Taxonomic Studies of Calliptaminae and Coptacridinae (Acrididae: Orthoptera) in Uttar Pradesh (India)

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Abstract
Survey was made to collect the grasshoppers from different ecosystems in Uttar Pradesh, India, during the period of 2011-2013. Grasshoppers are the members of family Acrididae, which constitute an important group of pests causing significant damage to agricultural crops, grasslands and pastures all over the world. *Acorypha insignis* of the subfamily Calliptaminae and *Eucoptacra praemorsa* of the subfamily Coptacridinae have been recorded from Uttar Pradesh. Grasshoppers of these subfamilies are regarded as minor pest of agricultural crops and also destroy the vegetations. Both the grasshoppers are extensively found in mixed vegetations of herbs, shrubs and grasses and causes damage to agricultural crops and grasses through defoliation.
Keywords: Taxonomy, Calliptaminae, Coptacridinae, Acrididae, grasshoppers, Uttar Pradesh, India

Introduction

Orthoptera is one of the largest order of insects, constituting 26,330 valid species and are found throughout the world (http://Orthoptera. SpeciesFile.org>. Dated 20.3.2014) and out of that 1033 species, 400 genera and 21 families are known from India (Shishodia *et al*., 2010). The Order is divided into two suborders *i.e*. Caelifera called short horned grasshoppers and Ensifera called long horned grasshoppers (Ander, 1939). Acrididae is the family under the Caelifera called grasshoppers and locust, comprising 8,000 species around the world and out of that136 species and 28 genera are endemic (Chandra and Gupta, 2013).

In general members of the family Acrididae are called grasshoppers and locusts. Grasshoppers are medium to large sized insects found all over the world and best known for their ability to jump incredible heights and distances. They can migrate over long distances when the weather gets too cold. They live in grassy areas such as fields and meadows and forest and woodland. Atennae of the grasshopper are known to be remarkably long in order to make sense of their surroundings. Grasshoppers have six jointed legs that are incredibly powerful for such a small creature, as grasshoppers are able to jump extraordinary distances. The two back legs of the grasshopper are long and powerful and are just for jumping, where the four front legs of the grasshopper are primarily used to hold onto prey and to help it to walk. Despite their large size, grasshoppers are herbivores and have a diet that consists solely of plant matter. Grasshoppers eat grasses, weeds, leaves, shrubs, bark and numerous other species of plants that surround them. They cause considerable damage to agricultural crops, pastures and forests (Joshi *et al.,* 1999). The primary diet for grasshoppers are grasses and forbs (Behmer and Joern, 1993). It is primarily graminivorous, feeding on several common grasses and sedges (Mulkern, 1967).

Grasshoppers belonging to subfamily Calliptaminae not recorded as a major pest from the country, they cause minor damage to agricultural crops thus regarded as minor pest. The taxonomy of these grasshoppers have been done by Kirby (1914), Uvarov (1943), Bei-Beinko and Mishchenko (1951), Termier (1991), Soomro and Wagan (2005) Indian Sub-Continent. Recently taxonomy of these species have been done by Usmai *et al*., (2010) from Western Uttar Pradesh, Usmani and Nayeem (2012) form Bihar, Nayeem and Usmani from Jharkhand (2012),

Kumar and Usmani (2013) from Rajasthan and consolidated study of the subfamily in Uttar Pradesh not known and present authors tried to find out these grasshoppers in order to make the record up to date.

Members of the subfamily Coptacridinae also have been described by Kirby (1914), Bei-Beinko and Mishchenko (1951) from Sub Continent whereas Chandra *et al*., (2007), Shishodia and Gupta (2009), Usmai *et al*., (2010), Usmani and Nayeem (2012), Nayeem *et al*., (2013), Shishodia *et al*., (2010) and Chandra and Gupta (2013) recorded from different parts of country. Above study indicates that subfamily Calliptaminae and coptacridinae has not been subject to recent study thus taxonomy and distribution of these species should be undertaken in order to make data of Uttar Pradesh, India, up to date.

Uttar Pradesh located at 26.8500° N, 80.9100° E has a humid temperate climate, demarcated into three distinct regions: the Himalayan region in the north, the Gangetic plains in the centre and the Vindhya hills and plateau to the south. The state is bordered by Rajasthan to the west, Haryana and Delhi to the northwest, Uttarakhand and the country of Nepal to the north, Bihar to the east, Jharkhand to the southeast, and Madhya Pradesh to the southwest. The climate varies from moderately temperate in the Himalayan region to tropical monsoon in the central plains and southern upland regions. In the plains, the average temperatures vary from 12.5°C to 17.5°C in January and 27.5°C to 32.5°C in May and June. Rainfall in the state ranges from 40-80 inches in the east to 24-40 inches in the west. It is the second largest state of India by economy, the leading sector is agriculture and majority of the population depends upon farming as its main occupation. The western region of the state is more advanced in terms of agriculture. Majority of the population depends upon farming as its main occupation. Wheat, rice, sugar cane, pulses, oil seeds and potatoes are its main products.

Taxonomic and ecological work on the grasshoppers fauna of Aligarh Fort have been done by Akhtar *et al*., (2012) and ecological work done by Usmani *et al*., (2012) whereas taxonomic and ecological work on the grasshoppers fauna of Aligarh have done by Usmani *et al*., (2012), Pulses of Uttar Pradesh by Usmani *et al*., (2012), rice ecosystem of Uttar Pradesh by Akhtar *et al*., (2012), Singh and Singh (2014) from Eastern Uttar Pradesh (Rafi and Usmani , 2013) from Poorvanchal region of Uttar Pradesh but there is not any description of these two species, it is therefore vital to make the study on the taxonomy of these species to update the record.

Material and Methods

A. Collection and killing

During survey in Uttar Pradesh, authors collected *Acorypha insignis* and *Eucoptacra praemorsa* from agricultural fields and grasses. They were caught by the ordinary aerial insect net and through hand picking as well. The collected specimens were killed in bottles having soaked cotton with ethyl acetate.

B. Identification

Specimens were identified with the help of binocular stereoscopic microscope (Nikon SMZ 1500) upto species level on the basis of characters like size, colour and texture, and available literature and keys. Thereafter, specimens were relaxed stretched and pinned on stretching box, and left for three days to dry to avoid odour.

C. Morphometry

Measurement in mm of four important differentiating parts of body (Body length, pronotum, tegmina and hind femur) has been done with the help of Vernier Calliper. Mean value, Standard Deviation of male and female of both the species.

D. Genitalic studies

For these studies apical parts of male and female were cut off and boiled in 10% KOH, clearing was done in clove oil and were mounted separately on cavity slides in Canada balsam. Slides were examined under the microscope

and drawings of the structures (Supra anal plate, Sub genital plate, Epiphallus, Aedeagus, Ovipositor and Spermatheca) were made with the help of Camera Lucida of the conventional microscope.

E. **Preservation**

Pinned specimens labeled with reference number, locality, date of collection and name of host plants were kept in store boxes and cabinets for further studies on their morphological structures. Naphthalene balls were kept in boxes to prevent decomposition of dry specimens and for wet preservation specimens are stored in plastic vials using 70 % ethyl alcohol.

Results

One species of grasshopper of the subfamily Calliptaminae i.e., *Acorypha insignis* and another species i.e., *Eucoptacra praemorsa* of the subfamily Coptacridinae have been recorded for the first time from Uttar Pradesh.

Taxonomic Account

***Acorypha insignis* (Walker, 1870) (Fig. 1)**

Caloptenus insignis Walker, 1870. *Catalogue of the Specimens of Dermaptera Saltatoria in the Collection of the British Museum*.4:701.

Caloptenus spissus Walker, 1871. *Catalogue of the Specimens of Dermaptera Saltatoria in the Collection of the British Museum Supplement.* 70. Syn.by Kirby, 1910. *A ynonymic Catalogue of Orthoptera (Orthoptera Saltatoria, Locustidae vel Acridiidae).* 3 (2): 551.

Acorypha insignis (Walker); Nayeem & Usmani. 2012. *Munis Entomology & Zoology.* 7(1):410.

Acorypha insignis (Walker); Kumar, H. & Usmani. 2014. *Journal of Entomology and Zoology Studies* 2(3):134

Diagnostic characters: Body medium sized; pronotum apparently smooth, metazona finely tectiform and considerably longer than prozona, median carina prominent, crossed by two transverse sulci; mesosternal interspace open, lobes rounded, wider than long, lower margin considerably angular; antennae uniformly filiform, nearly equal to head and pronotum together; fastigium of vertex elongate, narrow, sloping downwards, parabolic, with weak longitudinal concavity bordered by lateral carinulae, carinula of vertex easily perceptible; tegmina fully developed but comparatively shorter, more distinctly narrowed distally, discoidal area hyaline beyond middle, apex round; wings hyaline; arolium small.

Distribution: India: Bihar, Jharkhand, Madhya Pradesh, Rajasthan, Uttarakhand, Uttar Pradesh and West Bengal.
Elsewhere: Saudi Arabia, Oman and Pakistan.

Material Examined: India: Uttar Pradesh: Ghazipur, 2♂,1♀, 09-X-2010, On grasses; Deoria, 3♂,1♀, 12 -X-2010, On grasses; Kushinagar, 1♂,2♀, 13 -X-2010, On grasses; Gorakhpur, 4♂,2♀, 14 -X-2010, On grasses; Faizabad, 1♂,1♀, 24-X-2010, On grasses; Sultanpur, 2♂,4♀, 25 -X-2010, On grasses; Hamirpur, 3♂,2♀, 04 -IX-2011, On grasses; Fatehpur, 3♂,4♀, 11 -IX-2011, On grasses; Meerut, 2♂,3♀, 21 -VIII-2012, On grasses; Saharanpur, 2♂,2♀, 23-VIII-2012, On grasses.

Male: Supra-anal plate acutely angular, longer than wide with attenuate apex; cercus robust, incurved, longer than supra-anal plate with bilobate apex; sub-genital plate short, triangular, wider than long, basally broad with obtusely rounded apex; epiphallus bridge shaped, wide and undivided, ancorae small, lophi absent; aedeagus flexured, apical valve narrow, apex acute, connected with basal valve with flexure, basal valve slightly broad basally, narrowing towards its obtuse apex (Plate 1, A-D).

Female: Supra-anal plate angular, longer than wide, apex obtuse angular; cercus short and conical, apex obtuse; sub-genital plate short with posterior margin not straight rather wavy, postero-lateral margin setose, egg-guide short, broad and narrowing apically, apex obtuse; spermatheca with apical diverticulum long, narrow, tubular with dilated apex, narrower and shorter than pre-apical diverticulum; pre-apical diverticulum long, broad and curved back. ovipositor with dorsal valve broad and curved, apical tip long, curved and blunt, external edge dentate while ventral valve broad and curved with apical tip small, curved and blunt (Plate 1, E-H).

Table 1. Morphometry of *Acorypha insignis*

Measurement (mm)	Male	Female	Mean ± SD	
			Male	Female
Body length	20.68-22.89	22.56-24.39	21.87±0.78	23.94±0.58
Pronotum	03.78-04.81	04.79-05.68	04.46±0.33	05.09±0.28
Tegmina	17.82-19.25	21.34-22.28	18.41±0.50	21.81±0.32
Hind Femur	16.71-18.37	18.45-19.54	17.62±0.61	18.88±0.44

Standard deviation of 0.33 in case of male pronotum, 0.50 in case of tegimna, 0.61 in case of hind femur and 0.78 in case of body length indicates that size of pronotum, hind femur, tegmina and body length are not of much variable and may vary with little fractions among individuals of the species. Standard deviation of 0.28 in case of female pronotum, 0.32 in case of tegimna, 0.44 in case of hind femur and 0.58 in case of body length indicates that size of pronotum, tegmina, hind femur and body length are not of much variable and may vary with little fractions among individuals of the species.

