
microbial contamination of drinking water (Hisam et al., 2014). The outbreaks of gastroenteritis and waterborne disease have become common in urban areas. Annually more than 3 million citizens are affected from which 0.1 million do not survive (Haydar, 2009). In Pakistan, water borne diseases especially diarrhea is the leading cause of infant and children death affecting every fifth citizen (Haydar, 2009).

Providing safe and consumable drinking water is an important public health concern. Ignorance of potential risks and inappropriate trainings, faulty water drinking management systems result in unnecessary outbreaks of water borne diseases (Ashbolt, 2015).

In cities of Peshawar, Mardan, Charasada and Nowshehra of Khyber Pakhtunkhwa, more than 50 % of the total population have no access to clean drinking water. They use the drinking water from tube wells and shallow wells that are liable to be contaminated with biological pollutants from the surrounding sources such as toilets, underground damaged sewerage lines and seepage/percolation of contaminated surface water (Khan et al., 2013). Therefore, the present study aimed to achieve the objectives of water quality investigation through; Analysis of water samples for biological contamination and to identify the reasons for such pathogenic contamination.

**MATERIALS AND METHODS**

Water samples were collected for bacteriological analysis from different villages of Oghi i.e. Jalalabad, Bazargay, Manchora, Rasheeda, Belasyedabad, Kathai and Shamdara in duly sterilized bottles of one liter as per APHA guidelines of 2005 for drinking water. Water samples were collected from tap water, distribution network water, open well water, stream water and spring water.

For microbial study, growth mediums like selective and differential medium were used. Furthermore, for determining microbial quality of water sources, Coliform indicator was used. Generally, all Gram-negative bacteria which are able to conduct lactose fermentation and gas production and grow for 48 hours between 32 to 37°C are considered Coliform. Counting and presence of Coliforms were carried out by MacConkey agar growth medium. Growth medium was placed in incubator at temperature of 37 °C for 24 hours. Water samples were grown in positive lactose growth medium such as Eosine Methylene Blue (E.M.B) and MacConkey agar (M.C.) for confirmation test. In case of bacterial contamination such as Escherichia coli and Enterobacter, pink colonies (showing positive lactose) were observed after 24 hours of incubation at 37 °C on mentioned growth media.

Water microbiological tests were carried out by Pour plate method, Membrane filtration method, MacConkey broth/agar for the isolation of enterobacteracea/coliforms and identification test by using different selective and differential media.

To assess the impacts of water sanitation with relation to water borne diseases, a cross sectional study through self-administrative questioner was conducted among the local community of the study area. Different parameters like water handling practices, water channels safety, use of toilets, domestic waste water discharge into streams and open water sources were kept under consideration to check sanitation levels of different studied areas. Random House to house visits were conducted in which one person from each house was interviewed. After filling the questionaire from respondents, analyses were done through Microsoft excel.

**RESULT AND DISCUSSION**

Microbiological analysis of water samples from the five sources (spring, well, distribution network, stream and tap water) in seven sites of Tehsil Oghi showed that all water sources were positive for total coliforms and faecal coliform in two rounds of triplicate sampling except a few samples of well water. Shegilla and Pseudomonas spp. were found in very few samples. Salmonella were randomly found in different samples. Staph.aureus spp. were not found in most of the samples of Tehsil Oghi (Fig.,1).
Sanitation measures were found by survey method with the help of a cross sectional self-administrative questionnaire. Different parameters like water handling practices, water channels safety, use of toilets, domestic waste water discharge into streams and open water sources were kept under consideration to check sanitation levels of different studied areas.

There are three main water sources on which tehsil Oghi citizens are dependent and are using it for drinking purpose. One is scheme water including tap and distribution network water while the second one is open well water and other includes spring water. Analysis showed that 66.6% of the total population living in Jalal Abad use scheme water while other user of scheme water are 46.6% in Bazargay, 40% in Manchora, 26.6% in Rasheeda, 33.3% in Bela Syed Abad while 20% and 60% in Kathai and Shamdhara respectively. Survey shows that open well users are highest in Rasheeda (53.3%) while they are minimum in Bela Syed Abad where they are 26.6%. Other water sources are highly used in Kathai (53.3%) while in Jalal Abad and Shamdhara no one uses other water sources.

It can be seen from table (1) that 100 percent samples were heavily contaminated with E. coli and the presence of coliform indicates the low sanitary measures and lack of awareness about disposing sewerage lines into safe manner. This kind of contamination is an evidence of mixing of sewerage line with drinking water. It was either because of open defecation practice or discharge of sewerage line into water source.

### Table I. Microbiological analysis of water samples in different villages of district Oghi.

#### (A) TAP WATER

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Jalalabad</th>
<th>Bazargay</th>
<th>Manchora</th>
<th>Rasheed a</th>
<th>Belasyedaba d</th>
<th>Kathai</th>
<th>Shamdara</th>
</tr>
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<th>Belasyedabad</th>
<th>Kathai</th>
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(E) SPRING WATER

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</table>

- Sign indicates no bacterial species found;  
+ Bacterial species found  

The results from the laboratory work showed that 98 % of the water sources did not meet the safe limits for drinking water of the country as well as the WHO guidelines (zero=safe, 1-10 equitable quality, 11-100 contaminated water, 101-1000 hazardous and >1000 very hazardous), while well water samples of Bela Syed Abad and Jalalabad are safe, meet the safe limits for drinking water as well as the WHO guidelines, which are zero (00) CFU/100ml. Well water of Kathai having 1 CFU/ml is of equitable quality as per WHO standard
(WHO, 2004a). Salmonella spp. was detected in (7.42 %) of water samples having range 1-20 CFU/100 ml, while E.coli & coliforms was detected in (91.4%) of water samples having colonial population between 1-33 CFU/100 ml. Highly polluted water is stream water of Jalalabad and spring water of Bela Syed Abad and Kathai (mean value 33cfu100 ml\(^{-1}\)).

Questionnaire survey revealed that people of the study are mostly using open wells water and scheme water for their drinking purposes. Water sources are generally not covered from the surroundings and even sewerage and drainage lines are released in to bodies water like streams etc. In most cases septic tank found near the boring or well which contaminate the water source. It was observed that people do not have enough awareness about health and hygiene even not about the importance of cleanliness. In majority of the cases people use open defecation which is again a vital threat to water quality.

**CONCLUSION**

The present study was conducted in Tehsil Oghi for the purpose to analyze microbial contamination in drinking water and assess the sanitation status of study area. Different water sources such as tap water, open wells, stream water, spring water and water supplies network in rural communities of the study area were selected. Microbial contamination and chemical parameters were determined through intensive laboratory work while sanitation measure and status were measure through questionnaire, field survey and meeting with different communities group to make reliable and clean picture of studied area regarding water quality and sanitation measures. A total of 175 water samples were collected from the study area with 35 samples from each source.All samples count about 100 %E.coli+ coliform. The minimum count was found for pseudomonas and Stap.aureus. In all cases it was found that majority of water sources were either of improper quality or biologically contaminated.

The resulting biological contamination of drinking water cause dangerous health effects to local communities, who were suffering from coliform bacteria (E. Coli) related diseases i.e., vomiting, (typhoid and paratyphoid), Jaundice, diarrhea, hepatitis, skin diseases, cholera and dysentery. The results of this study are in line with other studies conducted in different cities of Pakistan (Hussain et al., 2011; InamUllah, 2014; Shedaiy et al., 2015). On the basis of conclusion it is suggested that the concerned authorities should take every possible measure to provide safe and clean water to every household. Filtration plants, proper chlorination and awareness program should be carried out among the communities for safe drinking water. Safety of water sources supplemented by sanitation and hygiene elevation programs can recover the quality of rural water sources, where decontamination is not possible.

**REFERENCES**


Hussain et al., 2011. Enumeration of Coliform bacteria in drinking water of Mughalpura, Lahore. BIOLOGIA (PAKISTAN), 57(1&2), 75-80.


New Fossils of Bovids (Bovidae, Mammalia) from Dhok Pathan Formation of Siwaliks

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Dr. Abu Bake Fossil Display & Research Centre, Department of Zoology, University of the Punjab, Quid-e-Azam Campus, Lahore 54590

ABSTRACT

New dental elements comprising upper and lower dentition of bovids have been recovered from Dhok Pathan Formation of Siwalik Group. The fossiliferous sites are situated in the Punjab province, ranging from Late Miocene to Middle Pliocene in age. On the basis of the comparative morphology and measurements, the material can be assigned to Pachyportax latidens, Selenoportax vexillarius and cf. Gazella. The new dental material is presented in this article.

Key Words: Taxonomy, Palaeontology, Gazella, Pachyportax, Selenoportax.

INTRODUCTION

The fluvial sequences of Siwaliks have been studied in detail (Colbert, 1935; Andrews and Cronin, 1982; Pilbeam, 1982; Acharyya, 1994). They consist of sandstones, mudstones and coarsely bedded conglomerates deposited during Middle Miocene to Upper Pleistocene times when the area was a vast basin (Chauhan, 2003). The Siwalik sediments in Pakistan are particularly widespread in the Potwar Plateau (72°30'E, 33°00'N). The Potwar Plateau is a high area of 20,000 km² attached to a belt ranging from the Salt Range (south) to the Margala Hills (North) and from the Jhelum River (east) to the Indus River (west) (Barry et al., 2002).

The studied specimens came from the deposits of the Dhok Pathan type locality (Lat. 33°07'S; Long. 72°14'E), district Chakwal, Punjab, Pakistan (Fig. 1) in the Potwar Plateau. The type locality is situated at Soan River about 75 km from Rawalpindi on Rawalpindi-Sargodha road and it is surrounded by extensive Neogene freshwater sedimentary rocks. The actual Dhok Pathan fossiliferous site is situated in the vicinity of the Dhok Pathan Rest House near the Soan River at an altitude of 1073 feet. The region of the Dhok Pathan exposes the most complete sequence of the Siwalik Group and yields a diversified assemblage of Middle Siwalik faunas. The magnetic polarity and stratigraphic dating has controlled the age of the Dhok Pathan Formation to be between 10.1 Ma and ca. 3.5 Ma (Khan et al., 2010). The Dhok Pathan bed near the Dhok Kundrali is one of the most fossiliferous beds of the Siwaliks (Akhtar, 1992).

The additional fossils of the Late Miocene bovids are documented in this article. The aim of the article is to provide the description of new fossils from the Late Miocene of the Siwaliks.

METHODOLOGY

The samples were collected in various field tours. The primary means of collecting the bovid remains was surface collection. The measurements are in millimeters (Table 1). The collected fossils represent good preservation. The specimens are stored in the Dr. Abu Bakr Fossil Display and Research Centre, Zoology Department, University of the Punjab, Lahore, Pakistan. The diagnostic characters of the recovered fossils are discussed. Terminology follows Gentry & Hooker (1988) and Akhtar (1992).