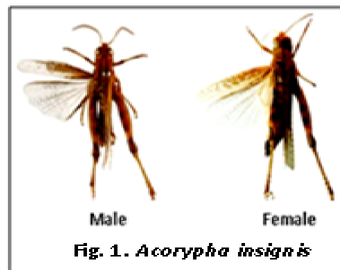

Fig. 1. *Acorypha insignis*

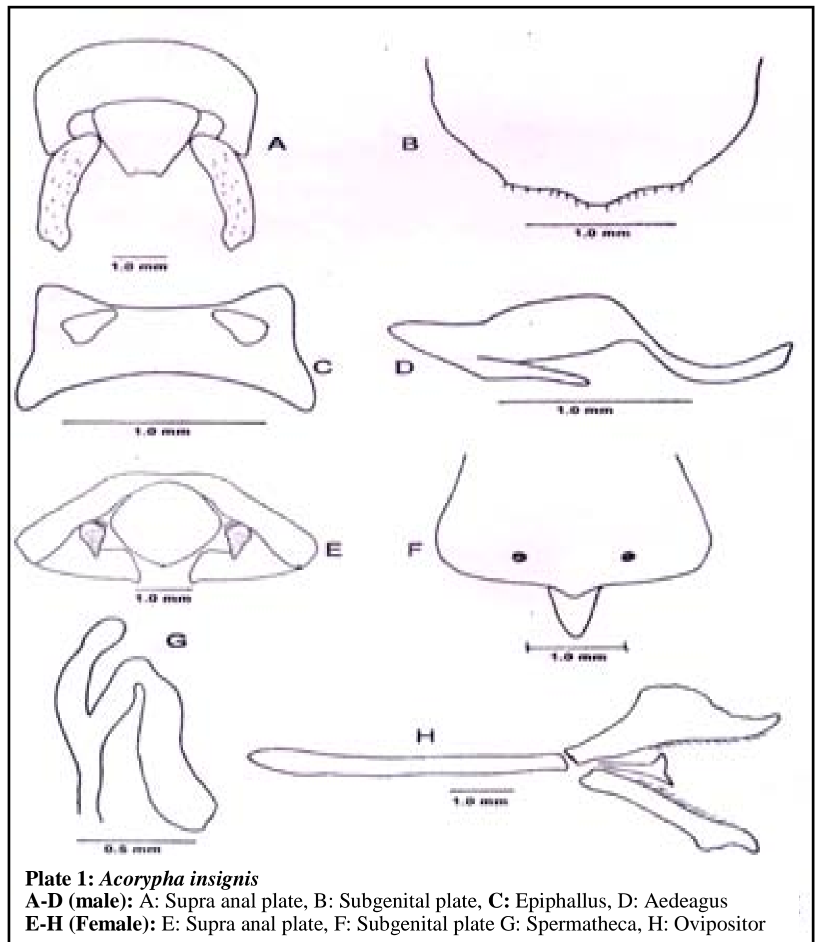

Plate 1: ***Acorypha insignis***
A-D (male): A: Supra anal plate, B: Subgenital plate, **C:** Epiphallus, D: Aedeagus
E-H (Female): E: Supra anal plate, F: Subgenital plate G: Spermatheca, H: Ovipositor

***Eucoptacra praemorsa* (Stal, 1861) (Fig.2)**

Acridium (Catantops) praemorsum Stal, 1861[1860]. *Kongliga Svenska fregatten Eugenies Resa omkring jorden under befal af C.A. Virgin aren 1851-1853 (Zoologi)*. 2 (1): 330.

Acridium saturatum Walker, 1870. *Catalogue of the Specimens of Dermaptera Saltatoria in the Collection of the British Museum*. 4: 704. Syn. by Bolivar, 1917. *Rev. Real Acad. Cienc. Exact., Fisic. Natur.* 16: 404.

Caloptenus obliterans Walker, 1870. *Catalogue of the Specimens of Dermaptera Saltatoria in the Collection of the British Museum*. 4: 712. Syn. by Bolivar, 1917. *Rev. Real Acad. Cienc. Exact., Fisic. Natur.* 16: 404.

Caloptenus sinensis Walker, 1870. *Catalogue of the Specimens of Dermaptera Saltatoria in the Collection of the British Museum*. 4: 704. Syn. by Bolivar, 1917. *Rev. Real Acad. Cienc. Exact., Fisic. Natur.* 16: 404.

Caloptenus strigifer Walker, 1871. *Catalogue of the Specimens of Dermaptera Saltatoria in the Collection of the British Museum Supplement*. 66. Syn. by Bolivar, 1917. *Rev. Real Acad. Cienc. Exact., Fisic. Natur.* 16: 404.

Coptacra cyanoptera Brunner, 1893. *Ann. Mus. Civ. Stor. Nat. Genova*, 2 [13] (33): 159. Syn. by Bolivar, 1917. *Rev. Real Acad. Cienc. Exact., Fisic. Natur.* 16: 404.

Eucoptacra praemorsa (Stal); Nayeem & Usmani. 2012. *Munis Entomology & Zoology*. 7 (1): 401.

Diagnostic characters: Body medium sized; antennae filiform, longer than head and pronotum together; head sub-conical; fastigium of vertex wide, slightly depressed; frontal ridge wide; pronotum rugose, dorsum crossed by three transverse sulci; prosternal process small, conical with obtuse apex; mesosternal interspace open, wide, lobes rounded, slightly wider than long; metasternal pits deep and not so close; tegmina fully developed; wings slightly parchment-like rather than hyaline, wingspan wide; hind femora widened basally, abruptly narrowed distally, upper carina serrated with black tipped spines, lower carina smooth, external upper carinula sharp with intermittent tubercles in middle part, lower one comparatively thick or obtuse and smooth; hind tibiae straight, moderately hairy; arolium of medium size.

Distribution: India: Andhra Pradesh, Arunachal Pradesh, Assam, Chhattisgarh, Himachal Pradesh, Jharkhand, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Manipur, Meghalaya, Orissa, Sikkim, Tamil Nadu, Tripura, Uttar Pradesh, Uttarakhand and West Bengal. **Elsewhere:** China, Myanmar, Taiwan and Tenasserim.

Material Examined: India: Uttar Pradesh: Allahabad, 2♂,3♀, 06-X-2010, On grasses; Azamgarh, 3♂,3♀, 08 -X-2010, On grasses; Kushinagar, 2♂,3♀, 13 -X-2010, On paddy & grasses; Sultanpur, 2♂,3♀, 25 -X-2010, On pulses & grasses; Jhansi, 3♂,2♀, 01 -IX-2011, On forest & grasses; Lalitpur, 3♂,3♀, 02 -IX-2011, On forest & grasses; Hamirpur, 4♂,3♀, 04 -IX-2011, On paddy & grasses; Jalaun, 1♂,2♀, 05 -IX-2011, On grasses; Kanpur Dehat, 2♂, 2♀, 06-IX-2011, On grasses; Meerut, 3♂,3♀, 21-VIII-2012, On grasses; Saharanpur, 4♂, 3♀, 23-VIII-2012, On grasses.

Male: Supra-anal plate longer than wide with medially curved lateral margins, apex round; cercus compressed laterally shorter than supra-anal plate with obtusely rounded apex, sub-genital plate short with obtusely rounded apex, epiphallus bridge narrow and divided medially, ancorae large and incurved, anterior projection broad with obtuse apex, posterior projection broad with rounded apex, lophi large and lobiform; aedeagus flexured, apical valve narrow, apex obtuse, connected with basal valve with flexure, basal valve slightly broad, narrowing towards its apex (Plate 2, A-D).

Female: Supra-anal plate longer than wide with obtuse- angular apex; cercus short and conical; sub-genital plate broad, posterior margin angled medially, setose marginally; egg-guide short with pointed apex; spermatheca with long apical diverticulum, slightly bent apically, pre-apical diverticulum tubular, bent in the middle; ovipositor with dorsal valve long and broad, longer than lateral apodeme, apical tip long and blunt, ventral valve narrow and curved, mesial valve curved apically with acute apex (Plate 2, E-H).

Table 2. Morphometry of *Eucoptacra praemorsa*

Measurement (mm)	Male	Female	Mean ± SD	
			Male	Female
Body length	16.89-18.22	21.94-23.40	17.70±0.52	22.63±0.51
Pronotum	03.71-04.24	04.12-04.58	03.91±0.18	04.37±0.15
Tegmina	18.48-19.22	22.54-23.62	18.84±0.31	22.74±0.36
Hind Femur	10.56-11.72	14.62-15.95	11.19±0.47	15.32±0.58

Standard deviation of 0.18 in case of male pronotum and 0.31 in case of tegimna 0.47 in case of hind femur and 0.52 in case of body length indicates that size of pronotum, tegmina, hind femur and body length are not of much variable and may vary with small fractions among individuals of the species. Standard deviation of 0.15 in case of female pronotum and 0.36 in case of tegimna 0.58 in case of hind femur and 0.51 in case of body length indicates that size of pronotum, tegmina, hind femur and body length are not of much variable and may vary with small fractions among individuals of the species.

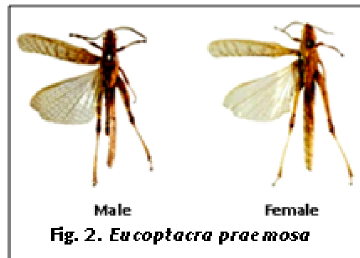

Fig. 2. ***Eucoptacra praemosa***

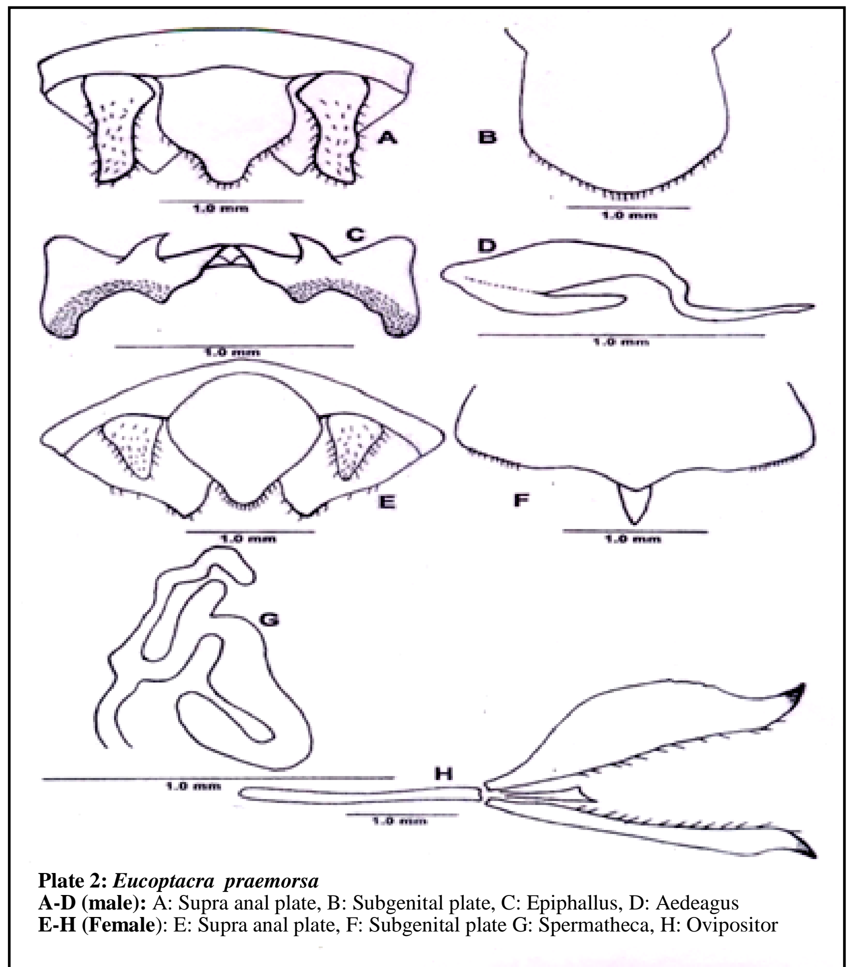

Plate 2: ***Eucoptacra praemorsa***
A-D (male): A: Supra anal plate, B: Subgenital plate, C: Epiphallus, D: Aedeagus
E-H (Female): E: Supra anal plate, F: Subgenital plate G: Spermatheca, H: Ovipositor

Discussion

Grasshoppers feed on plant foliage, with a particular fondness for grasses and spurges. When grasshoppers populations increase to the point of crowding, can completely defoliate grasslands and agricultural crops over large areas. They are dominant ground invertebrates in cultivated crops and natural vegetations, cause considerable damage to agricultural crops, pastures and forests and are well reputed for their destructiveness all over the world. The primary diet for grasshoppers are grasses and forbs. It is primarily graminivorous, feeding on several common grasses and sedges. Three special type of vegetation namely Savanah, Tropical rain forest and Alpine forest, faster the grasshopper population.