SYSTEMATIC PALAEONTOLOGY

Ruminantia Scopoli, 1777
Bovidae Gray, 1821
Genus Pachyportax Pilgrim, 1937
Pachyportax latidens Pilgrim, 1937
New material: PUPC 16/55, rM1; PUPC 16/52, IM2; PUPC 16/51, right mandible fragment with p4-m2.
Description and comparison: The upper molars are well preserved, representing seleno-hypsodonty
(Fig. 2). The molars are in early wear with slender styles. The median basal pillar is strong and expanded transversely. The teeth are almost quadrate and the cavities are simple in outline. There is no sign of posthypocrista and neocrista. The precristae and postcrista of the principal cones show selenodonty very well. The enamel is thick and moderately rugose. The major cusps are pointed. The p4 is asymmetrical in shape. The protoconid is well developed. A small eyelet is present in the entoconid. The cingulum is distinguishable at the base of the protoconid (Fig., 2). The median basal pillar is well developed in the lower molars (Fig., 2). The molars have pointed conids. The metaconid and enoconid are higher than the protoconid and hypoconid. The metastylid is less developed than the entostylid.

The studied specimens reflect the morphology of the genus Pachyportax. However, they are of appropriate size to match that of P. latidens. The molars show all the basic features of this species: strong styles and median ribs, no constricted crown neck, which is also common in Selenoportax and Tragoportax, transversely extended entostyle and comparatively large size (Pilgrim, 1937, 1939; Bibi, 2007; Khan et al., 2009a). Genus Selenoportax Pilgrim, 1937
Selenoportax vexillarius Pilgrim, 1937
New material: PUPC 16/53, left maxillary fragment with P4-M1; PUPC 16/54, IM1.
Description and comparison: The 4th premolar has a deep valley labially (Fig. 2). The para style and mesostyle are present in the P4. The fossette is deep. The preprotocrista is smaller than the postprotocrista. The M1 is quadrate in shape (Fig. 2). The median basal pillar is strongly developed. The para style is more prominent than the metastyle. The mesostyle is moderately developed. The cones are crescentic in appearance. The median ribs are strong. The spur is present in the fossettes.

The studied specimens show rugosity of enamel, strong median ribs, constriction of labial lobes and strong and divergent styles. These are the main features on the basis of which specimens were placed in the genus Selenoportax and conversely excluded the genus Pachyportax (Pilgrim, 1937; Khan et al., 2009a). The genus Selenoportax includes two species S. vexillarius and S. lydekkeri. In the studied specimens the P4 shows a deep posterolingual fold and a posterior re-entrant. The comparative study of these specimens revealed that the configuration of the premolar and molars as well as their dimensions correspond to that of S. vexillarius (Pilgrim, 1937).

Genus cf. Gazella Blainville, 1816
New material: PUPC 16/56, IM2.
Description and comparison: PUPC 16/56 is an upper molar. The tooth is quadrate in shape (Fig. 2). The major cusps are pointed and the cones are crescent in shape. The protocone is extended lingually. The metacone is higher than the other cones. The median basal pillar is absent. The mesostyle is more developed than the parastyle and metastyle. The posthypocrista is longer than the praehypocrista. The fossettes are narrow and crescent. The studied sample shows resemblance with the Siwalik gazelle, however, the exact identification based on a single molar is uncertain. The collected specimen is assigned to cf. Gazella on the basis of metric and morphological features (Table 1) (Pilgrim, 1937, 1939; Akhtar, 1992).

DISCUSSION

The fossils of Selenoportax, Pachyportax and Gazella had been continuously excavated from the Siwaliks by the researchers of Dr. Abu Bakr Fossil Display and Research Center, Zoology Department, University of the Punjab, Lahore, Pakistan (Bakr & Akhtar, 1985; Akhtar, 1992, 1995, 1996; Khan et al., 2006; Khan, 2007). More than 500 teeth of the genera Selenoportax, Pachyportax and Gazella have been collected from the Potwar Plateau of Pakistan. Pachyportax, Selenoportax and Gazella were present abundantly in the Middle Siwalik Subgroup. The second most abundant taxon after boselaphines is Gazella lydekkeri (Pilgrim, 1937, 1939; Khan et al., 2008, 2009a, b, 2010, 2011, 2012, 2013, 2014, 2015).

Pachyportax and Selenoportax were large bodied animals during the Late Miocene of the Siwalik. These animals got extinct before the end of the Pliocene (Barry et al., 2002). At the end of the Late Miocene, bovini or bovine-like bovids originated from the boselaphines (Gentry, 1999). The genus Pachyportax was recorded from the Middle Siwaliks (Lydekker 1876, 1884; Pilgrim, 1937, 1939; Akhtar, 1992, 1995, 1996; Khan, 2008; Khan et al., 2009a). Pachyportax is represented by two species P. latidens and P. nagrii, relatively small size (Pilgrim, 1937, 1939; Khan et al., 2009a). Selenoportax also represented by two species; S. vexillarius and S. lydekkeri. Gazella was abundantly found in the Hasnot region of the Middle Siwaliks (Pilgrim, 1937, 1939; Akhtar, 1992; Barry et al., 2002; Khan, 2007, 2008, Khan et al., 2009, 2010).
Table I: The comparative measurements (mm) of the cheek teeth referred to *Pachyportax nagrii*, *Slenoportax vexillarius* and *cf. Gazella.* The studied specimens. Referred data are from Pilgrim (1937, 1939), Akhtar (1992) and Khan et al. (2009b).

<table>
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<td>11.0</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
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<td>21.7</td>
<td>10.0</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>PMNH 85/09</td>
<td>M1</td>
<td>22.0</td>
<td>24.0</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>PMNH 83/91</td>
<td>M2</td>
<td>25.7</td>
<td>23.8</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>PMNH 83/93</td>
<td>M2</td>
<td>23.0</td>
<td>22.6</td>
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</tr>
<tr>
<td></td>
<td>PMNH 83/638</td>
<td>M2</td>
<td>23.0</td>
<td>24.0</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>PMNH 85/07</td>
<td>M2</td>
<td>23.5</td>
<td>22.0</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>PMNH 85/12</td>
<td>M2</td>
<td>24.6</td>
<td>25.6</td>
<td>1.04</td>
</tr>
<tr>
<td><em>cf. Gazella</em></td>
<td>PUPC 16/56*</td>
<td>IM2</td>
<td>10.1</td>
<td>10.6</td>
<td>1.05</td>
</tr>
<tr>
<td><em>Gazella lydekkeri</em></td>
<td>PUPC 97/22</td>
<td>IM2</td>
<td>13.5</td>
<td>13.0</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>PUPC 84/65</td>
<td>IM2</td>
<td>18.0</td>
<td>17.3</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>PUPC 97/21</td>
<td>IM2</td>
<td>12.0</td>
<td>12.0</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>AMNH 19663</td>
<td>IM2</td>
<td>13.5</td>
<td>11.5</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>PUPC 84/133</td>
<td>rM2</td>
<td>15.5</td>
<td>10.0</td>
<td>0.64</td>
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<tr>
<td></td>
<td>PUPC 02/37</td>
<td>rM2</td>
<td>14.5</td>
<td>9.00</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>AMNH 19663</td>
<td>rM2</td>
<td>13.0</td>
<td>7.50</td>
<td>0.57</td>
</tr>
</tbody>
</table>
Fig., 1: Map of Potwar Plateau showing the Dhok Pathan type locality (enboxed).

Fig., 2: *Pachyportax latidens*: 1. PUPC 16/55, M1; 2. PUPC 16/52, M2; 3. PUPC 16/51, p4-m2. *Selenoportax vexillarius*: 4. PUPC 16/53, P4-M1; 5. PUPC 16/54, M1.cf. *Gazella*: 6. PUPC 16/56, M2. a = occlusal, b = lingual, c = buccal. Scale bar 10mm.
CONCLUSIONS

New fossils of *Pachyportax latidens*, *Selenoportax vexillarius* and cf. *Gazella* were recovered from the Middle Siwalik Subgroup. The studied site is situated in district Chakwal, Punjab, Pakistan. The new fossils provide evidence of the diverse bovid faunal elements in the Siwalik Middle Miocene to Pliocene of Pakistan

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Comparative Analysis of Biosurfactant Production Assays by Five Indigenous Oil Sludge Bacteria

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ABSTRACT
Although there are several screening assays available for screening and evaluating the presence of biosurfactants but many of these show variable results under the same conditions and for the same organism. Most commonly applied screening assays including oil spreading technique (OST), tilted glass slide assay (TGS), drop collapse assay, emulsification index E24 and emulsification assay were used to evaluate production of biosurfactants in five biochemically identified strains isolated from oil contaminated sites. All strains used were known producers of biosurfactants but showed variable results for different tests. Four of five strains produced similar results for OST, TGS and emulsification assay. Drop collapse assay produced negative results when small amounts of biosurfactants were present. So it was concluded that a single primary screening test alone cannot identify an organism’s ability to produce biosurfactants and these tests should be used in combinations to get a reliable picture.

Key Words: Biosurfactants, screening assays, Oil Spreading Technique, Tilted Glass Slide Test, Emulsification index E24, Emulsification assays.

INTRODUCTION
Microbial flora of oil sludges produce surface active agents to lower oil and water as well as oil and soil interfacial tension that makes oil available for consumption or solubilization. Such chemical agents are called biosurfactants or bioemulsifiers, depending on their interaction with the hydrocarbon moiety and physicochemical properties (Uzoigwe et al., 2015). Hydrocarbonclastic bacteria produce biosurfactants and therefore this can be considered as a survival mechanism in oil-polluted environment (Pacwaha-Plociniczak et al., 2011).

Oil-polluted areas have always been a grave concern in regard to the environmental pollution, and thus microbial activity to produce biosurfactants holds an important area for research (Colwell et al., 1977; Atlas, 1981). Biosurfactants with their wide diversity and substrate specificity have functionality in the areas of biodegradability and inactivation/sequestering (Makkar & Cameotra, 2002). These properties account for their desirability in agriculture, food, textiles, cosmetics, petrochemicals etc (Haferburg et al., 1986; Georgiou et al., 1992; Prince, 1993). In addition, biosurfactants are helpful in microbial enhanced oil recovery (MEOR) (Brown et al., 1985).

To harness the capability of microbes in biodegradation or rehabilitation of oil polluted areas; various assays have been developed. Initial testing of microbial production of biosurfactants is done by any one of the most commonly performed assays including oil spreading technique (OST) (Morikawa et al., 2000), emulsification index E24 (Cooper & Goldenberg, 1987), emulsification assay (Patil & Chopade, 2001), tilted glass slide (TGS) assay (Persson & Molin, 1987) and drop collapse test (Bodour & Miller-Maier, 1998).

MATERIALS AND METHODS
Selection of Bacterial Strains: Soil samples from different oil contaminated areas of Lahore, Pakistan were collected. Five strains, namely G, H, J, K and L, of different phenotypic and morphological characteristics were selected and isolated on L-agar culture media. These isolates were then biochemically identified according to the identification schemes of Bergey’s Manual (Garrity G. et al., 2006).