There is no previous record of theses grasshoppers from Uttar Pradesh. Present study indicates that these species are frequently distributed throughout the state in grasses and paddy fields. When present in large numbers they feed on leaves and severe defoliation is caused , followed by arrested growth and size of plants, which results in low yield or no yield at all. Hoppers are more dangerous than adults and no more differences observed among them except wing, hoppers are usually wingless which later transforms into developed wings. Population of these species of grasshoppers fluctuates on first shower of monsoon in the month of June/July relatively becomes low with decreasing temperature from the month of November. In the present study taxonomy and mode of damage have been discussed and distribution revealed that these grasshoppers extensively found in grasses than crops. On the absence of grasses feeds upon crops, thus cultivation techniques should be modified in such a way that grasses which support population of grasshoppers may be grown around the crop field to reduce damage.

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Effect of Monocrotophos on Histopathological Changes in Gills of an Air Breathing Fish *Channa gachua* (Ham.)

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Abstract

Gills are the most vital organs in all aquatic animals including fishes which carry out respiration and osmoregulation. The gills are the first target site organ which is affected severely on exposure to various aquatic pollutants as they are continuously bathed in the surrounding water. Since gills have a key role in the transport of oxygen for the metabolic activities, hence they serve as an ideal material for studies on the effect of toxic pollutant on respiration. Thus, the study of gill pathology is important in understanding the biological response of aquatic animals to a variety of aquatic pollutants. *Channa gachua* is a fresh water fish, but occurs also in muddy water. In aquatic resources, fishes are sensitive to the toxic substances mixed into water and deleterious effect of metals or heavy metals on fishes. The toxic substances caused damage to the organism and degree of the cell damage reflects the various concentrations of the pollutants. In the present study an attempt has been made to study the effects of monocrotophos, an industrial as well agricultural effluent on *Channa gachua*. 50% mortality was exposure to monocrotophos on 0.4 ppm at 96 hours respectively. The impact of monocrotophos on the gill of *Channa gachua* showed gill filaments were twisted and primary axis was in filtered. The gill exhibited a film of coagulated mucous over the gill surface.

Keywords: Monocrotophos, histopathology, toxicity, *Channa gachua.*

Introduction

Oxygen, the most primary necessity for all living organisms for survival and it provides energy for vital activities of organisms. The toxic substance alter the chemical properties of aquatic body, thereby brings about behavioural, physiological and biochemical changes in fishes. The organophosphate insecticides are liquids of liphophilic character, and some volatile and a few are solids. The organ phosphorous inhibits the variety of esterases but associated with cholinesterase inhibition. Increasing industrialization leads to continuous addition of harmful pollutants in the environment especially in water. Monocrotophos, one of the organophosphate is becoming the serious pollution threats to public health. The insecticides were affected the aquatic ecosystem, especially fishes. This biochemical changes inhibited the slow blood flow as compared to the cardiac output as well as association of hepatocytes in mammal (Hinton and Lauren, 1990).The domestic sewage, agricultural pesticides, industrial waste are harmful to the threatened status of fishes or aquatic life. The fish population was hampered because of the daily used agricultural pesticides. The world wide uses of chemicals are very hazardous to high risk of toxicity and environmental pollution of the other organism (Rao *et.al.*, 2005). The polluted water is not suitable for drinking by physico chemical properties and microbial activities which shows microbial content infection. The deeper depth of water should carry out at the tar sand in which presence of potential elements (Parihar *et.al.*, 2012; Odunaike *et.al.,* 2013). Pollution has been occurred by most of the human interference and daily used products which are hazardous to the environment (Abbai and Sunkad, 2013). Uncontrolled discharged of pollutants into any water body degrades the water quality to such an extent that it produced lethal effects on the fish fauna. The extent and degree of their harmful effect on fish can be gauged to a greater extent by experimental studies in a laboratory

(Sonaraj *et. al.*, 2005). In the environment, metals are anthropogenic sources and natural spectrum (Sajid and Muhammad 2006). The submerged and industry less zone surrounding supply of water was tolerant to fish cultivation (Hossain *et.al.,* 2013) the water conductivity total dissolve oxygen (TDS) and carbon oxygen demand (COD) was significant (Kushwah *et. al.,* 2012). Fishes come in contact to the various metals which are very hazardous to the aquatic environment. The aquatic life, fishes, are highly sensitive to a toxic substances present in water and deleterious effects of the metals on fishes can be easily established (Ayyappan, 2000; Revathi *et.al.,* 2003; Baird and Girard 1853; Wooten *et.al.,* 1988; Fuller *et.al.*, 1999). The *Channa gachua* is introduced as a bio-control for various countries and is commonly present in ponds and lakes and are easy to maintain in the aquarium. Gills perform various functions like respiration, osmoregulation and excretion of nitrogenous wastes. Hence gills are an important biomarker of water pollution and good indicator of water quality. The histopathological studies of gills in *Channa gachua* on exposure to malathion shows primary makers gills are aquatic pollution (Cengiz and Unlu, 2001; 2003).

Materials and Methods

Live specimens of *Channa gachua* were procured from local fish dealers at Hazaribag (Latitude 25º 59'N and Longitude 85º 22'E) and maintained in large glass aquaria size (90x60x60cm) with continuous flow of water. The specimens were fed on chopped goat liver daily during a minimum acclimation period of 15 days in the laboratory. Thus the fish could exchange gases with water by way of its gills as well as with the air using the supra branchial chamber. The fish were acclimatized to the respirometers for at least 12 hours before the readings were taken. The experiments were conducted at 29.0 ± 1.5°C. However, sexually mature fishes of almost same weight group (40-50g) were used. The water was stored for 15 days so as to be free from chlorine. The aged water was used for acclimatization and for making test solution. After acclimatization, fishes were collected, weighed on weighing balance and divided into six groups having ten fishes, out of six groups, one group was considered for control and remaining five groups were exposed to 0.2, 0.4, 0.6, 0.8 and 1.0 ppm concentration to chronic duration of 24, 48, 72 and 96 hours (Pandey *et al*., 2011). The tests were carried out again for about 10 times and the results were calculated. After stipulated time, the fishes were sacrificed, tissue was fixed in 10% formalin and processed for histological studies (Godkar and Godkar , 2003).

Results and Discussion

The impact of monocrotophos on the mortality of *Channa gachua* shows that 0.4 ppm at 96 hours exposure, the gills exhibited a film of coagulated mucous over the gill surface (figure-4). The gills of *Channa gachua* showed lamellar epithelial cells changed, twisted tips of gill filaments and primary axis infiltration of cells respectively (figure-2 and 3). The epithelial cells of secondary gill filaments also degenerated by changed effect of monocrotophos (figure- 1 and 2). Also, fusion and shortening of secondary lamellae was observed (figure-3). Edematous separation of gill epithelial and desquamated secondary lamellae was observed (Figure-2). Degenerated secondary lamellae and pycnotic nuclei were also observed. All the changes were pronounced in 0.4 ppm at 96 hours monocrotophos exposed in *Channa gachua* depend on the quantity of the pollutants reaching the gill ventilation volume as well as concentration of the pollutants in the water (Llyod, 1960). Since gills are continuously bathed by polluted water that brings more pollutants in contact with gill epithelium due to heavy metal salts, detergents, and phenol toxicity (Hughes and Morgan , 1973) respectively.

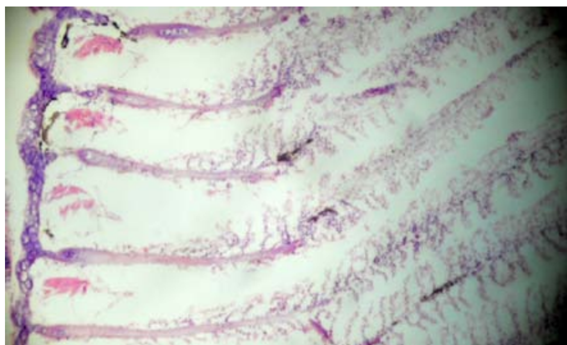

Fig. 1. Control gills showing, secondary gill filament (SGF), respiratory gill filament (RGF), primary axis (P) and respiratory filament (RF) in *Channa gachua*.

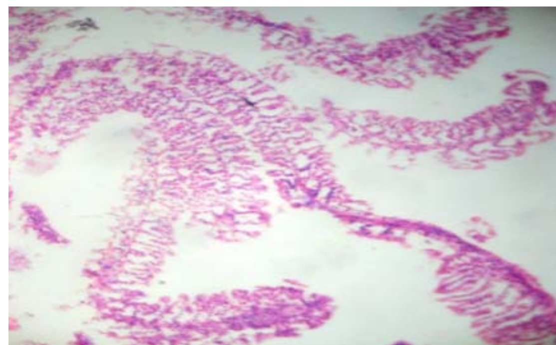

Fig. 2. Experimental gills showing secondary gill filament (SGF) and respiratory filament (RF) in *Channa gachua*.

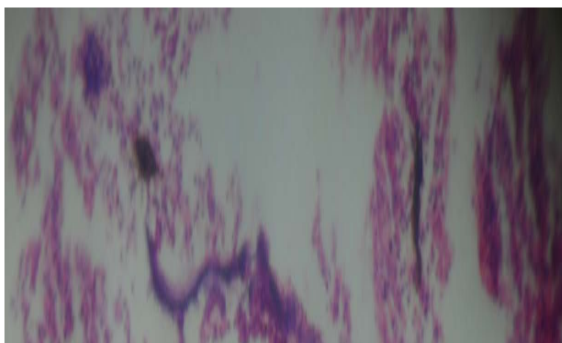

Fig. 3. Gill showing, hemorrhages in secondary gill filaments (HGSGF) and necrosis in secondary gill filaments (NGSGF) in *Channa gachua*.

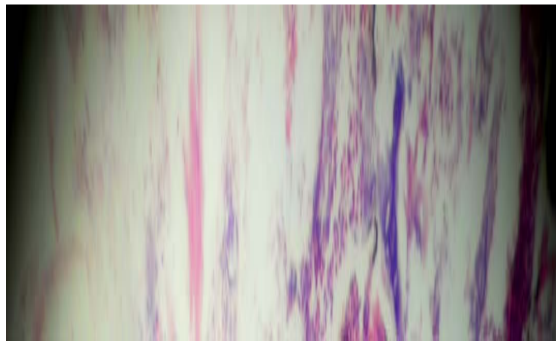

Fig. 4. Gill showing, clubbing of secondary gill filaments (CSGF), severe necrosis in inter lamellate space (SNILS) and separation of epithelial layer in SGF and infiltration of respiratory lamellae in *Channa gachua*.

In the present investigation the monocrotophos induced architectural changes in gill of *Channa gachua*. It includes bulging of lamellae and structural disorganization of primary gill lamellae. There is fusion and destruction of secondary gill lamellae, which is main seat of gaseous exchange. In respiratory epithelium hyperplasia is observed. Excess of mucus secretion and disorganization of gill lamellae took place. The fish exposed to monocrotophos indicates that disturbance in proper gaseous exchange and also affect osmoregulation. Swelling in the gill epithelia, unusual enhancement in the rate of mitosis which stimulate epithelial cells to give effect to bulged out or swollen condition. Vacuoles and damaged gill lamellae and damage to respiratory epithelium hematomas. The epithelial lesions observed on the respiratory surface, due to collapse of pillar cells, increasing number of mucous openings and mucus secretions are due to the hyperplastic condition. In gills clubbing, hyperemia, and edema were observed. The toxic substances or pesticides normally attacked the respiratory organ (Ferguson, 1967). If oxygen decreases, the gills probably consume more pesticide through the polluted water (Ferguson and Bingham, 1966). The intimate contact of the gills with the polluted water may lead to alterations in normal respiratory mechanism, lower the diffusion mechanism through the gill and thereby oxygen consumption is reduced, which creates a

physiological imbalance to the organism (Finney and Berman,1976). After some time they showed vigorous fin movement with fast swimming than normal. The fish remained at the corner of the bottom part of container with continuous gill or operculum movements. They loss balance and went deep by keeping head down in position and touching the bottom of container. If fishes were disturbed, they showed sudden body pulses. When they died they appeared slimier than control due to the secretion of large amount of mucus on their body. There are also little changes in body weight and water content of body due to susceptibility of fish to monocrotophos pesticide. Supportive cartilage was also observed in secondary gill lamellae, in primary gill filament of cartilage or fusion of supportive cartilage. Destruction of epithelial lining of gill lamella and supporting cartilage directly affect on the respiration of fish. Due to accumulation of monocrotophos in the nucleus of cells, it is densely staining and this can affect nuclear function. Due to the secretion of mucus which filled the space between gill filament and gill lamellae, ultimately affecting the gaseous exchange leading to stasis of blood and death of the fish. In support of this, done in laboratory conditions, proved that heavy metal mixtures cause the histopathological changes (Vinodhini and Narayanan, 2009) respectively. The present result concluded that the histopathological changes found in gills of the examined freshwater fish are typical for the clinical finding in aquatic habitats polluted with heavy metals. Influence of water pollution is not only devastating to human being, animals, insects but also aquatic organisms. The more polluted industrial water destroys the aquatic ecosystem and reduces its biodiversity. The decrease in the rate of oxygen consumption after exposure to monocrotophos is due to the sluggishness of the fish, as a result of the pesticide stress and also the secretion of excessive mucous, which formed a thin film over the gill thereby preventing absorption of oxygen during the process of gaseous exchange. The present study also suggests that, the monocrotophos pesticide is very harmful to the aquatic life especially to the fishes, and there is urgent need to control this water pollution.

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New Sphingolipid from G*ylcine max*

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Abstract
In a study of the chemical profiling of plants used in dietary supplements to investigate the possible misidentification and authentication, the methanolic extract of *Glycine max* was evaluated chemically; one new sphingolipid was isolated by normal phase liquid chromatography. The compound was characterized by spectroscopic techniques including 2D NMR spectroscopy.
Keywords: *Glycine max*, fabaceae, sphingolipid

Introduction

The relationship between diet and human health has become a very active area of research and debate. Identification and characterization of specific diet-derived chemicals, and an increased understanding of their biological activity have given rise to increased interest in the role for natural products in disease prevention and treatment (Willer, 1994). Legumes play a pivotal role in the traditional diets of many regions throughout the world (Messina, 1999). Soybeans are unique among legumes because they are a primary source of soysaponins, flavonoids and other secondary metabolites (Kitwaga *et al.,* 1985; *Shiraiwa et al*., 1991; Fliegmann *et al.,* 2010; Akihisa *et al.,* 1994; Jay *et al*., 1983; Berlag *et al.,* 1988). Plant-derived saponins are considered to play a significant role in plant defense systems against pathogens and herbivores. Numerous reports emphasize the fungicidal (Lee *et al*., 2001;), allelopathic (Waller *et al*., 1993), insecticidal (Nielson *et al*., 2010) and molluscicidal (Huang *et al.,* 2003) activity of various saponins. The presence of saponins in soybeans has also attracted considerable interest because of both their health benefits and adverse sensory characteristics. Soysaponins are the primary dietary sources of saponins from foods. Soysaponins have been demonstrated to possess multiple health-promoting properties, such as lowering of cholesterol by inhibiting its absorption, being anticarcinogenic, antihepatotoxic, promoting antiinfectivity of HIV, antimutagenic and immunostimulatory activities (Kinjo *et al*., 1998; Miyao *et al.,* 1998; Okubo *et al*., 1994; Baxter *et al*, 1990; Hu *et al.,* 2004; Berhow *et al*., 2000; Hostettmann and Marston, 1995). As a part of our programme to phytochemically investigate medicinal plant derived

dietary supplements, the methanolic extract of soybean led to the isolation of new sphingolipid (Fig. 1).

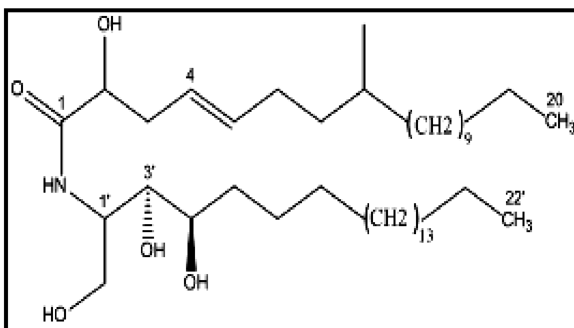

Fig. 1. Structure of compound 1

Material and Methods

General

NMR spectra were recorded on a Varian AS 500 NMR spectrometer instrument using TMS as internal standard. Chemical shifts were reported in δ units and coupling constants (J) in Hz. ESIMS was obtained on Agilent Series 1100 SL mass spectrometer. IR spectra were recorded using KBr pellet on a Bruker Tensor 27 FT-IR spectrometer. Optical rotations were measured on a Rudolph Research AutoPol IV polarimeter. Column chromatography was performed by using silica gel (40 µm mesh, JT Baker). TLC analysis was carried out on silica gel 60 F_{254} plates (Merck) and spots on TLC plates were observed under UV light (254/365 nm). Spraying reagents *p*-anisaldehyde-H_2SO_4 (Sigma-Aldrich), 10% H_2SO_4 in ethanol and water, followed by heating were used for the detection of spots.

Plant source

The soybean seeds (*Glycine max*) purchased from market and were properly identified by running the TLC/Co-TLC with the standard sample of soybean seeds.

Extraction and isolation

The finely powdered *Glycine max* seed extract (70 g) was mixed with an equal amount of silica gel and subjected to column chromatography (CC) over a silica gel (1.0 kg) column (135×6.0 cm) and eluted with $CHCl_3$-MeOH (9:1) to obtain fractions SS-A (33.2g) and SS-B (15.1g). The polarity of eluent was changed to $CHCl_3$-MeOH-H_2O (13:7:2; lower layer, labelled as solvent system A) to afford seven fractions labeled as SS-C to SS-J. Fraction SS-C (5.1 g) after recolumn-chromatography afforded **1** (58.7 mg).

Compound 1: Green amorphous powder; IR (KBr): ν_{max} = 3270 (-N-H), 2884 (-C-H) 1740 (-CO); 1281, 764 cm^{-1}; ESIMS (positive mode): *m/z* 682.1271 $[M+H]^+$ (calcd. for $C_{42}H_{83}O_5N$); ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) data, see Table 1.

Table 1. ^{1}H- (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$) of 1

Position	1		Position	1	
	δ_C	δ_H		δ_C	δ_H
1	175.0		10-19	27.1-31.9	1.25-1.31
2	78.2	4.20	20	15.1	0.90
3	39.1	2.20	1'	55.2	3.75
4	123.3	5.25	2'	62.1	3.50
5	134.4	5.41	3'	75.6	3.91
6	28.2	1.92	4'	71.1	3.29
7	36.5	1.50	5'	32.1	1.42
8	39.1	1.58	6'-21'	25.0-31.9	1.25-1.31
9	19.1	0.96	22'	15.0	0.92
N - H		8.02			

Chemical shifts are in ppm, J in parentheses are in Hertz

GC/MS

GC/MS data obtained on Varian Mass Spectrometer using VF-5 column (60 m × 0.32 mm i.d.; 0.25 μm film thickness). Column temperature programmed 5 min. at 60 °C, then rising at 2 and 3 °C upto 240 °C. "Injector temperature, 240 °C"; "ion source temperature, 250 °C", "interface temperature, 270 °C; acquisition mass range 700-40 amu; ionization energy, 70 eV. Helium was used as carrier gas with a flow rate 0.5 ml/min. The identification of peaks was accomplished by comparison of the mass spectra with those reported in the NIST library. Identification of the aliphatic chains was also done by comparison of their linear RI with those from Mass Finder library.

Results and Discussion

Compound **1** was obtained as greenish amorphous powder. The ESI/MS depicted the *m/z* 683.1271 $[M + H]^+$, corresponding to the molecular formula $C_{42}H_{83}O_5N$. The series of interconected signal in ^{1}H NMR spectrum between δ_H 1.25-1.31 revealed the presence of side chain, which was supported by the fragementation pattern of long chains at *m/z* 311.5310 $[M + H]^+$ and *m/z* 374.4508 $[M + H]^+$, which depicted the presence of icosane and docosane aliphatic moieties respectively, which were identified by GC/MS by using the corresponding standards. As the IR showed the typical N-H stretching at 3280, 1550 cm^{-1} indicating the presence of amide group interconnected two long side chains, supported by the typical signal at δ_H 8.02 in

^{1}H NMR spectrum. The resonances in ^{13}C NMR at δ_C 175.1, 123.3, 134.1 revealed the presence of carbonyl and side chain double bond. The position of the double bond was assigned to icosane side chain positioned between C_4-C_5, supported by obtaining the long chain fragement of C-16 (hexadecane) in ESI/MS/MS at *m/z* 227.4302 $[M + H]^+$. As the molecule is polyhydroxylated revealed by the ^{13}C NMR resonaces at δ_C 78.2, 75.6, 71.1 and 62.1. The position of the hydroxyls was given on the basis on fragementation pattern obtained both in ESI/MS/MS and GC/MS. Based on the above description, structure 1 (Fig. 1.) was assigned to compound **1**.

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Wood Anatomical Features of Stems of *Salix alba* L. from Temperate Climate of Kashmir Himalaya

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Abstract
Willow *(Salix alba* L.) is regarded as an important natural resource almost throughout the temperate regions of the world but relatively little is known about anatomical characteristics of its wood. For this purpose sections were cut in three different planes and also maceration was done in order to know about wood anatomical features viz. fiber length, fiber diameter, fiber wall thickness, vessel element length and vessel element diameter, growth ring.
Keywords: *Salix alba*, fiber, wood, anatomy.

Introduction

Willow belongs to the genus *Salix* and family *Salicaceae,* comprising deciduous and dioecious prostrate shrubs to large trees over 30 meters high, but most are shrubs or small trees. The word *Salix* is derived from Celtic 'Sal' meaning near and 'lis' meaning water. They are usually found on damp ground or along river and stream margins. There are about 450 species of *Salix* mainly distributed in Asia, Europe and North America (Argus 1997, 2010) but there is still disagreement among authors regarding the number of species (Fang *et al*., 1999, Skvortsov 1999, Ohashi 2001, Heywood *et al*., 2007). In India, there are around 33 species and most of them are categorized as shrubs except *S. tetrasperma*, *S. alba, S. daphnoides, S. fragils* and *S. babylonica*.