Tests for biosurfactants: Primary screening tests were checked for the efficacy and accuracy in determination of biosurfactants, using coconut oil, in five bacterial strains i.e; G, H, J, K and L which are known producers of biosurfactants at different levels (Bento et al., 2005) (Hamed et al., 2012). Each test was performed in duplicates and mean results were noted.

Oil spreading technique (OST) The assay was performed according to the technique of Morikawa et al., 2000. Briefly, a Petri plate was filled...
with 20 ml of distilled water and on it 8 μl of crude oil was placed forming a uniform oil layer. 6 μl of bacterial culture was added to the center of plate; on top of the oil layer, and clear zones were observed after 30 seconds. Quantity of biosurfactant formation per cm² of oil displaced was defined as one BS (biosurfactant) unit (Thaniyavarn et al., 2003).

Drop Collapse assay Bodour & Miller-Maier (Bodour & Miller-Maier, 1998) drop collapse assay developed from Drop collapse assay by Jain et al (Jain et al., 1991), was used. Glass slides were rinsed using hot water, treated with ethanol and dried after washing with distilled water. These were then coated with oil and equilibrated to form a thin oil coating. 5 μl of bacterial culture supernatant was placed onto the glass slide and results were noted as a positive if the drop collapsed from its beaded shape or a negative if it remained in its initial form.

Tilted glass slide test: A colony of each strain was mixed with normal saline (0.9% NaCl) at an end of the glass slide which was cleared of any oil or possible surfactants by carefully washing with ethanol or heat dried. The slide was tilted and the drop was observed for collapsing or any kind of similar changes like dipping down or flowing down of the drop (Persson & Molin, 1987).

Emulsification index E24: Equal volumes of (2ml) bacterial culture and oil were mixed and vortexed at high speed for 2 minutes. These were then left to stand for 24 hours for ensuring a stable emulsified layer of oil. E24 index was calculated by dividing the height of emulsification “he” by the total height “ht” and multiplied by 100 to get percentage emulsification (Cooper & Goldenberg, 1987). Clear distilled water was used as a negative control.

\[ E24 = \left( \frac{he}{ht} \right) \times 100 \]

Fig., 2: Tubes are showing the Emulsification index test (E24) using coconut oil. A: Strain H, B: Strain J, C: Strain K, D: Strain G, E: Strain L, F: Negative control.

Emulsification assay: Twenty four hours fresh cultures grown under optimum conditions were centrifuged at 10,000 rpm for 15 minutes to ensure settling of cells. Three ml of each culture was collected and mixed with 0.5 ml of the test-oil. The mixture was vortexed for 2 minutes and incubated at 37 °C for 1 hour to allow separation of aqueous and oil phase. After that oil layer was removed and absorbance was measured for the aqueous phase at 400nm for each strain using uninoculated sterile growth medium as a blank. One emulsification unit (emulsification activity per ml [EU ml⁻¹]) is equal to absorbance of 0.01 at 400nm multiplied by the dilution factor (in the case of diluted emulsifier preparation) (Patil & Chopade, 2001).

RESULTS AND DISCUSSION

Selected isolates were identified as Aeromonas spp (Strain G), Pseudomonas aeruginosa (Strain H), Escherichia coli (Strain J), Bacillus pumilis (Strain K) and Staphylococcus spp (Strain L). All five biochemically identified strains were checked for biosurfactant production and activity by five screening assays. These screening assays were selected for their simple technique and rapid identification of results. Oil displacement assay utilizes the ability of biosurfactant to change the oil-water interface angle. The diameter of the zone was considered directly related to the activity of biosurfactant (Morikawa et al., 1993). Using E.coli as a test strain highest activity was observed (Fig., 1) with a diameter of 0.6 cm and biosurfactant unit of 0.28. Previously reported studies have also shown that E.coli is one of strongest candidates for production of biosurfactants (Pruthi & Cameotra,
Aeromonas was the weakest producer with diameter of 0.3cm and biosurfactant unit of 0.07.

Drop collapse and tilted glass slide assays also showed E.coli as the strongest while Aeromonas spp. as the weakest producer. Drop collapse assay was considered a bit non-specific as only positivity and negativity could be observed but the level to which biosurfactant is being produced was difficult to evaluate. Furthermore Staphylococcus spp. showed negative results in tilted glass slide assay but was positive in all others, this also questioned reliability of tilted glass assay as the sole technique for evaluating biosurfactant production (Table I).

Table I: Comparative analysis of screening techniques (+ positive, ++ strong positive, +++very strongly positive, - negative)

<table>
<thead>
<tr>
<th>S train</th>
<th>OST</th>
<th>DC</th>
<th>TGS</th>
<th>Emulsificatin E_{24}</th>
<th>Emulsification Assay (EUml^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas spp.</td>
<td>0.07</td>
<td>-</td>
<td>+</td>
<td>40</td>
<td>79.6</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>0.195</td>
<td>+</td>
<td>++</td>
<td>47.5</td>
<td>103.5</td>
</tr>
<tr>
<td>Bacillus pumilis</td>
<td>0.195</td>
<td>+</td>
<td>++</td>
<td>40</td>
<td>112.4</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.28</td>
<td>+</td>
<td>+++</td>
<td>42.5</td>
<td>123.8</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td>0.125</td>
<td>+</td>
<td>_</td>
<td>37.5</td>
<td>105.7</td>
</tr>
</tbody>
</table>

OST: Oil spreading technique; DC: Drop collapse assay; TGS: Tilted glass slide assay. While using E24 it was observed that Pseudomonas aeruginosa showed best results with E24 of 47.5%, E.coli with 42.5% was the second strongest while Staphylococcus showed the least ratio.

Fig., 2 shows the amount of biosurfactant produced for each strain and it is one of the first screening methods applied for the detection of biosurfactants (Willumsen & Karlson, 1996) (Christova et al., 2004) (Bento et al., 2005).

The results are summarized in table I facilitating comparative analysis of the tests that were performed and their consequent efficacy. The tests appear to be consistent for Pseudomonas aeruginosa, Bacillus pumilis and Escherichia coli, as they show good activity in E24 and emulsification assay, but with the exception of Pseudomonas as it showed poor emulsification per ml capacity, even lower than Staphylococcus spp. The tests were variable in the case of these two strains as well in the case of Staphylococcus spp. Negative for TSG, lowest activity in OST after Aeromonas spp., lowest emulsification capacity, it appeared positive for drop collapse and showed emulsification per ml slightly higher than Pseudomonas aeruginosa. Similarly, in the case of Aeromonas spp., it has lowest results in OST and emulsification per ml, negative for drop collapse as well, but positive for TSG. Its emulsification paralleled with that of Bacillus pumilis.

Our results showed that E.coli a predominant strain for biosurfactant activity as it was consistent with highest activity in OST, TGS and EA, except in E24 essay, where P. aeruginosa was dominant. So non-pathogenic strains of E.coli are good source of biosurfactant productivity and they can be used for different purposes (Banat et al., 2010) (Segura et al., 2014; Thies et al., 2014). Pseudomonas aeruginosa and Bacillus pumilis were the second most biosurfactant producing strains. One showing greater activity in E24 while other shows the greater activity in EA simultaneously. Hence, Pseudomonas aeruginosa and Bacillus pumilis also proved to be a good source of biosurfactants and these results are consistent with (Priya & Usharani, 2009) (El-Sheshtawy & Doheim, 2014). In our results Aeromonas spp and Staphylococcus spp showed the least activity for biosurfactant production. Aeromonas spp showing the negative results for TGS assay (Ilori et al., 2005). In other tests like OST, E24 and EA, they also showed the least activity, possibly because of their small size and gram negative nature.

Our results also show that TGS and drop collapse assays can be used as nonspecific initial screening assays. Their results are less reliable. Compared to these E24 and EA are more specific and can be used with efficacy and much reliability for testing biosurfactant production. It should be noted that none of these tests measured amounts of biosurfactants in specific culture so these test could only be applied as initial screening assays.

Hence, bioemulsification is not consistent for the biosurfactant’s activity on different substrates, rather only points to the presence of surfactants (Plaza et al., 2006) (Segura et al., 2014).

CONCLUSION

In the present study some primary screening tests, which are most frequently used for
the analysis of biosurfactants, were applied for those strains which are known to produce biosurfactants, to check the reliability of these tests. Variability suggested that only one test is not sufficient to determine presence or absence of biosurfactants. Therefore, it is recommended to use a combination of the most reliable tests for screening purposes.

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Prevalence of Anaemia in Children of Rural Areas of Punjab

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ABSTRACT

Anaemia is one of the significant public health problem among children in the world especially in those who live in rural communities having low socioeconomic status. This cross sectional study was conducted to determine the prevalence of anaemia in children of rural communities of Punjab from January 2016 to July 2016. The children were selected from Services hospital, Lahore and District hospital Sharqpur, Dist. Sheikhupura. A total of 500 samples of children were collected. Questionnaire was designed to collect information for studying variables regarding age, gender, father’s occupation, parent’s education, child’s family income etc. Their blood samples were collected and analyzed to find out the level of Haemoglobin, RBC’s, WBC’s, platelets, MCV, MCH and haematocrit specifically. Statistical analysis was done by using SPSS (version 13). Among the 500 subjects, according to haemoglobin level 130 subjects (26%) were normal whereas 370 (74%) were anaemic. It was found that 120 (24%) were mild anaemic, 151 subjects (30%) were moderate anaemic, and 99 subjects (20%) were severely anaemic. It has been concluded that the prevalence of anaemia was considerably high in children below the age of 12 years living in rural communities. It has been concluded that haematological parameters were greatly altered in anaemic patients.

Key Words: Anaemia, Children, Risk factors, rural areas

INTRODUCTION

Anaemia is one of the most serious public health problems affecting people living in developing and industrialized countries. It is one of micronutrient’s deficiency which has serious public health consequence in the world (Aikawa et al., 2006). Aanemia can occur at anytime and at all stages of the lifecycle but young children and pregnant women are at greater risk (Woldie et al., 2015). Like other developing countries, Pakistan is also facing a problem of high population growth and limited resources which affects socioeconomic development in the country (Parvez et al., 1997; Grant et al., 1995). Anaemia is usually defined as a reduction in haemoglobin concentration and haematocrit, or the number of circulating red blood cells below that is considered normal (Nestel, 2002).

Anaemia has been shown to be a public health problem that affects low, middle and high income countries. Anaemia can occur due to nutritional and non-nutritional factors. There is different classifications of anaemia which include nutritional anaemia, anaemia of infection, anaemia of chronic diseases and pernicious anaemia (Mary et al., 2005). The most common and prevalent cause of anaemia is an iron deficiency; however, the condition may also be caused by deficiencies in folate, vitamin B_{12} and protein. Some condition of anaemia is not caused by nutritional factors; other factors can be parasitic diseases such as malaria and congenital factors (Sowunmi et al., 2010).