Wood anatomy an important branch of wood science; which is suitable for predicting the varied utility of woods for different purposes, the present work is an attempt to elucidate wood anatomical patterns in *Salix alba,* from temperate climate of Kashmir Himalaya, as its wood is suitable for Veener, pulp, plywood, laminated wood, reconstituded wood products, artificial limbs, fruit boxes, agriculture implements, furniture, tool handles and sports goods like cricket bat, polo balls etc.

Material and Methods

Source of Material

The present study was carried out on hardwood trees *Salix alba* L. with deciduous type of habit, belonging to the family *Salicaceae* from natural provenances of Kashmir Himalaya for their anatomical characteristics.

Samples of approximately 1 cm^3 in size were taken at breast height (1.3 m) and were softened by boiling in water for 10-15 minutes. Cross, radial and tangential sections of 15–20 μm thick were prepared by using Reichert microtome, as well as sharp razor for studying various wood anatomical microscopial features. These features were identified as per International Association of Wood Anatomists (IAWA) list of microscopic features for hardwood identification (Wheeler *et al*., 1989).

Maceration

Maceration was done as per Jeffery's method (Johansen, 1940) for all the sample studies. In this method, small slivers of wood were taken from the samples collected and put into the test tube and then filled with 10% chromium trioxide and 10% nitric acid and left for one to several days at room temperature and the process was

hastened by heating up to approximately 60^0C for few minutes. After that, the material was thoroughly washed with distilled water till traces of the acid were removed. The mixture was teased/shaken thoroughly to separate the wood elements and stained with 1% Safranin and mounted in glycerine on microscopic slides.

Staining Procedure for Sections

Twenty micron thick; transverse, radial and tangential sections were stained in Heidenhains haematoxylin and safranin for 20 minutes. The stained sections were washed in acetone and xylene of 1:1 ratio for 10 minutes to ensure complete dehydration and subsequently in Xylene. Finally they were mounted in Diphenyl Pthalate Xylene (DPX) mountant to make the permanent slides.

Photomicrography

Photomicrography involves combination of the principles of microscopy and photography; is a technique of recording microscopic image. By this method, the object was focussed under microscope and the photographs were taken with the help of Olympus Clinical CH20I microscope model CH20 BIMF 200; on the top of which Olympus camera was fitted with photomicrographic attachements.

Wood Element Measurements

The measurements/dimensions of different wood elements viz., vessel elements and rays were made from macerated and transverse section materials, with the help of ocular-stage micrometry. Twenty-five measurements were made from unbroken fibers and vessel elements for lumen diameter, wall thickness, length and width.

Colour

The colour feature was determined when logs were cut across, showing two distinct regions of sapwood and heartwood (Chowdhury and Ghoush, 1958) and (Rao and Junjea, 1971).

Some timbers when cut on the surface have some characteristic smell or odour due to resins, oils or chemical deposits. It may be asset or a liability in its utilization for a given purpose. This feature was determined by exposing fresh wood sample or by adding moisture through breathing already cut wood sample (Wheeler *et al.,* 1989).

Results

Sapwood white or nearly so, merging into the heart wood; heart wood uniformly light red at first turning light reddish brown to light with age, frequently irregular in contour in the transverse section; rather lustrous (especially the sapwood) with a silky sheen when first exposed but becoming dull with age, without characteristic odour or taste.

Wood diffuses porous; growth rings distinct. Vessels numerous (170-340 per sq mm), mostly in short to long radial multiples of 2-4 (10) and small clusters, occasionally solitary, vessel diameter 30 (20-45) µm in tangential diameter. Vessel elements 320 (180-420) µm long, with simple perforations and alternate intervessel pits, 7-10 µm in diameter; vessel ray pits with much reduced borders and rounded to angular outline, 6-9 µm in diameter. Libriform fibers thin walled, 540 (410-650) long, with simple pits mainly restricted to radial walls, occasionally with gelatinous wall layers. Axial parenchyma in narrow and discontinuous terminal bands and very scanty paratracheal. Rays uniseriate (more rarely with biseriate portions), weakly heterocellular with square central cells, 4-40 cells or 500-1920 µm tall. Perforated ray cells restricted to upright ray cells, with perforations of about 53 x 59 µm in horizontal x vertical diameter.

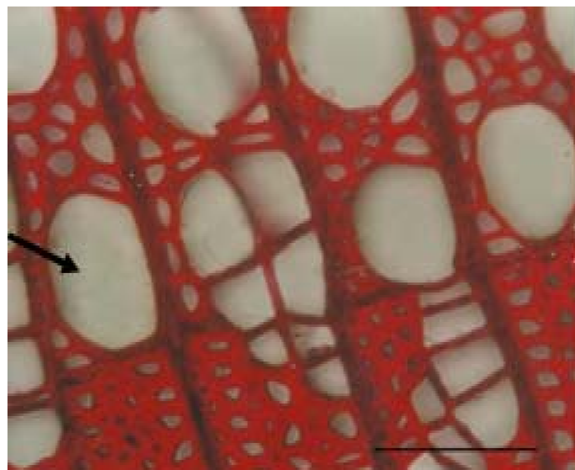

Fig. 1 . T.S. showing pores and angular shaped fibers 40X

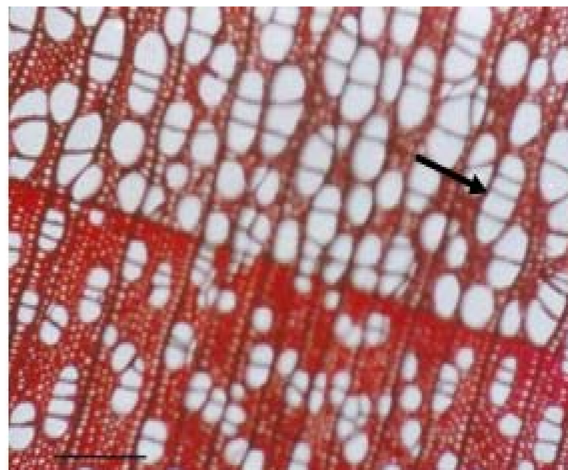

Fig. 2. T.S. showing pores in radial multiple of 2-4 10 X

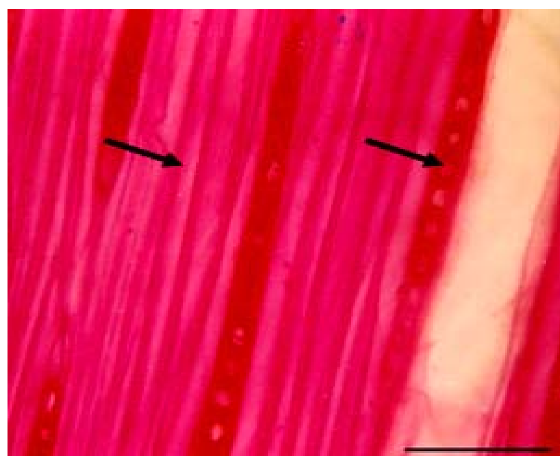

Fig. 3. T. L. S. showing exclusively uniseriate rays and cluster of fibers

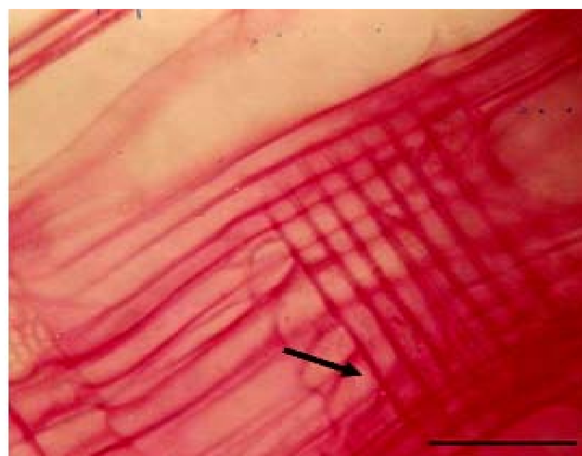

Fig. 4. R. L. S. showing weakly heterocellular rays and vessel

[Scale bar Fig. 1, 3 and 4 = 40 µm and Fig. 2 = 50 µm]

Discussion

Wood anatomical structure of *Salix alba* L. growing in the Kashmir Valley was investigated. According to the records in the literature, there have not been any studies on the wood anatomy of willows in Kashmir except few studies by Wani and Khan (2010 a, b). Further these studies were mainly oriented towards the wood variability and cambial activity in *Salix alba*. The most detailed information on wood anatomy of *Salix* is given by Metcalfe and Chalk (1965) in *Anatomy of Dicotyledons.* Also Yazganet *et al.,* (1986) provided a cross section of *Salix alba* with schematic and anatomical illustrations. In the present study, the Sapwood of *Salix alba* was found white or nearly so which merges into the heartwood. The sap wood of a new wood provides a pipeline for the movement of water and nutrients through the trunk and into the leaves (Medhurst and Beadle, 2002). The heartwood was found uniformly light red at first and afterwards turns light reddish brown with passage of time and having irregular contour in the transverse section. Also in the present study, wood of *Salix alba* was found to be diffuse porous with distinct growth rings. Vessels were numerous mostly in short to long radial multiples of 2-4(10). Vessel

elements were found to be long with simple perforations and with alternate intervessel pits. Libriform fibres were thin walled ,long with simple pits mainly restricted to radial walls . Axial parenchyma was narrow and with discontinous terminal bands. Rays mostly uniseriate. The similar results were obtained by Metcalfe (1939), Lyr and Bergmann (1960), Sacre (1974), Sennerby- Forse (1989), Arihan and Guvenc (2011) and Isabelle *et al.,* (2013)

Conclusion

Wood anatomical studies of the *Salix alba* Linn**.** a versatile natural resource of Kashmir Himalaya which is chiefly used for the manufacturing of high quality cricket bats have been carried out . The dimensions of cellular structures viz., vessels, fibers, rays, parenchyma present in the *Salix alba*, which have direct bearing on the properties of wood been discussed.

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Morphological Characteristics and Taxonomic Position of α-Amylase Producing *Penicillium* Species Isolated from Soil

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Abstract
This study reports the morphology, colony forming unit (CFU) and taxonomy of the α-amylase producing *Penicillium* species isolated from soil. Soil samples were collected from the study sites under sterile conditions and processed in the laboratory for isolation of fungal isolates. The soil samples were diluted by serial dilution method and 0.1 ml innoculum was inoculated onto the Potato Dextrose Agar (PDA) plates and incubated for 3-7 days in an incubator at 37° C to develop the fungal colonies. *Penicillium* species isolated from the soil were screened for α-amylase activity on starch agar medium through iodine test. *P. chrysogenum, P. purpurogenum, P. caesicolum, P. funiculosum* showed positive α-amylase activity and were further selected for production. During the study the morphological characteristics like appearance, colour, elevation and margins were observed for *Penicillium* species and it was found that *Penicillium* species showed a varied morphology, some were circular, rhizoidal and some were filamentous in appearance. The taxonomic position of four isolated *Penicillium* species indicated that they all belong to division Ascomycota and class Trichocomaceae of kingdom Fungi. Among all the isolates, P. chrysogenum showed the colony forming units of 2.8×103 at site I in the month of June and colony forming units of 3.5×103 at site II in the month of August.
Keywords: Soil, morphology, *Penicillium*, α-amylase, colony forming units (CFU)

Introduction

Soil is the best medium not only for the growth of plants but also for the micro-organisms. It is defined as a natural body consisting of layers (soil horizons) of mineral constituents of variable thicknesses, which differ from the parent materials in their morphological, physical, chemical and mineralogical characteristics. Soil microbial population is the key element in the bio-geochemical cycling of nutrients in nature (Pelczar *et al.,* 1993). The role of fungi in the soil is extremely complex and is fundamental to the soil ecosystem. They perform ecological services that strongly impact the quality of human life and have enormous potential for providing economic benefits. It is estimated that there are 1.5 million fungal species on earth, of which only about 70,000 have been described till recent (Hawksworth and Rossman, 1997). Different species of genus *Aspergillus* and *Penicillium* serve in the production of a number of biotechnologically produced enzymes and other macromolecules, such as gluconic, citric, and tartaric acids, as well as several pectinases, lipase, amylases, cellulases, and proteases (Akpan *et al*., 1999). Cellulases and amylase have been obtained both in the wild and mutant strains of *Aspergillus* sp such as *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus nidulans* and *Aspergillus niger* (Dar *et al*., 2014). Amylases are the hydrolytic enzymes, widespread in nature with potential application in a number of industrial processes constitute a class of industrial enzymes representing approximately 25-33% of the world

enzyme market (Nguyen *et al.*, 2002; Van der Maarel *et al.,* 2002) can be obtained in bulk from different species of this genus. Evaluation of morphological characters and population of different species of genus *Penicillium* for the production of α-amylases will help in the proper channelization of the agro industry based wastes into the world enzyme market in addition to the solution of the solid waste generation and disposal problems in state like that of ours (Dar *et al.*, 2014) and furthermore it will help us in the optimization of the conditions for the production of these hydrolytic enzymes from these species. In recent years, the potential of using micro organisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extra cellular enzymatic activity in several microorganisms and in this regards, the present study dealing with the Morphological characteristics and Taxonomic position of α-amylase producing *Penicillium* species isolated from soil was taken up.