Approximately 50% cases of anaemia are considered to be due to iron deficiency. Iron deficiency anaemia (IDA) affects 43% of preschool children all over the world especially in developing countries, which shows that prevalence rates four times higher than those found in industrialized countries. This high prevalence is associated with some conditions like poor sanitation conditions, low socioeconomic conditions and high morbidity among infants (Finch, 1977;)

A risk factor is an element linked to a person, a behavior, lifestyle or environment that increases the chance of developing the condition and has been found correlated with the condition in epidemiological studies. Some of the factors that are found to be associated with both anaemia and poor cognitive development are low socioeconomic status, poverty, lack of stimulation in the home, lack
of maternal warmth; poor maternal education and intelligence, maternal depression, low birth weight (<2.5 kg), parasitic infection, elevated blood lead levels and under nutrition. Our goal is to evaluate the prevalence of anaemia in children ≤12 years of age living in rural communities and identify its associated factors in children.

MATERIALS AND METHODS

Study Design and Population

The present cross sectional study was designed to investigate the prevalence of anaemia in children of rural areas of Punjab. The study was carried out at Services Hospital, Lahore which is a tertiary care hospital and Tehsil Head quarter, hospital Sharqpur, Sheikhpura. Services hospital is a tertiary care hospital and subjects from nearby rural area visited. Subjects were assessed via personal interview. So, a total of 500 samples were collected from those children who belonged to rural communities. Questionnaire was framed to collect information regarding age, gender, father's occupation, parent's education, socioeconomic status, family history of anaemia, lifestyle, duration of breastfeeding and consumption (frequency and amount) of animal meat, fish, and other traditional foodstuffs.

Inclusion criteria

All children of age 1 month to 12 years admitted in children ward were included in the study and it was made sure that all the children of either gender should be ≤12 years of age.

Exclusion criteria

Children were excluded if they were chronic / seriously ill and who were above 12 years of age and had any other disease.

Laboratory measurement

Blood samples were collected in EDTA tubes and the samples were immediately run on haematological analyzer (Model 'KX-21 Sysmex'', Germany) to assess various parameters including WBC (White Blood Cells) count, RBC (Red Blood Cells) count, Hgb (Haemoglobin) concentration, Hct (Haematocrit), MCV (Mean corpuscular volume), mean corpuscular haemoglobin (MCH), and platelet count (PLT).

Statistical analysis

The data of all the subjects was entered on MS excel work sheet. The statistical analysis was then conducted on the data using statistical software packages SPSS version 13.0 for window. Demographic data was presented as mean ± S.E.M. F-test was used for comparison of numerical data. Prevalence was determined by percentage.

RESULTS

This cross sectional study was designed to investigate the prevalence of anemia in children of rural areas of Punjab. The major risk factors found in our research were parent's education (11%), lack of breast feeding (20%), low family income (23%), use of cow's milk (25%), irregular heart beat(9%), use of unboiled water and obesity(12%). They are suspected to increase the chances for development of anaemia in children.

Among the 500 subjects, according to haemoglobin level 130 subjects (26%) were normal whereas 370 (74%) were anaemic. The anaemic subjects were further categorized on the basis of severity of anaemia. 120 (24%) children were mild anaemic, 151(30%) were moderate anaemic and 99 (20%) children were severe anaemic (Fig., 1)

![Fig: 1. Prevalence of different categories of Anaemia](image-url)

When 500 samples of children were categorized according to age as presented in table I. It was found that out of 500 samples 29% children were anaemic who were >1 year in age. In our study population 50% children were anaemic who were in between 1-6 years of age. Children in between the age of 6-12 years were 21% anaemic.
Table I: Prevalence of anaemia in children of different age group

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Age groups</th>
<th>Anaemic n (%)</th>
<th>Not anaemic n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;1 year</td>
<td>130 (29%)</td>
<td>5 (10%)</td>
<td>135 (27%)</td>
</tr>
<tr>
<td>2</td>
<td>1-6 years</td>
<td>227 (50%)</td>
<td>18 (40%)</td>
<td>245 (49%)</td>
</tr>
<tr>
<td>3</td>
<td>6-12 years</td>
<td>97 (21%)</td>
<td>23 (50%)</td>
<td>120 (24%)</td>
</tr>
<tr>
<td>4</td>
<td>Total</td>
<td>454</td>
<td>46</td>
<td>500</td>
</tr>
</tbody>
</table>

Assessment of haematological parameters in different categories of anemic subjects

Different haematological parameters were assessed and presented in table II which shows that they were altered in anaemic patients. The average values of Haemoglobin, RBCs, WBCs, PLT, Hct, MCV, MCH found in various group are given in table II.

Table II: Mean of Haematological parameters in anaemic subjects

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>HEMATOLOGICAL PARAMETERS</th>
<th>MILD (n=149)</th>
<th>MODERATE (n=168)</th>
<th>SEVERE (n=137)</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RBC count (10^6/µL)</td>
<td>4.61±0.1</td>
<td>4.13±0.1</td>
<td>4.51±0.1</td>
<td>0.429<strong>NS</strong></td>
</tr>
<tr>
<td>2</td>
<td>WBC count (10^3/µL)</td>
<td>18.0±1.0</td>
<td>14.4±1.0</td>
<td>13.7±1.0</td>
<td>0.203<strong>NS</strong></td>
</tr>
<tr>
<td>3</td>
<td>PLT count (10^3/µL)</td>
<td>343.1±11.4</td>
<td>411.7±11.4</td>
<td>335.7±11.4</td>
<td>0.026*</td>
</tr>
<tr>
<td>4</td>
<td>Hematocrit %</td>
<td>29.6±0.4</td>
<td>27.8±0.4</td>
<td>25.3±0.4</td>
<td>0.001*</td>
</tr>
<tr>
<td>5</td>
<td>HGB (g/dl)</td>
<td>10.2±0.1</td>
<td>8.65±0.1</td>
<td>5.99±0.1</td>
<td>0.000*</td>
</tr>
<tr>
<td>6</td>
<td>MCV (fL)</td>
<td>67.7±0.6</td>
<td>69.7±0.6</td>
<td>65.7±0.6</td>
<td>0.061<strong>NS</strong></td>
</tr>
<tr>
<td>7</td>
<td>MCH (pg)</td>
<td>24.8±0.5</td>
<td>22.9±0.5</td>
<td>23.0±0.5</td>
<td>0.267<strong>NS</strong></td>
</tr>
</tbody>
</table>

*Significance at p≤0.05
**Significance at p≤ 0.01

DISCUSSION

The present study addressed the high prevalence of anaemia in children and influence of different factors on anaemic patients which are suspected to increase the chance for development of anaemia in children living in rural communities. It is common among school age children and children living in rural areas as our study support this. The prevalence of anaemia in this study was 74%. This rate is relatively higher than that obtained by Asobayire et al., 2001 in children with the same age group (46 %). This could be explained by the fact that the study was extended to the rural population with different demographic characteristics.

In this study among 500 subjects, 130 subjects (26%) were normal whereas 370 (74%) were anaemic. By following the criteria of WHO (2008) it was found that 120 (24%) were mild anaemic, 151 subjects (30%) were moderate anaemic, and 99 subjects (20%) were severely anaemic. Similar study was conducted by Mazher., 2015 in which total of 450 girls were studied and among these 43% subjects were severely anemic and a majority out of these (36%) belonged to low and middle income class. 28% were mild anemic, 24% were moderate anaemic and 5% were normal. But in present study 26% were normal where as in her study 5% were normal the difference may be because of age group and gender as in our study both gender were enrolled and age group is less than 12 years. Another study conducted by Sop et al., 2015 the overall prevalence of anemia was 66.67%. Among these children, 5.08 %, 22.60 % and 36.16 % were suffering from severe, moderate and mild anaemia respectively.

In this study besides Haemoglobin (Hb), other haematological parameters were also measured like red blood cell (RBC), haematocrit (HCT), packed cell volume (MCV), mean corpuscular haemoglobin (MCH), mean platelet volume (MPV), platelet, and white blood cell (WBC) and were compared among different categories of anaemia. Significant difference was observed in PLT, Haematocrit and Hb levels. Whereas Mujib et
al., 2014 studied complete blood count in iron deficient anaemic children and he compared the sex difference instead of comparing mean values among the anaemic groups.

We have also identified several factors that are correlated with anaemia status. One factor which affects 60% of children in our study is use of cow’s milk. It was observed that consumption of cow’s milk consistently presents as a risk factor for anaemia in children. Cow’s milk affects many of its users as revealed by Sathish et al., 2015 in which 57% children were anemic because they were using cow’s milk at an early age and not feeding on breast milk. In his study the infants who were not breast fed were more likely to have IDA (Iron Deficiency Anemia) as compared to breast fed infants. Lack of breast feeding also affects children at early age usually before 6 months. Our study indicates that lack of breast feeding affects 48% of children. Exclusive breast-feeding for 6 to 8 months of age is recommended because it is thought that it protects against IDA. After this period, even with the excellent bioavailability of iron in human milk, it is necessary to use complementary foodstuffs that are rich in this micronutrient i.e., iron.

The indicated increased risk of anaemia in children below the age of 24 months is consistent with findings from other countries (Austin et al., 2012). This is mostly due to the increased need for iron at this age and inadequate introduction of iron-rich foods. Foods most commonly consumed by the population are cereals which do not have adequate quantity of iron.

In term of family income our data indicate that children whose family income is low had higher anaemia rates. Low family income affects 54% of child in this study. It was observed that mother’s education and family income had greater impact on the status of IDA among infants. In our studied population both mother’s education and family income were associated risk factors of anaemia among infants. It was seen that the infants of uneducated mothers and of those who belonged to low income families <10,000 were more likely to have IDA as compared to infants of literate mothers and of those who belonged to high income families. Similar results were shown by Oliveira et al 2007, Pasricha et al., 2010 and Park et al., 2006, reporting low socioeconomic status as a significant risk factor for anaemia. According to Soekarjo et al., (2001) socioeconomic status is the root line cause for anaemia among children. Iron deficiency anaemia is a vicious cyclical problem in community having low income.

In our studied population, most of children were anaemic and they belonged to rural communities. Poor bioavailability of dietary iron is a major factor for anaemia. 50% anemic children were from 1-6 years of age because at this stage they require more iron for their growth which was not provided sufficiently to meet their needs. The study provides evidence that the prevalence of anaemia in these areas starts at very young age normally in those who were less than 12 years of age. Iron deficiency (ID) in these areas needs to be prevented in order to enable the children to develop and grow normally.

CONCLUSION

The present study demonstrates that the prevalence of anaemia is considerably high in children below the age of 12 years, living in rural communities. 50% anaemic children were from 1-6 years of age. It has also been concluded that hematological parameters were greatly altered in anemic patients. Breast feeding practices need to be strictly followed to prevent incidence of anemia targeting children aged less than two years of age.

REFERENCES


ABSTRACT

Production of biofuels at commercial level has been perceived as sustainable solution to the problem of energy and economic crisis. Of all the liquid biofuels, an important one is biodiesel, the fatty acids methyl esters. Due to beneficial decomposition of bio-waste resources towards clear environments, oils from single cell microbes (yeasts) are gaining attention. Further cost reliance of these microbes to produce single cell oils (SCOs) could be reduced by using lignocellulosic waste (LCW) biomass produced in millions of metric tons annually all over the world covering 60% of annual crop produced. This review presents the yeasts SCO production from LCW, and its favorability towards commercialization in developing countries like Pakistan, which is eighth largest country among GDP sector for farm production.

Key Words: Oleaginous yeast, Single cell oil, Lignocellulosic wastes, LCW, Biodiesel, Yeast extract.

INTRODUCTION

Biofuels have taken the stern attention of researchers due to the increasing demands and depleting reserves of petroleum oils-based fuels. There is an immense need to recognize appropriate sustainable alternatives to conventional fossil fuels. Volatile as well as non-volatile, flammable hydrocarbons derived from plant or animal wastes or synthesized by autotrophic microbes such as microalgae oils able to be utilized as a rich source of energy represent biofuels. A variety of cellulytic microorganisms capable of utilizing different lignocellulosic materials including agro-industrial wastes has been documented for the production of biofuels. Lignocellulosic biomass is broken down into cellulose or hemicellulose oligomers and monomers yielding ultimately sugars including pentoses and hexoses (Hahn et al., 2007). Among all the biofuels, biodiesel, due to its clean burning, low viscosity, non-toxic and biodegradable nature low CO emissions and slightly higher NOx with reduced production of SOx is an efficient biofuel. Therefore, biodiesel production from different feedstocks like animal fats and plants oils has gained much attention all over the world. Plants such as sunflower, rapeseed, soybean, olive, jatropha and palm oils have been employed so far to produce biodiesel. Animal processing unit wastes and tallow have also been used to generate biodiesel. But the oils such as vegetable and cooking oils are expensive and food competitive. To commercialize the process of biodiesel production, feedstock used must be abundant and more sustainable. In this scenario, the most abundant feedstocks are the lignocellulosic wastes (LCWs). These LCWs are produced in huge quantities all over the world throughout the year. LCWs are employed to fermentation using oleaginous yeasts to produce microbial lipids mainly consisting of triacylglycerides (TAG), which can be transesterified into biodiesel. The biodiesel derived from lignocellulosic biomass including certain crops’ residues like wheat straw, sugarcane bagasse, corn stover, rice husk, municipal solid wastes and peels of fruits and vegetables are under colossal consideration.

Current progress and limitations in Biodiesel production

In the running scenario of the fossil fuels utilization and depletion, these fuels are no more questionable economically and ecologically. Production of sustainable and renewable biofuels has been under consideration since twentieth century. Transportation is the fastest growing energy consumption sector all over the world especially in US after electric energy sector. Alternative petroleum fuels are therefore under consideration of world energy producing companies to cope this crisis. Biodiesel is non-toxic, highly degradable and sustainable replacement of petroleum diesel accompanying existing petroleum engines with least modifications because of its chemical nature. Over the last decade, global
biofuel production increased rapidly; in 2008, about 15 billion liters of biodiesel were produced globally almost all of which was first-generation biofuel. In the European Union, biodiesel accounts for the major share of total biofuel production and is mainly derived from oil crops (canola and sunflower) as feedstock (Sustainable Production of SECOND – Generation Biofuels Potential and perspectives in major economies and developing countries, 2010). Some theoretical examples illustrate the vast amount of plant oil production necessary to replace conventional diesel. Converting the entire 2005 USA soybean crop to biodiesel would replace only 10% of conventional diesel consumed. Even the total world plant oil production of 2005 (approximately 120 million metric tons) would only satisfy approximately 80% of USA diesel demand (Weiss et al., 2012; Velmourougane et al., 2013). As discussed previously, devoting a greater proportion of plant oils for the production of biodiesel has already contributed to higher vegetable oil prices not only making biodiesel production more expensive but also having an impact on other sectors of the economy, such as food prices.

First generation biodiesel production includes the feed stocks like vegetable oils as well. As oils extracted from corn, soybean, coconut, palm, sunflower etc. These feed stocks have proved to be renewable as well as environment friendly emitting lesser amounts of CO₂ resulting in remediation of Global warming. The amount of CO₂ emitted as a result of burning is directly balanced by the amount of CO₂ consumed during photosynthesis of plants thus resulting in comparatively cleaner environment (Osamu & Carl, 1989; Stevens & Verhi, 2004). These oils contains higher levels of fatty acids like oleic acid, linoleic acid and steric acid as compared to other fatty acids. More oleic acid containing oils are preferred for biodiesel production (Firestane, 2006). These vegetable oils are of less worth as biodiesel feedstock for they are expensive and food competitive.

Non-edible oil seed plants have major significance for biodiesel production as they are not edible and could be grown in abundance over lands surrounding canal banks, road green belt and cultivator’s leftover land during seasonal crop and naturally destructed forests area without the issue of food-fuel competition. Non-edible oil plants like Polanga (Calophyllum inophyllum), Mahua (Madhuca indica), Karanja (Pongamia pinnata), Rubber plant seed, Cotton seed, Jatropha, Jojoba, Neem, Linseed and Tobacco might be cultured in additional lands for cost-effective provision of second generation biodiesel feedstocks (Chhetri et al., 2008; Mustafa, 2011; Atabini et al., 2013; Ong et al., 2013; Ashraful et al., 2014). But the oil produced from these seeds requires greater engine modifications as well as higher temperature zones to be applicable in transport sectors. In addition many categories of such oils are also non-favorable because of their higher viscosities.

For third generation biodiesel production, both prokaryotic and eukaryotic oleaginous microorganisms have been documented by researchers (Duong et al., 2007; Li et al., 2008; Chatzifragkou et al., 2010; Gorenberg et al., 2013). These microbial oils/lipids are said to be Single Cell Oils (SCO). SCO can be made as best demanding and responsive because of their greater efficiencies, higher yields and productivity. The SCO are also independent of the issues regarding land, venue, season and climate (Ratledge & Evans, 1984; Li et al., 2008; Moona et al., 2008; Galafassi et al., 2012; Tsigie et al., 2012). Microalgae species of these genera like Chlorella, Scenedesmus, Anabanae, Rhizocolonium and many other have been reported to produce biodiesel based on the mechanism of CO₂ utilization giving out excess biomass able to be utilized as animal feed or fertilizers. The biodiesel from microalgae needs greater amounts of CO₂ and light as well as longer time period to yield the product.

Microalgalae are on a great verge for biodiesel production having greater yields but the choice differs in the following aspects as they need hectors of area for their cultivation and maintenance as well as long cultural durations (Chisti, 2007). Further their oil composition differs mostly from that of vegetable oils being quite rich in mono as well as polyunsaturated fatty acids having four/ more double bonds (Belarbi et al., 2000) making the oils more susceptible to oxidation when stored and acceptability for biodiesel production might face restrictions (Chisti, 2007). Although the process of microalgae biodiesel production has been commercialized in many countries both heterotrophically and photosynthetically (to reduce cost of carbon source) but the process needs serious attention for provision of culture able land as well as stability of microalgal oils as they are significantly better as compared to other microbial lipids because these oils are highly unsaturated and more prone to degradation at high temperature (www.Biofuel.org.uk). Bacterial cells, being much smaller than the yeasts, attain comparable intracellular lipid/oil contents for biodiesel conversion. While owing to their fast metabolic rate, rapid growth and their abilities of utilizing complete bio waste resources such as cellulose might make them appealing candidates for biodiesel production at least in select situation such as addressing the
lignocellulosic mass utilization. Table I shows produced from different feedstock.
different advantages and disadvantages of biodiesel

Table I: Advantages and drawbacks of different biodiesel feedstock

<table>
<thead>
<tr>
<th>Category</th>
<th>Feedstocks</th>
<th>Worldw ide oil Production</th>
<th>Advantages</th>
<th>Drawbacks</th>
<th>References</th>
</tr>
</thead>
</table>
| First Generation Biodiesel       | Palm oil, Raps seed oil, Cocunut oil, Sunflower oil, All vegetable oils | More than 95% of biodiesel production all over the world | i. Environment friendly  
ii. Social and economic security | i. Expensive  
ii. Food competitive  
iii. Used in blends with petroleum biodiesel  
iv. High viscosity  
v. Lower volatility | Demirbas, 2003; Gui et al., 2008; Naik et al., 2010; Sitepu et al., 2014 |
| Second Generation Biodiesel      | Non-edible oil seed plants  |                            | i. Cheap  
ii. Non-food  
iii. No special nutritive requirements for growth | i. Cultureable land  
ii. High viscosity  
iii. Require engine modifications  
iv. Used in warmer areas  
v. Only annual or seasonal production | Fengrui et al., 1999 |
| Third Generation Biodiesel       | Microalgae                  | Thousand Gallons of oil/month | i. Strong oil producers  
ii. Strong CO₂ sequesters  
iii. Yields much biomass able to be utilized as animal feed or water purifier  
v. Similar fatty acids as that of vegetable oils  
v. Similar fatty acids as that of vegetable oils  
v. Similar fatty acids as that of vegetable oils  
vi. Higher growth periods (7-14 days) | i. Require more land area as well as water bodies for cultivation.  
ii. More susceptible to Bacterial or protozoan contamination in open ponds system.  
ii. Light required all the time for photosynthetic cultivation  
v. Most lipids are of lower fuel values as compared with diesel fuel  
v. Higher cultivation cost compared to plant oils | Solazyme, 2013; Meng et al., 2009; Chisti 2007; Huang et al., 2013 |
| Yeast                            |                             |                            | i. Quick growth to higher densities  
ii. Able to grow in variety of substrates  
iii. Able to control bacterial contaminations by low pH growth conditions in open culture systems  
v. Also could be i. Heterotrophs  
ii. Needs Simple sugar to ferment, therefore require pretreatment of lignocellulosic biomass  
iii. Commercially not well known  
iv. Pathogenic nature of some of yeast species should be considered before | Sitepu et al., 2014; Qi et al., 2013 |
In this era overburdened land agricultural practices have directed the scientists to look for such microbial cultivations which can be managed in small area/volume owing to their rapid rates of multiplication coupled with their ability of using waste biomass as feedstock. Thus through the development of a process a value added product is obtained with concomitant consumption of waste(s). Therefore, in this review our main focus is on yeast biodiesel production using lignocellulosic wastes. In this regard certain species of oleaginous yeasts have much potential. The yeast can be cultivated on certain agri/food industrial wastes and byproducts. Depending upon the nature of complexity and recalcitrance of the waste substrates physicochemical and biological pretreatment is mandatory, however.