Material and Methods

Location and site description

Harwan area of the Srinagar city was choosen for the collection of soil samples. This place is situated in Srinagar, Jammu and Kashmir, India. Its geographical coordinates are 34° 10′ 0″ North, 74° 54′ 0″ East. Harwan-situated at an altitude of about 2743m asl, lying in the Srinagar District of Jammu and Kashmir state, is a small village set in the heart of mountains to the South East of Srinagar. Two study sites were selected, Site I was Orchard land and Site II was Fallow land area

Collection of Samples

Composite samples of soil from the sites were collected during the study period, from a depth of 5 inches. Samples were collected in sterile polythene bags and carried to laboratory for analysis (Dar *et al.,* 2013). The samples were processed using the soil plate method (Warcup, 1950) and Soil dilution plate Method (Waksman, 1922). The pure cultures of *Penicillium* isolates were prepared and stored

Soil plate method

About 1g of soil was scattered on the bottom of a sterile Petri dish and molten cooled (40-45°C) Rose Bengal Agar medium (RBA) was added, which was then rotated gently to disperse the soil particles in the medium. The plates were then incubated at 35±2°C for 3-7 days.

Soil dilution plate method

The soil samples were mixed with sterile distilled water and a series of dilutions were made. From the dilutions, 0.1ml inoculum was poured onto Potato Dextrose Agar and incubated at 35±2°C for 3-7 days. The number of colonies counted was expressed as cfu/g and were calculated by using the formula:

$$Cfu/g = n \times d$$

Where n= number of colonies; d = dilution factor = 1/dilution (10^{-1}, 10^{-2} etc.)

Identification of *Penicillium* species

The identification of *Penicillium* isolates was done on the basis of the micro and macro morphological features, reverse and surface coloration of colonies grown on Czapek's dox Agar (CZ), Malt Extract Agar (MEA), Czapek's Yeast Agar (CYA) and Potato Dextrose Agar (PDA) media and the Fungal morphology was studied macroscopically by observing colony features (Colour and Texture) and microscopically by staining with lacto

phenol cotton blue and observed under microscope for the conidia, conidiophores and arrangement of spores. They were further identified from Agharkar Research Institute Pune, India

Screening and Bioprospecting of *Penicillium* Isolates

Screening of α-amylase producing fungi

The isolated strains of *Aspergillus* were streaked onto the starch agar plate and incubated at room temperature for 72 hours. After incubation 1% of iodine solution was layered on the agar plates and zone of clearance was observed for screening the fungi (Pandey *et al*., 2006).

Submerged fermentation of Amylase

Submerged fermentation was carried out in the Ehlenmeyer flasks by taking 100 ml of amylase production medium (Bernfed, 1951); containing Peptone (6.0g/L), MgSO4 (0.5g/L), KCl (0.5g/L), Starch (1g/L). In addition to this certain agricultural waste products like Cocos nut meal (Cocos nut oil cake) will be used as a submerged fermentation medium. The medium was maintained at a pH range of 3, 6 and 9, at 30°C on a shaker with 120rpm for 6 to 18 days (Pandey *et al*., 1999).

Enzyme extraction

Crude enzyme was extracted by mixing a known quantity of fermented substrate with distilled water containing 0.1%, tween 80 on rotator shaker at 180 rpm/1 hr. The suspension was centrifuged at 7000xg at 4°C and the supernatant used for enzyme assay (Pandey *et al*., 2006).

α- amylase assay

α-amylase activity was determined (Pandey *et al*., 1999). Then reaction mixture containing 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 mM acetate buffer (pH 5.0) and 0.25 ml of crude enzyme extract will be incubated for 10 minutes at 50°C. After incubation the reducing sugar will be estimated by Dinitrosalicylic acid (DNS) method (Miller, 1959).

Results and Discussion

Among all the isolates, *Penicillium chrysogenum* showed the colony forming units of 2.8×10^3 at site I in the month of June and colony forming units of 3.5×10^3 at site II in the month of August. The two sites were having a marked difference in various biotic and abiotic factors. *Pencillium* species were isolated and selected for further study. The 4 isolates which showed positive amylase activity were present at both the sites. During the study the morphological characteristics like appearance, colour, elevation and margins were observed for *Penicillium* species and it was found that *Penicillium* species showed a varied morphology, some were circular, rhizoidal and some were filamentous in appearance. The taxonomic position of four isolated *Penicillium* species indicated that they all belong to division Ascomycota and class Trichocomaceae of kingdom Fungi (Table 1), and the colony forming units (cfu/g) of *Penicillium* species obtained during the study are shown in the Tables 2-5 and Fig 1. Fungal cultures were isolated from soil sample by serial dilution on Potato Dextrose Agar medium (PDA). Four cultures of *Penicillium* species isolated from soil at the two sites showed positive amylase activity. All the *Penicillium* isolates were tested for positive amylase production by starch hydrolysis through iodine test. On the basis of the area of clearance, all the four *Penicillium* isolates were selected for further studies on amylase production. The monthly population (cfu/gm) was found highest for *P. chrysogenum* as 2.8×10^3 and 3.5×10^3 in the months of June and August respectively at the two sites under consideration and the diversity of *Penicillium* species was highest at Site II. This may be attributed to the difference in various biotic and abiotic factors like pH and temperature that have been found to influence the composition and diversity of soil microbial communities and the results obtained are in consonance with the findings of Piao *et al*., (2000), Fierer and Jackson, (2006), who worked on various biotic and abiotic factors influencing the microbial communities. Since the temperature is found to be highest in the months of June and August in Kashmir valley, which might have increased the

reproductive rate of microbial communities. These results are in accordance with the studies of Murphy (2000) and Dar *et al.,* (2013), who evaluated a correlation between temperature changes and microbial communities. The total count (cfu/g) was highest at site II which was dominated by cattle activities. The cattle activities thus might have induced changes in the microbial community structure which is in concurrence with the studies carried out by Kohler *et al.,* (2005) and Dar *et al.,* 2013 in which they have reported the effects of cattle grazing on microbial communities in pastures and has shown that microbial community changes due to simulated effects of cattle grazing. *P. funiculosum* and *P. chrysogenum* were obtained in the present study which were also reported by Bandh *et al.*, (2011), from Dal lake and reported that *P. funiculosum* was the most abundant (28.7%) followed by *P. chrysogenum* (27.04%), which also concurs with the present study. The highest colony forming units (cfu/g) was observed for *P. chrysogenum* in the month of August at site II while as no colony forming unit (cfu/g) was recorded in the months of Feb for *P. chrysogenum* and *P. purpurogenum* where as *P. caseicolum* and *P. funiculosum* showed no cfu/g in the months of Dec, Apr and Oct. at site I respectively, but all these species were present at site II. A comparison graph shown in Fig. 1 indicates that the colony forming unit (cfu/g) was highest at site II which was a fallow land area.

Table 1. Morphological characteristics and taxonomic position of isolated *Penicillium* species

S. No.	Isolated species	Taxonomic Position				Morphological Characteristics			
		Kingdom	Division	Family	Genus	Appearance	Margin	Elevation	Colour
1.	*Penicillium chrysogenum* Thom	Fungi	Ascomycota	Trichocomaceae	*Penicillium*	Circular	Entire	Flat	Green
2.	*Penicillium purpurogenum* Stoll					Filamentous	Filamentous	Convex	White
3.	*Penicillium caseicolum* Bain					Circular	Filamentous	Convex	Cream
4.	*Penicillium funiculosum* Thom					Rhizoidal	Filamentous	Flat	Green

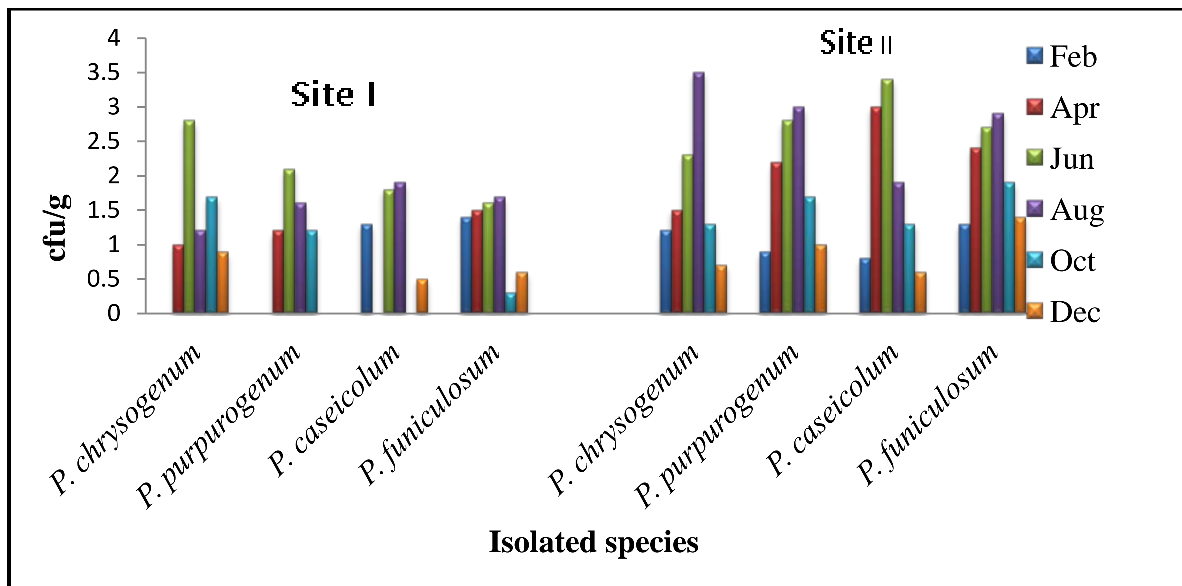

Fig. 1. Comparison of colony forming units (cfu/gm) of *Penicillium* species at different sites

Table 2. Colony count of isolated *Penicillium* species at Site I

S. No.	Isolated species	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1.	*P. chrysogenum*	0	10	28	12	17	9
2.	*P. purpurogenum*	0	12	21	16	12	0
3.	*P. caseicolum*	13	0	18	19	0	5
4.	*P. funiculosum*	14	15	16	17	3	6

Table 3. Colony forming units (cfu/g) of isolated *Penicillium* species at Site I

S. No.	Isolated species	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1.	*P. chrysogenum*	0	1×10^3	2.8×10^3	1.2×10^3	1.7×10^3	0.9×10^3
2.	*P. purpurogenum*	0	1.2×10^3	2.1×10^3	1.6×10^3	1.2×10^3	0
3.	*P. caseicolum*	1.3×10^3	0	1.8×10^3	1.9×10^3	0	0.5×10^3
4.	*P. funiculosum*	1.4×10^3	1.5×10^3	1.6×10^3	1.7×10^3	0.3×10^3	0.6×10^3

Table 4. Colony count of isolated *Penicillium* species at Site II

S. No.	Isolated species	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1.	*P. chrysogenum*	12	15	23	35	13	7
2.	*P. purpurogenum*	9	22	28	30	17	10
3.	*P. caseicolum*	8	30	34	19	13	6
4.	*P. funiculosum*	13	24	27	29	19	14

Table 5. Colony forming units (cfu/g) of isolated *Penicillium* species at Site II

S. No.	Isolated species	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1.	*P. chrysogenum*	1.2×10^3	1.5×10^3	2.3×10^3	3.5×10^3	1.3×10^3	0.7×10^3
2.	*P. purpurogenum*	0.9×10^3	2.2×10^3	2.8×10^3	3.0×10^3	1.7×10^3	1.0×10^3
3.	*P. caseicolum*	0.8×10^3	3.0×10^3	3.4×10^3	1.9×10^3	1.3×10^3	0.6×10^3
4.	*P. funiculosum*	1.3×10^3	2.4×10^3	2.7×10^3	2.9×10^3	1.9×10^3	1.4×10^3

Conclusion

The taxonomic position of four isolated *Penicillium* species indicated that they all belong to division Ascomycota and class Trichocomaceae of kingdom Fungi. Among all the isolates, *P. chrysogenum* showed the colony forming units of 2.8×10^3 at site I in the month of June and colony forming units of 3.5×10^3 at site II in the month of August.