**Oleaginous yeasts as renewable feedstock’s for biodiesel production**

The role of oleaginous precursors for the production of biodiesel depends upon their efficiency and environmental favorability. So the choice of oleaginous precursors should be according to the environmental concerns and coping competition of depleting fossil fuels. The biodiesel obtained from the respective precursors should also have the similar molecular structures as well as the energy density to that of petro diesel fuels. Yeast lipid production is a biphasic process: firstly the SCO produced are very much similar to vegetable oils and are highly compatible to be converted into the biodiesel and secondly, the cell biomass could be harvested and employed as a rich protein supplement to humans as well as aquaculture. Feasibility to this LCW based SCO production also leads to clear environments and surroundings improving health status of the local people.

Microbes like bacteria, yeasts and microalgae have up to 60% tendency of their dry cell mass to accumulate lipids/oil in them, while the yeast Cryptococcus curvatus has been reported to accumulate 82.7% lipid contents per 104.1 g/L of biomass utilizing glucose as sole source of carbon (Ykema *et al*., 1988). The complex substrate utilization by yeast can be made possible by co-culturing cellulolytic bacteria and oleaginous yeast (Uchida *et al*., 2004; Zuroff *et al*., 2013).

While oleaginous yeasts have many advantages as being unicellular, having high growth rates with lipid accumulation in discrete lipid bodies in lesser time durations and are accomplishment within relatively short period of time. On the other hand usage of low-cost fermentable media including agricultural and industrial residues renders the process economically lucrative (Malisorn & Suntornsuk, 2007; Angerbauer *et al*., 2008; Yousuf *et al*., 2010; Koutinas *et al*., 2014). Furthermore the accumulation of higher lipid contents depends mainly on the higher C/N ratio of the cultivation media (Angerbauer *et al*., 2008; Saenge *et al*., 2011). Papanikolaou *et al*., 2003 reported 150 C/N ratios for giving out higher yields upto 68% of dry

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>grown on same conditions as heterotrophically grown algae</th>
<th>industrialization</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Fast growth rate</td>
<td>v. Can accumulate upto 70-80% of lipids in four days</td>
<td>i. Smaller size</td>
</tr>
<tr>
<td>ii. No added vitamins or supplements required</td>
<td>vi. Resistant to fermentative inhibitors upto 0.5g/l of HMF, furfurals, and 2.5g/l acetic acid</td>
<td>ii. Yields complicated lipoids instead of lipids</td>
</tr>
<tr>
<td>iii. No hectors of areas required for growth</td>
<td>vii. Resistant to fermentative inhibitors upto 0.5g/l of HMF, furfurals, and 2.5g/l acetic acid</td>
<td></td>
</tr>
<tr>
<td>viii. No added vitamins or supplements required</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Huang <em>et al</em>., 2013</td>
</tr>
</tbody>
</table>

Huang *et al*., 2013
cell mass of the yeast *Lipomyces starkeyi* utilizing pretreated sewage sludge. Moreover, *Yarrowia lipolytica* has been reported for 70% lipid accumulation utilizing agro-industrial wastes like stearin (waste industrial animal fats) and technical glycerol and glucose ultimately resulting in the increased production of citric acid and cocoa butter like lipids upto 14g/L and 3.4g/L, respectively. Papanikolaou and Aggelis (2011) found that the lipids accumulation in oleaginous yeasts increases in the nitrogen or to a lesser content on phosphorus or sulfate deficiency in media and is termed as *de novo* lipid accumulation which starts degrading soon after the depletion of carbon in the media, while *ex novo* lipid accumulation is independent of the nitrogen content of the media. Lipid accumulation is also influenced by the factors like carbon source, nitrogen source, C/N molar ratios and physical growth parameters such as temperature and pH. Organic nitrogen (peptone and yeast extract) supplementation is more advantageous over ammonium sulfate [(NH₄)₂SO₄] and ammonium nitrate [NH₄NO₃] in gaining higher cell mass as well as lipid yield from *Trichosporon fermentans*. Decrease in biomass using inorganic nitrogen source [(NH₄)₂SO₄ and NH₄NO₃] might be due to the formation of inhibitor (HNO₃ and H₂SO₄) acids after 72 hours growth of *Y. lipolytica* Po1g in sugarcane bagasse medium resulting the decrease in pH upto 6 and 5.8, respectively (Zhu et al., 2008; Tsige et al., 2011). So a medium with surplus of carbon and other limiting supplements like nitrogen is required for higher lipid yields (Papanikolaou et al., 2006). Arachidonic acid, a polyunsaturated fatty acid produced by yeasts and is of great importance as dietary supplement in infant formula (Ratledge & Wynn, 2002; Ratledge, 2004). Fatty acid composition of lipids from different sources varies greatly. Meng et al. (2009) have summarized ratio of different fatty acids constituents of lipids produced by microalgae, yeast, fungi and bacteria as shown in table II.

### Table II: Fatty acid profiles of different microorganisms (Meng et al., 2009)

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Lipid Composition (w/total lipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palmitic acid C16:0</td>
</tr>
<tr>
<td>Microalgae</td>
<td>12-21</td>
</tr>
<tr>
<td>Yeast</td>
<td>11-37</td>
</tr>
<tr>
<td>Fungi</td>
<td>7-23</td>
</tr>
<tr>
<td>Bacterium</td>
<td>8-10</td>
</tr>
</tbody>
</table>

The typical biodiesel with maximum efficiency is defined as a mixture of long chain fatty acid methyl esters (typically C₁₄- C₂₂) being a nontoxic, biodegradable pollution reducer (Monoz et al., 2014). The relevant fatty acid profiles of different yeast strains have been shown in the following table III.

### Table III: showing fatty acid profile of different oleaginous yeast species/ strains on glucose containing medium (adapted from Ratledge & Wynn, 2002; Liu & Zhao, 2007 & Beopoulos et al., 2011)

<table>
<thead>
<tr>
<th>Yeasts species/strain</th>
<th>Lipid accumulation (%D.W)</th>
<th>Major Fatty acid Residues (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Palmitic acid C16:0</td>
</tr>
<tr>
<td>Cryptococcus curvatus</td>
<td>58</td>
<td>25</td>
</tr>
<tr>
<td>Cryptococcus albidus</td>
<td>65</td>
<td>12</td>
</tr>
<tr>
<td>Candida sp 107</td>
<td>42</td>
<td>44</td>
</tr>
<tr>
<td>Lipomyces starkeyi</td>
<td>63</td>
<td>34</td>
</tr>
<tr>
<td>Rhodotorula glutinis</td>
<td>72</td>
<td>37</td>
</tr>
<tr>
<td>Rhodotorula graminis</td>
<td>36</td>
<td>30</td>
</tr>
<tr>
<td>Rhizopus arhizus</td>
<td>57</td>
<td>18</td>
</tr>
<tr>
<td>Trichosporon pullulans</td>
<td>65</td>
<td>15</td>
</tr>
<tr>
<td>Yarrowia lipolytica</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td>Rhodotorula toruloides</td>
<td>58</td>
<td>24.3</td>
</tr>
</tbody>
</table>
Recently, Santamauro et al. (2014) have reported an oleaginous yeast Metschnikowia pulcherrima capable of utilizing waste lignocellulose derived sugar mixture (glucose, xylose, arabinose and cellobiose) to accumulate lipids up to 40% under non-sterilized conditions. Active growth rate was observed in stressed physical conditions like pH and temperature followed by nitrogen-starved medium. The heterotrophic organisms such as yeast may prove beneficial alternative to microalgae as biodiesel feedstock with an elevated production potential of lipids on per cell per 24 hours basis yielding up to 65% lipid/dry cell mass within 3-7 days from 10-100g/L of sugars (Li et al., 2008) comparing with microalgae giving only 0.15-0.25 g/L per day in open ponds (Sharma et al., 2011). Moreover, unlike microalgae yeast does not require light enabling 24 hours production of lipids on equal extent rendering further economical fermentation of both C6 and C5 sugars from lignocellulosic waste substrates (Sun & Chang, 2002; Larsen et al., 2008; Santamauro et al., 2014).

**Lucrative substrates for yeast oil production**

1. **Lignocellulosic Wastes**

Lignocellulose, a generalized term used to describe the three major components of plant material namely lignin, cellulose and hemicelluloses held together in complex matrices composed of several different polysaccharides, phenolic polymers and proteins. Large energy reserves are present in cellulose which is also the major portion of lignocellulosic biomass having existent potential for catalytic conversion into biofuels. Lignocellulosic biomass is the most abundant renewable organic source which could be exploited for biofuels production. It is produced around 200 billion tons annually comprising 60% of lignocellulosic wastes as crop residues, forest left over and industrial residues following food and vegetable processing (Silva et al. 2012; Gracia et al., 2014). Oleaginous yeasts produce a variety of SCOs having different fatty acid profiles depending upon the variety of nutrients/ carbon source. This property of the oleaginous yeasts for biodiesel production is under consideration since last few decades. To improve economics of the product by using low-cost/no-cost lignocellulosic wastes commonly called as crop/industrial residues are under consideration (Candia et al., 2014).

However, oleaginous yeasts are less active in cellulolytic activity and needs an efficient pretreatment of LCW for biodiesel production. Different pretreatment methods like acid/alkali treatments, steam and sulfur dioxide explosions, ammonia fiber explosion, ionic liquids and others are used to convert this lignocellulosic biomass into fermentable sugars. Many structural, compositional and physico-chemical parameters hinder conversion of the biomass into sugars. Further the pretreated lignocellulosic waste biomass is to be detoxified by over liming etc. for bypassing the inhibitory effects of certain LCW pretreatment/ hydrolysis derived molecules such as furfurals and 5-hydroxymethyl furfural (HMF) (Haung et al., 2011; Bochmann & Montgomery, 2013; Behera, et al., 2014).

Typical ratio of hexoses (glucose and mannose) to pentoses (xylose and arabinose) in LCW is reported to range from 1.5:1 to 3:1 (Balan et al., 2008; Balan et al., 2009; Lau & Dale, 2009). Pretreatment is also important so that the naturally occurring or genetically engineered or modified oleaginous yeasts could be able to hydrolyze sugars other than glucose such as xylose (second abundant sugar) to produce biodiesel. Xylose is well thought out for active consumption to produce biodiesel by oleaginous yeasts because it is less reported as fermentable for bioenergy production processes (Rahman et al., 2006). Microbial utilization of xylose will subsequently lead to the maximum utilization of LCW biomass. A thumbnail sketch of the whole process designed for converting LCW into biodiesel through the lipid accumulating metabolism of oleaginous yeasts is depicted in Fig.1.
Fig.1: A generalized overview and sequential organization of various steps involved in the process of upgrading agri/food industrial wastes to biodiesel routed through the eukaryotic microbial metabolism (Xuet al., 2013) EMP pathway; Embden-Meyerhof-Parnas pathway.