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Intervarietal Differences of Barley (*Hordeum vulgare* L.*) i*n Response to Hydrazine Hydrate.

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Abstract

Assessing the impact of mutagens for creating variations in crops like Barley (*Hordeum vulgare* L.) is an important criterion in the contemporary world where food insecurity and malnutrition is alarming at the doors of various nations. In the present research, mutations were induced in two varieties (BH-393 and PL-172) of *Hordeum vulgare* L. by treating the seeds with hydrazine hydrate (Hz) at 0.01%, 0.02%, 0.03% and 0.04 % concentrations. It was found in both varieties (BH-393 and PL-172) that seed germination, seedling height, number of branches, spike length and yield increased at lower concentrations (0.01 and 0.02)% and per cent injury increased with increasing concentration of mutagen (14.14 % - 86.82% in BH-393 and (8.69% -19.56% in PL - 172.

Keywords: Food insecurity, mutations,hydrazine hydrate, yiel, *Hordeum vulgar* L.

Introduction

Food insecurity is becoming major constraint for the development of national building programmes in various countries, including India. With an increase in human population the ghost of hunger are making its impact among millions of people all around. The rescue lies in tailoring the better varieties of crop plants, high in nutrition and yield, and induced mutagenesis is one of those novel techniques, which impart variations in subject crops through sustainable approach. By inducing mutations, vast amount of genetic variabilities have been generated in crop plants which have played a significant role in plant breeding and genetic studies (Kumar and Singh, 2003). More than 3200 mutant cultivars have been developed through this approach (Pierre, 2012). Induced mutations have been utilized to achieve success in improving plant yield as well as plant architecture. Both dwarfness and compactness of a genotype ensure more plants per unit area, thereby significantly contributing the production and productivity. Induction of chemical mutation has been studied by many researchers in the past (Haque and Godward, 1985). Keeping in view the above facts, the present study was carried out in the Department of Botany, Aligarh Muslim University, Aligarh.

Material and Methods

Seeds of both the varieties of Barley (BH-393 and PL-172) were presoaked in distilled water for 9 hours and were treated with freshly prepared solution of 0.01, 0.02 ,0.03 and 0.04 percent hydrazine hydrate (Hz)), a base analogue manufactured by Qualigens Fine Chemicals, Mumbai, for 6 hours. Treated seeds were thoroughly washed in tap water .Controls were maintained by treating the seeds in distilled water only. After mutagenic treatments, seeds from each treatment and control were sown in pots. Data on seed germination and on certain quantitative traits, viz., plant height (in cm), days to maturity, fertile branches per plant, total yield per plant were analyzed statistically to find out variation in M1 population. Three replications were maintained for each treatment. Each replication had a total of seven seeds. Ten separate seeds for each concentration were spread over

moist cotton in petriplates. These petriplates were then kept in BOD incubator at 20°C in order to find out seed germination and seedling growth.

The statistical analysis was done to find out Mean, Standard error, Standard deviation and Co-efficient of variation in the control and treated population.

Results and Discussion

The germination started second day after sowing in control and in mutagen treated population of the Varieties PL -172 and PH -393. Lower concentrations 0.01% & 0.02 % of Hz enhanced seed germination whereas the higher concentrations 0.03% & 0.04 % decreased the germination considerably in both the varieties. In the control of variety PL-172, seed germination was recorded to be 19.04%. The percentage of seed germination was 47.61% and 4.76 % at 0.01 % and 0.04% respectively (Table 1). The percentage of seed germination was 33.33 and 4.76% with the same concentration in the variety PH-393. 14.28% seed germination was recorded in control of variety BH-393 and PL-172. Data on seed germination was recorded in both pots as well in petriplates. In both the cases, variety BH-393 was found to be more sensitive than the variety PL-172.

Data recorded on seedling height (cm) of the two varieties of barley are given in the Table 2. The study of seedling height in petriplates after 9 days of sowing showed that various Hz treatments caused reduction in seedling height in barley. The percentage injury in seedling height increased with mutagen concentration. Variety PH-393 was found to be more sensitive than the variety PL-172. In the variety PH-393, the % injury ranged from 14.14 -86.82 % whereas it was 8.69 -19.56 % in the variety PL-172.

Various types of morphological varieties namely dwarf, tall, increased number of branches ,variation in size and colour of leaves and spike length (Plate I) were isolated from Hz treated population of barley varieties PL-172 & PH-393. The maximum number of variants was noticed for plant height and leaf morphology. Variety PH-393 produced higher number of morphological variations than the variety PL-172(PLATE-1)

Fig. 1. Higher no of branches (0.02% Hz) in PL-172

Fig. 2. Tall plants (0.03 %Hz) in BH-393

Fig. 3. Spike of control and mutagen treated plant . (PL-172)

Fig. 4. Leaves of control and mutagen treated plant showing variation in size. (BH-393)

Fig. 5. Leaves of control and mutagen treated plant showing variation in colour (PL-172)

The effect of various treatments of Hz on certain quantitative traits namely plant height (cm), fertile branches per plant and yield per plant are presented in Tables 3-5.Data revealed that mean values and co-efficient of variation differed with various mutagenic treatments. Mean values for plant height (cm) ,fertile branches per plant and yield is decreased with increase in mutagenic concentration in both the varieties .Co-efficient of variation was in general recorded higher for fertile branches per plant followed by yield and plant height. Lower concentration of the mutagen were found to be more effective in increasing the variation in the quantitative traits in both the varieties of Barley, where as the higher concentration of Hz produced injurious effects on various traits.

In the present study, the dose dependent reduction in various biological parameters viz., seed germination and seedling height was noticed with increasing concentration of Hz in M1 generation of barley. The reduction in seed germination might have arisen due to inhibition of physiological and biological processes including enzymatic activity (Kurobane *et al.*, 1979), hormonal disproportion (Chrispeels and Varner, 1967)and hampering mitotic processes (Ananthaswamy, 1971). In M_1 generation, the seedling growth also showed a declining trend from lower to higher concentrations. The inhibition of seedling growth was reported due to auxin obliteration and change in ascorbic acid content (Usuf and Nair, 1974), destruction of apical meristem (Petal and Shah, 1974), transitory deferral of cell division (Evans and Scott, 1964) and reduction in the level of amylase activity (Reddy and Vityavathi, 1985). The separation in seedling growth may be the consequences of either physiological or due to chromosomal abnormalities caused by the mutagen.

Morphological variants Viz. plant height, number of fertile branches, leaf size and colour are among the most common variations noticed in Hz treated population of Barley. The possible cause of these morphological variations may be due to chromosomal aberrations or most probably gene mutation.

The mutagen induced genetic variability for quantitative traits in different crops plants such as chickpea (Wani *et al.*, 2012), *Vicia faba* (Parveen *et al.*, 2012)**,** *Lathyrus sativus* (Waghmare and Mehra, 2000) has been already assessed in the past.In the present study, there was a considerable increase in mean number of fertile branches per plant. The increase in variability for number of branches following mutagenesis has been reported in various crop plants. (Gottschalk and Kaul, 1980; Khan and Wani, 2005; Khan and Goyal, 2009; Kharkwal and Khan, 2003; Kozgar and Khan, 2009; Wagmera and Mehra, 2000). The increase in yield per plant in present study was due to an increase in number of fertile branches. Similar results have been reported by Wani *et al.*, (2012)

Conclusion

The extent of variation in mean values and C.V for various quantitative traits studied in present study was not same in the two varieties showing the varietal differences. Variety PH-393 was found to be more sensitive than the variety PL-172. Higher dose of Hz produced adverse effects on all the parameters studied. On the other hand, lower concentrations of the mutagen had beneficial effects. Therefore, it is suggested that lower concentration of Hz should be used for any breeding programme of Barley. Mean and co-efficient of variation for quantitative traits of Barley provides ample evidence that mutagenic treatments could alter the mean value and create additional genetic variability for polygenic traits.

Table 1. Effect of mutagen on seed germination in control and treated population of barley in M_1 generation

Variety PL-172				
Seed Germination			% Of Inhibition	
Treatments	In Pots	In Petriplates	In Pots	In Petriplates
Control	19.04	50	-	-
0.01 % Hz	47.61	60	+ 150.05	+20.00
0.02 % Hz	19.04	70	0.00	+40.00
0.03 % Hz	19.04	50	0.00	0.00
0.04 % Hz	4.76	50	-75.00	0.00
Variety PL-393				
Seed Germination			% Of Inhibition	
Treatments	In Pots	In Petriplates	In Pots	In Petriplates
Control	14.28	50	-	-
0.01 % Hz	33.33	50	+ 133.40	0.00
0.02 % Hz	23.80	30	+66.66	+40.00
0.03 % Hz	14.28	50	0.00	0.00
0.04 % Hz	4.76	30	-66.66	-40.00

Table 2. Effect of mutagen on seedling height (cm) in control and treated population of barley in M_1 generation

Variety PL-172				
Treatments	Root length (cm)	Shoot length (cm)	Total length (cm)	% of inhibition
Control	18.00	5.00	23.00	-
0.01 % Hz	17.00	5.00	22.00	-4.34
0.02 % Hz	16.50	4.60	21.00	-8.69
0.03 % Hz	15.00	6.30	21.30	-8.69
0.04 % Hz	14.00	4.50	18.50	-19.56
Variety BH-393				
Treatments	Root length (cm)	Shoot length (cm)	Total length (cm)	% of inhibition
Control	15.50	5.00	20.50	-
0.01 % Hz	15.50	5.00	20.50	0.00
0.02 % Hz	17.00	6.00	23.00	+12.19
0.03 % Hz	13.00	4.60	17.60	-14.14
0.04 % Hz	4.50	2.30	6.80	-66.82

Table 3. Effect of mutagen Hz on plant height (cm) of barley in M_1 generation.