2- Industrial wastes
Increasing energy demands by world’s population necessitate to recycling of every possible waste substrate. Industry is developing with the passage of time all over the world. Billions of tons of wastes like molasses, vegetable and fruits
processing wastes, glycerol, whey and waste waters including sewage sludge and from oil mills are rich sources of fermentable sugars. The feedstock from wastes could be utilized as potential substrates for SCO production worldwide (Haung et al., 2013). The potential of SCO production using the low-cost substrates from wastes by variety of yeasts have been tested so far and the strains of *L. starkeyi, Rhodotorula glutinis, Y. lipolytica, C. curvatus, Apiotrichum curvatum* and *T. fermentans* have been found potentially active to produce SCO as biodiesel feedstocks (Akhtar et al., 1998; Papanikolaou & Aggelis, 2003; Zhu et al., 2008; Yousuf et al. 2010; Wu et al., 2011). Lipid fermentation is not dependent upon the substrate selection solely. It also bases on microbial lipid production and suitability of the lipid produce for biodiesel production. Substrate should be cost-competitive and should not influence the quality of microbial oil.

**Significance and abundance of wastes derived SCOs from yeasts**

Microbial lipids, being an important feedstock for biodiesel production from oleaginous yeasts are of momentous significance around the world (Meng et al., 2009). During last decade oleaginous yeasts *Rhodotorula glutinis, C. albidus, L. starkeyi*, and *Candida curvata* (currently called as *Cryptococcus curvatus*) capable of yielding higher quantities of SCOs utilizing acid/alkali hydrolyzed lignocellulosic wastes as carbon sources like wheat straw, corn stover, rice husk, sugarcane bagasse, corn cob, populus and Eucalyptus leaves, grass etc. have been documented. The SCOs are transesterified into biodiesel and other value added products. Some of the oleaginous yeast species are able to ferment xylose, a pentose sugar too, utilizing most of residual portion of lignocellulosic waste adding a positive clue to enhance the production of biodiesel (Haung et al., 2013). Ability of xylose metabolism enhances the composition and nature of fatty acids chain lengths and degree of saturation. One of the most important selection criteria for oleaginous yeast strains is the chemical nature of fatty acids present in biolipids produced to ascertain their suitability for biodiesel production (Tanimura et al., 2014).

Oleaginous yeasts could be preferable than fatty acid methyl esters (biodiesel) producing plants because they do not need any arable land and could be grown on low-cost degradable wastes using their carbon as energy source to accumulate lipids as well as non-food competitors resulting in cost effective biodiesel production (Bautista et al., 2012). The nature of yeasts SCOs is similar to that of plant/vegetable oils as they are most commonly occurring saturated fatty acids. The major fatty acids of SCOs comprises of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and (C18:3) which is similar to vegetable oils and is species specific (Beopoulos et al., 2009). Another way to favor SCOs from yeasts is the temperature and environmental favorability. Being rich in biodiversity yeasts are found in almost all habitats including hypertonic as well as hypotonic environments in aerobic as well as anaerobic conditions. Yeasts also favorably grow in a temperature range from 23-37°C within 48-72 hours in aeration. pH also ranges in a long range from 2-9 (Avalione et al., 2001; Jackels & Jackels, 2005). Velmourougane (2013) reported that during the process of fermentation when 60% glucose is utilized the pH of the microbial culture is lowered because of the production of raw substances like acetic acid, lactic acid and ethanol which could further be used as supplement by yeast. Some of the criteria to choose yeast as SCO producer biodiesel feedstock have been reported by Pereira et al. (2014) and Sitepu et al. (2014). They have ability to grow in a wide range of lab media (LCW/low-cost biomass as carbon source), fast growth log phase attained within 24-48 hours, faster lipid accumulation within 96-120 hours, maximum substrate utilization, 80% maximum product yield of dry cell mass, higher metabolites’ tolerance (12-15% ethanol, 2% lactic acid and 2% acetic acid), osmotic pressure tolerance because of rigid cell wall (upto 50% glucose or fructose), pH tolerance during fermentation (pH 2-8), heat tolerance (upto 43°C), lipids completely similar to plants/oil biodiesel, production all the year through- No seasonal boundary, environment friendly biodiesel, Xylose utilization from lignocellulosic waste, less viral vulnerability and lesser chances of bacterial contamination because of fermentation at low temperatures.

**Effects of wastes derived carbon sources on yeast’s lipids**

Oleaginous yeast species are able to utilize diverse range of carbon sources for the production of maximum cell biomass and lipids. These sources can be glucose, xylose, glycerol, starch, hydrolyzed lignocellulosic biomass and others including industrial and municipal organic wastes. Independent of the carbon source lipid accumulation depends upon the limited supply of some nutrient other than carbon. *Trichosporan fermentans* produces lipids on a number of carbon sources (glucose, xylose, arabinose, mannose, galactose and cellobiose) derived from detoxified sulfuric acid treated hydrolyzate of rice straw (SARSH) and hydrolyzed pine/aspen lignocellulosic residues. The highest lipid contents were observed as 40.1% with
a cell biomass of 28.6g/L with a lipid yield of 11.5g/L utilizing detoxified SARSH and 13.9, 11.5 and 10.4g/L utilizing galactose, mannose and cellobiose, respectively (Parajo et al., 1998; Ezeji et al., 2007; Haung et al., 2009). The fraction of fatty acids was dependent on carbon source containing 65% lipids of T. fermentans. With sweet potato vines enzymatic hydrolyzate (SVH) T. fermentans was found to be an efficient lipid producer with a net yield of 9.6g/L lipid contents having 35.6% of lipid yield after 7 days of incubation utilizing 90% of reducing sugars. While with a supplementation of SVH with fructose, the yield was increased upto 27.6g/L (Zhan et al., 2014). Wheat straw is an abundant lignocellulosic waste produced all over the world. Annual production of wheat straw is recorded to be 850 tons per annum based on straw/crop ratio of 1.3 (Talebnia et al., 2010) which are exclusively increasing with the passage of time. Lipid producing potential of five oleaginous strains namely Cryptococcus curvatus, Rhodospirillum toruloides, Rhodotorula glutinis, Yarrowia lipolytica and Lipomyces starkeyi was tested using detoxified and non-detoxified acid hydrolyzed wheat straw having composition of 24.3 g/L of pentose and 4.9g/L of hexoses. Rest of the hydrolysate contained inhibitors like furfurals, acetic acid and hydroxyl methyl furfural (HMF). The resultant liquid hydrolysate contained 3g of glucose, 16g of xylose, 3.8g of arabinose, 1g of galactose and 3.3g of acetic acid per 100g of wheat straw. Out of these five oleaginous strains C. curvatus was found an efficient lipid producer having 33.5% and 27.1% lipid yield from 17.2g/L and 15.6g/L of dry cell mass on non-detoxified and detoxified liquid hydrolysate of wheat straw, respectively. All other strains also showed positive results on detoxified as well as non-detoxified liquid hydrolysate except R. toruloides for the non-detoxified liquid hydrolysate (Yu et al., 2011). Chen et al. (2013) reported corncob acid hydrolysate as potential source of carbon for the growth of yeast Trichosporon cutaneum. The hydrolysate contained sugars namely glucose, xylose and cellobiose. This strain yielded 45.5% lipids with a lesser biomass of 22.9g/L. They also confirmed the effect of higher C/N ratio on the lipid accumulation resulting in reduced cell growth. Lipid production potential of sweet potato starch by Lipomyces starkeyi has been studied. These cells can consume dissolved starch in batch cultures fermentation processes containing 40% lipids with a dry cell mass of 0.41g of cells per g of starch (Wild et al., 2010). L. starkeyi is also capable of giving higher lipid yields up to 6.4% in pretreated organic rich sewage sludge from 9.4g/l of cell biomass (Angerbauer et al., 2008). Lipomyces starkeyi is a distinctive strain for having ability of not utilizing its own lipids (Holdsworth et al., 1988). Crude glycerol is the by-product of the industrial plants converting oils into biodiesel, constituting 10% of the oils fed as substrates. The increase in the production of biodiesel has tremendously increased the production of crude glycerol (Dasari et al., 2005; Johnson & Taconi, 2007). This by-product cost high prices for its purification. Therefore, there is a need to utilize its crude form for producing SCOs. Oleaginous red yeast Rhodotorula glutinis has been reported to ferment this crude glycerol into valuable lipids for biodiesel production having 6.10g/L of lipid yield from 10.05g/L of biomass in a fed batch fermentation process (Saenge et al., 2011). Another lignocellulosic waste of industrial sector is sugarcane bagasse. It is the major raw product of sugar industry. The presence of higher carbohydrate and lower lignin values makes it a suitable substrate for yeast fermentation. Sugarcane bagasse can yield 13.59 g/L xylose, 3.98 g/L glucose, and 2.78 g/L arabinose when treated with 2.5% of HCl. Detoxified sugarcane bagasse hydrolysate was found to be suitable substrate for yeast Yarrowia lipolytica yielding 6.68g/L lipids (Tsigie et al., 2011). These authors also confirmed the higher lipid and biomass production utilizing peptone (organic nitrogen source) as compared to ammonium nitrate as a source of nitrogen. Molasses is also an industrial raw-product of sugarcane industry. It basically contains glucose, fructose and sucrose. This low-cost material has been found as a cheap medium formulation material for the fermentation of SCO from yeast. But its higher nitrogen contents prevent lipid accumulation in higher amounts (Zhu et al., 2008 & Chatzifragkou et al., 2010). It is concluded that conversion of agro-industrial by-products and wastes having quantitatively large amounts of carbon is a sustainable alternative for recycling and conserving resources. These raw materials could be recycled in the same industry they are being produced. The only need is to establish an extra plant for their fermentation. So the biodiesel fermentation by oleaginous yeast is a key step to be followed by the local governments to overcome the energy crisis at domestic levels ultimately making nations self-sustainable in industrial and transport sectors. Yeast biodiesel production potential in Pakistan from wastes

In order to certify a sustainable energy prospect, Pakistan’s prerequisite to broaden its stock blend cannot be overemphasized. Manipulation of native resources of energy should be the soul of future strategies to enhance the
energy security of the country through subsidizing reliance on energy imports. Renewable and sustainable energy might play a crucial role in the future of energy competence in Pakistan as the government had spent US $9 billion on energy import to fulfill current energy requirements during 2008-2009 (Asif, 2009; Rehman et al., 2013).

Geographically, Pakistan is located in the Southwest of Asia, lying just above the tropic of cancer having an area of about 796,095 Km² comprising of 97.13% land, of which only 4% is covered by forests. Annually, the country receives a heavy rainfall upto 150mm on an average. Pakistan is blessed with four seasons ranging the average temperature as low as 5°C to maximally 55°C. The optimum temperatures vary from 23-37°C residing most of the part of the country for maximum part of the year, especially from March- June and September- November (on an exception of mid- December since last few years) (Weiss et al., 2012). The climate conditions thus are quite favorable for the optimal growth and biolipid productivity of yeast (Chen et al., 2013; Haung et al., 2013; Zhan et al., 2013). Moreover, on the world ranking, Pakistan stands at 14th position in having arable land equal to 20,714,000 hectares by the year 2011 and 25% of this land is under cultivation according to the FAO report, 2011. According to the list of countries by GDP sector composition Pakistan ranks eighth worldwide in farm output. The same report also ranks Pakistan for cotton, date palm, sugarcane, wheat and rice production at 4th, 5th, 5th, 7th and 14th positions, respectively (Food and Agriculture Organization, 2011).

Pakistan’s economy is based on agricultural sector and is considered to be one of the world’s top exporters of mango, orange, apricot, sugarcane, wheat and rice. Safe disposal of crop residue is a great problem to be overcome and to subsidize this problem careful attention is to be needed for the safe and valued utilization of the residues as a feedstock to produce microbial lipid and ultimately biodiesel (Khan et al., 2010).

According to Pakistan Economic survey (2005-2006) the initial research for biodiesel production pilot scale projects are in progress. Mirza et al. (2008) reviewed all biodiesel power projects in Pakistan and presented that the biomass produced annually should be utilized for centralized power generation by providing appropriate awareness among the farmers and crop cultivators to initiate the production at pilot scale so that more research could be done on the hurdles coming in the way to progress the production of biodiesel and more ways could be explored for a complete utilization of LCW. In accordance to biofuel production, Pakistan Agricultural Research Council (PARC), Islamabad has launched many projects regarding to second generation biodiesel production from oil-crops like jatropha, salicornia and castor oil requiring no agricultural land and less water. The technology is cost effective as compared to ethanol production. Soon in coming few years when these projects would be in progress then it would be more easy to produce biodiesel using LCW that are majorly wasted and only one third is used in burning purposes by the rural population (PSC (Science Technology: Plant Biotechnology- Sustainable Bioenergy for Pakistan, 2011; Kurian et al., 2013; Anwar et al., 2014).

Regarding to biofuel significance in Pakistan, the country has the potential to produce all types of energy including solar, wind, tidal, thermal, biogas, geothermal, biodiesel and biomass/biowaste energy, out of which biodiesel or biomass/biowastes energy production is under main progressive execution, because if Pakistan become self-sufficient in biodiesel production then the country could be among one of the developed nations and be able to withdraw US $ loans up to 60% by saving foreign exchange utilized for energy fulfillsments (Sheikh, 2010). According to ministry of petroleum and natural resources by the Government of Pakistan, having highly favorable environments if Pakistan utilizes all uncultured land for biodiesel production then it would be able to produce 56 million tons of biodiesel per annum, however, the annual consumption including all sectors is just 8.5 million tons. Main consumer sector of petro diesel in Pakistan is transportation and power sector including all the industrial machinery with heavy engines. As modern economy is totally dependent upon the availability of cheap fuel and biodiesel is the only reciprocal petroleum fuel having same efficiency as fuel obtained from crude oil. Cheap fuel could be attained from cheap raw- materials otherwise there is no future of diesel engine after the depletion of fossil fuels (Khan & Dessouky, 2009).

Pakistan produces 69 million tons of only field based crop residues including wheat straw, rice husk, cotton sticks, maize stalks and sugarcane
While industrial based crop residues and animal manure have been excluded from this calculation. These residues are not utilized beneficially and are wasted because of lack of their demand (FAO). This waste biomass is a low cost raw material for biofuel production and comprises of 60-70% of the total crop. So this abundant residual biomass can be utilized in the production of biodiesel to decrease the import of petro fuels and ensure an extra income of 200-300$ per acre annually per farmer. Hence the crop residues are considered viable options for Pakistan to cope energy crisis (DAWN News, 2009).

Being a clean and low-emission fuel biodiesel with its higher octane number is also advantageous for increasing engine efficiency and life. Another sustainability of SCO biodiesel (methyl esters) production is that the biolipids produced are reacted with ethanol to form esters and glycerol which in the presence of acid or alkali catalyst gives higher yields. Use of ethanol is favorable as per annum production of ethanol in Pakistan has been quantified as 400,000 tons from 2 million tons of molasses in 21 distillery units reported by ministry of industries, Production and initiatives, Pakistan. The ester produced is viscous enough to replace petro diesel leaving no need to modify the present engine manufacturing technology (Khan & Dessouky, 2009). Thus due to the abundant availability of waste derived feedstocks for cultivating oleaginous yeasts, climatic favors and availability of ethanol from local sources to process the biolipids into biodiesel and ease in use of the biofuel in the diesel engines without any modification are suffice to advocate strongly for initiating development project in this sector in Pakistan. It can be visualized as fortunate that big cities of Pakistan release millions of tons of industrial as well as household, cattle and poultry wastes each year. Due to less developed solid waste management facilities, heaps of such wastes can be observed around industrial and suburban areas. Such dumps and open heaps have caused serious health problems to the local people. Lack of awareness about the proper management and disposal of these wastes and their utilization as potential energy source is one of the main barriers to promote renewable energy sector in Pakistan. Keeping in consideration all these aspects of energy subsidy in Pakistan there is an immense need to utilize all the gifted facilities in a proper way to put the country in the list of developed nations. **Economization and commercialization of yeast biodiesel production**

To economize and commercialize a biotechnological process it is necessary to make it cost-effective with abundant supply of feed stocks required to run the process. The cost of biodiesel production almost doubles the cost of petroleum diesel. This is so because of the high price of feedstock and their transport to biodiesel production plants. Biodiesel has many advantages over petroleum diesel because of its environmental benefits with its biodegradable nature, reduced or none sulfur and aromatic contents and lesser toxic emissions (Demirbas, 2005 a.b). But still the cost of biodiesel production hinders the process to be commercialized. Since far, vegetable oils and non-edible oil seeds like jatropha, jojoba etc. have been used to cope the diesel crisis but the cost for transesterification and purification alter the feedstock to be introduced commercially. Another main drawback is the availability of feedstock. All the above mentioned feed stocks are the seasonal crops and only available in the season. Further with the passage of time the food and feed requirements are increasing with the increase in population. This scenario will soon lead to the lack of agricultural and cultivatable land too. Therefore there is need to focus to economize the microbial oil production towards commercialization. Microalgal biodiesel production is on road towards commercialization but the doors shut when there comes the maintenance and land availability. Therefore, the focus should be transferred to the SCO production by yeast. Further SCO derived biodiesel from yeast could be viewed as an additional member of the co-product list so that the cell mass and the byproducts could be used as low-profit food or feed supplements as shown in the fig., 2.
The cost of yeast oil production has been estimated on an average of US$ 3,000 per ton recently excluding the cost of feedstock which is not economically viable till the increase in the prices of petroleum and vegetable oils further (Ratledge & Cohn, 2008). Another recent estimate by China claims the cost of SCO production from yeast and microalgae using lignocellulose biomass as US$ 1230 per ton including the cost of feedstock (Haung et al., 2013). This process will lead to much more benefits towards the industrialization if considered the following facts:

i. Non-pathogenic strain selection in case of yeast.

ii. Economization of the cost of lignocellulosic biomass hydrolyzate with minimum inhibitors production.

iii. Development of a genetically modified strain to tolerate the inhibitors during sugars fermentation.

iv. Valorization of the process end products other than the lipids such as biomass and glycerol making an improvement in economic viability of yeast biodiesel production process reducing the cost of additional supplements.

v. Lowering the cellulose enzyme cost to produce hydrolysates.

vi. Year-round production.

vii. Selection of Yeast strain able to grow without costly nutrients like Vitamins could also reduce the production cost of biodiesel. Recently, seven out of nine known species from basidiomycetous yeast of genus Rhodosporidium can grow on vitamin-free media and on a total account 38 out of 48 known oleaginous yeast strains can grow in vitamin-free media (Sampaio, 2011 & Sitepu et al., 2014).

**Construction of an economic process to improve yeast biodiesel production**

The cost of biodiesel production is strongly influenced firstly, by the nutrients needed for the cultivation of yeast up to 50% and secondly, by the solvent needed for the extraction and transesterification of lipids (25%) into useable biodiesel. SCO production from yeast needs higher improvements to become economically viable process. These improvements include the cost reduction relative to the production, transport, pretreatment and hydrolysis of the feedstock. This production cost could be economized by creating a consortium based process of lipid production or by...
using crude enzymes of bacteria to hydrolyze the lignocellulosic feedstock to become available for fermentation by yeasts. This natural remedy will reduce the cost of hydrolysis, pretreatment and detoxification of lignocellulosic biomass instead of using chemical based treatments. The recycling of waste lignocellulosic material and industrial effluents will also reduce the cost of waste disposal techniques. For further improvements the cost relevant to aeration and pH should also be controlled. Fig. 3. Illustrates the consortium based bioprocessing idea for yeast biodiesel production.

**Fig., 3:** Diagrammatic illustration of consortium based fermenter for oil recovery and yeast extract production

As a strong evidence of industrial interest in oleaginous yeast California- based Oil Company, Solazyme has been busy in applying several patents on use of yeast oil for food, chemical and fuel ingredients (Franklin et al., 2011; Trimbur et al., 2012). Other cost relevant improvements to the consolidated bio-processing of biodiesel production may include the improvement of the metabolic pathways in the selected yeast strains genetically. These improvements includes the incorporation of cellulase producing genes from bacteria, high oil and cell mass production, osmotolerance as well as inhibitors resistance, fast growth, simultaneous utilization of glucose as well as xylose to avoid time constrain, simultaneous fermentation and scarification and allowing desired array of fatty acid metabolism (Liu et al., 2012). Another way to economize the process of biodiesel production from yeast is to divert the carbon sources towards wastes such as municipal wastes, industrial wastes majorly glycerol from brewery industry, waste vegetable oils and other carbonaceous wastes (Fakas et al., 2009a, b). This is so because the 75% cost of biodiesel production depends on the feedstock. Once the feedstock is economized then this process is no more far from commercialization in next few years. *Lipomyces starkeyi* was checked for its biodiesel production in starchy wastes from sweet potato processing industry as sole source of carbon by Patil (2010). The economic analysis resulted in the production of microbial lipids produced at a factory gate price of $2.30 per gallon. This could support the biodiesel price at $3.00 per gallon with a
continuous subsidy of $1.00 per gallon which is quite reasonable to become commercialized.

CONCLUSION

From the above discussion it is concluded that there is a massive need to become self-sufficient in biofuel production. This is so because the development and economy of every nation is a direct reflection of its energy sources and their useful exploitation. To attain the target, the feedstock should be abundant and cost-effective. Lignocellulosic and industrial wastes could be used in biodiesel production processes as they are produced in abundance and problem of their disposal will be solved by recycling them. Further SCO from yeasts are same as vegetable oils so they are proved to be appropriate pre-biodiesel oils. The fermentation processes could be made lucrative by overcoming the cost consuming steps like pretreatment of waste substrates. This could be done so by consortia between valuable yeast and cellulolytic bacteria or by using bacterially saccharified sugar syrups of wastes as biologically treated substrates to produce yeast origin biolipids. To take the biodiesel production up to maturation in Pakistan the need is just to provide the general awareness to the local industrialists for sustainable production of biodiesel at domestic levels.

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