	Variety PL-172				Variety BH -393			
Treatments	Mean ± S.E	Shift in $\overline{x}$	S.D	C.V%	Mean ± S.E	Shift in $\overline{x}$	S.D	C.V %
Control	34.00±1.03	-	3.28	9.64	31.10±0.65	-	2.07	6.65
0.01 % Hz	48.30±1.62	+14.30	5.15	10.66	52.10±1.16	+21.00	3.67	7.04
0.02 % Hz	45.90±1.34	+11.90	4.24	9.23	51.10±1.01	+20.50	3.21	6.22
0.03 % Hz	57.70±0.64	+23.70	2.05	3.55	60.40±0.64	+29.30	2.05	3.39
0.04 % Hz	44.40±0.63	+10.40	2.00	4.50	59.80±1.12	+28.70	3.54	5.91

Table 4. Effect of mutagen Hz on fertile branches per plant of barley in M_1 generation.

	Variety PL-172				Variety BH -393			
Treatments	Mean ± S.E	Shift in $\overline{x}$	S.D	C.V%	Mean ± S.E	Shift in $\overline{x}$	S.D	C.V%
Control	12.60±0.39	-	1.24	47.69	3.40±0.61	-	1.95	57..35
0.01 %Hz	3.90±0.44	+1.30	1.41	47.00	2.80±0.44	+0.64	1.4	5.00
0.02 %Hz	2.80±0.74	+0.20	0.74	25.00	2.90±0.35	+0.50	1.13	38.96
0.03 %Hz	5.00±0.77	+2.40	0.77	15.40	4.00±0.70	+1.10	2.23	55.75
0.04 %Hz	2.90±1.30	+0.30	1.30	44.82	4.30±0.93	+0.90	2.96	68.83

Table 5. Effect of mutagen Hz on yield (g) of barley in M_1 generation.

	Variety PL-172				Variety BH -393			
Treatments	Mean ± S.E	Shift in $\overline{x}$	S.D	C.V%	Mean± S.E	Shift in $\overline{x}$	S.D	C.V%
Control	4.64±0.03	-	0.12	2.58	4.30±0.54	-	1.71	39.76
0.01 %Hz	3.89±0.004	+0.80	0.013	0.33	4.33±1.29	+0.30	4.69	108.30
0.02 %Hz	3.80±1.13	+0.84	3.60	94.73	3.88±0.11	+0.42	0.42	10.82
0.03 %Hz	3.90±1.44	+0.74	4.58	17.43	4.41±0.82	+0.11	0.026	0.58
0.04 %Hz	4.22±0.016	+0.42	0.053	1.25	4.21±0.31	+0.09	0.042	0.99

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Phytochemical Screening and Extraction: A Mini Review

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Abstract
Plants are a source of large number of drugs comprising of different groups such as antispasmodics, anti-cancer, antimicrobials etc. A large number of the plants are claimed to possess the antibiotic properties in the traditional system and are also used extensively by the tribal people worldwide. It is now believed that nature has given the cure of every disease in one way or another. Plants have been known to relieve various diseases in Ayurveda. Therefore, the researchers today are focusing on evaluation and characterization of various plants and plant constituents against a number of diseases based on their traditional claims of the plants given in Ayurveda. Extraction of the bioactive plant constituents has always been a challenging task for the researchers. In this present review, an attempt has been made to give an overview of certain extractants and extraction processes.
Keywords: Medicinal plants, phytochemicals, extraction, solvent, screening

Introduction

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube *et al*., 2008). The plants that possess therapeutic properties or exert beneficial pharmacological effects on the human body are generally designated as medicinal plants. In most of the traditional systems of treatment, the use of medicinal plant include the fresh or dried part, whole, chopped, powdered or an advanced form of the plant usually made through extraction with different solvents play a major role and constitute the backbone of the traditional medicine (Mukherjee *et al.,* 1986). Botanical medicines or phytomedicines refer to the use of seeds, berries, leaves, bark, root or flowers of any plant for medicinal purposes by significant number of people (Barret *et al.,* 1999). Although there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities or virtues that make them medicinally important. It has now been established that the plants which naturally synthesize and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatiles oils and contain minerals and vitamins, possess medicinal properties (Ghani, 2003). Accordingly, the World Health Organization (WHO) consultative group on medicinal plants has formulated a definition of medicinal plants in the following way: "A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs" (Goldstein *et al*., 1974). Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well

as important raw materials for the manufacture of traditional and modern medicine. Substantial amount of foreign exchange can be earned by exporting medicinal plants to other countries. In this way indigenous medicinal plants play significant role of an economy of a country (Ghani, 2003).

Extraction (as the term is pharmaceutically used) is the separation of medicinally active portions of plant using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered galenicals, named after Galen, the second century Greek physician (Remington and Beringer, 2006).

Extraction methods used pharmaceutically involves the separation of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity (Ncube *et al*., 2008).

The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products contains complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignin's (Handa *et al*., 2008).

The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion,decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydro fluorocarbon solvents). For aromatic plants, hydro distillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) may be employed.

Some of the latest extraction methods for aromatic plants include headspace trapping, solid phase micro-extraction, protoplast extraction, micro distillation, thermo-micro distillation and molecular distillation (Handa *et al*., 2008).

The basic parameters influencing the quality of an extract are (Ncube *et al*., 2008):

a. Plant part used as starting material
b. Solvent used for extraction
c. Extraction procedure

Effect of extracted plant phytochemicals depends on (Ncube *et al*., 2008):

a. The nature of the plant material
b. Its origin
c. Degree of processing
d. Moisture content
e. Particle size

The variations in different extraction methods that will affect quantity and secondary metabolite composition of an extract depend upon (Ncube *et al*., 2008):

a. Type of extraction
b. Time of extraction
c. Temperature
d. Nature of solvent
e. Solvent concentration
f. Polarity

1. Plant material

Plants are potent biochemists and have been components of phytomedicine since times immemorial; man is able to obtain from them a wondrous assortment of industrial chemicals. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc. i.e. any part of the plant may contain active components. The systematic screening of plant species with the purpose of discovering new bioactive compounds is a routine activity in many research laboratories. Scientific analysis of plant components follows a logical pathway.

Plants are collected either randomly or by following leads supplied by local healers in geographical areas where the plants are found (Parekh *et al*., 2006). Fresh or dried plant materials can be used as a source for the extraction of secondary plant components. But as many plants are used in the dry form (or as an aqueous extract) by traditional healers and due to differences in water content within different plant tissues, plants are usually air dried to a constant weight before extraction. The plant material is dried in the oven at about 40°C for 72 h. In most of the reported works, underground parts (roots, tuber, rhizome, bulb etc.) of a plant were used extensively compared with other above ground parts in search for bioactive compounds possessing antimicrobial, antifungal and antioxidant properties (Ncube *et al*., 2008; Das *et al*., 2010).

2. Choice of solvents

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants (Eloff, 1998). The choice of solvent is influenced by what is intended with the extract. Since the end product will contain the various solvents that are used in the extraction procedures are (Table-1):

1. *Water*: Water is universal solvent, used to extract plant products with antimicrobial activity. Though traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extract. Also water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics only important as antioxidant compound (Das *et al*., 2010).

2. *Alcohol:* The higher activity of the ethanolic extracts as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. It means that they are more efficient in cell walls and seeds degradation which have unipolar character and cause polyphenols to be released from cells. More useful explanation for the decrease in activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, whereas in methanol and ethanol they are inactive. Moreover, water is a better medium for the occurrence of the micro-organisms as compared to ethanol (Lapornik *et al*., 2005). The higher concentrations of more bioactive flavonoid compounds were detected with ethanol 70% due to its higher polarity than pure ethanol. By adding water to the pure ethanol up to 30% for preparing ethanol 70% the polarity of solvent was increased (Bimakr *et al*., 2010). Additionally, ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material (Wang *et al*., 2010). Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction (Cowan *et al*.,

1999). Methanol is more polar than ethanol but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results.

3. *Acetone:* Acetone dissolves many hydrophilic and lipophilic components from the plants used, it is miscible with water, is volatile and has a low toxicity to the bioassay used, it is a very useful extractant, especially for antimicrobial studies where more phenolic compounds are required to be extracted. A study reported that extraction of tannins and other phenolics was better in aqueous acetone than in aqueous methanol (Das *et al*., 2010; Eloff, 1998). Both acetone and methanol were found to extract saponins which have antimicrobial activity (Ncube *et al*., 2008).

4. *Chloroform:* Terpenoid lactones have been obtained by successive extractions of dried barks with hexane, chloroform and methanol with activity concentrating in chloroform fraction. Occasionally tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Cowan *et al*., 1999).

5. *Dichloromethane:* It is specially used for the selective extraction of only terpenoids (Cowan *et al*., 1999).

6. *Ether:* Ether is commonly used selectively for the extraction of coumarins and fatty acids (Cowan *et al*., 1999).

Structural features and activities of various phytochemicals from plants are given in table-2.

Table 1. Solvents used for active component extraction

Water	Ethanol	Methanol	Chloroform	Ether	Acetone
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Terpenoids	Flavonoids	Terpenoids	Flavonols
Tannins	Polyacetylenes	Saponins		Coumarins	
Saponins	Flavonol	Tannins		Fatty acids	
Terpenoids	Terpenoids	Xanthoxyllines			
Polypepdies	Sterols	Totarol			
Lectins	Alkaloids	Quassinoids			
		Lactones			
		Flavones			
		Phenones			
		Polyphenols			

Table 2. Structural features and activities of various phytochemicals from plants.

Phytochemicals	Structural features	Example(s)	Activities
Phenols and Polyphenols	C3 side chain, - OH groups, phenol ring	Catechol, Epicatechin, Cinnamic acid	Antimicrobial, Anthelmintic, Antidiarrhoeal
Quinones	Aromatic rings, two ketone substitutions	Hypericin	Antimicrobial
Flavones Flavonoids Flavonols	Phenolic structure, one carbonyl group Hydroxylated phenols, C6-C3 unit linked to an aromatic ring Flavones + 3-hydroxyl group	Abyssinone Chrysin, Quercetin, Rutin Totarol	Antimicrobial Antidiarrhoeal
Tannins	Polymeric phenols (Mol. Wt. 500-3000)	Ellagitannin	Antimicrobial, Anthelmintic, Antidiarrhoeal
Coumarins	Phenols made of fused benzene and α-pyrone rings	Warfarin	Antimicrobial
Terpenoids and essential oils	Acetate units + fatty acids, extensive branching and cyclized	Capsaicin	Antimicrobial Antidiarrhoeal
Alkaloids	Heterocyclic nitrogen compounds	Berberine, Piperine, Palmatine, Tetrahydropalmatine	Antimicrobial, Anthelmintic, Antidiarrhoeal
Lectins and Polypeptides	Proteins	Mannose-specific agglutinin, Fabatin	Antimicrobial
Glycosides	Sugar + non carbohydrate moiety	Amygdalin	Antidiarrhoeal
Saponins	Amphipathic glycosides	Vina-ginsenosides-R5 and -R6	Antidiarrhoeal

3. Methods of extraction

Variation in extraction methods usually depends upon:

a. Length of the extraction period,
b. Solvent used
c. pH of the solvent
d. Temperature
e. Particle size of the plant tissues
f. The solvent-to-sample ratio (Das *et al*., 2010)

The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction. Earlier studies reported that solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been used as ideal (Das *et al*., 2010).

3.1. Extraction procedures

3.1.1. Plant tissue homogenization: Plant tissue homogenization in solvent has been widely used. Dried or wet, fresh plant parts are grinded in a blender to fine particles, put in a certain quantity of solvent and shaken vigorously for 5 - 10 min or left for 24 h after which the extract is filtered. The filtrate then may be dried under reduced pressure and dissolved in the solvent to determine the concentration. Some researchers however centrifuged the filtrate for clarification of the extract [Das *et al*., 2010].After drying, crude extracts were stored in stock vials and kept in refrigerator for further use. Percent of Yield (Patel *et al*., 2010) was calculated as follows:

Extract yield % = $W_1/W_2 \times 100$

Where, W_1= Net weight of powder in grams after extraction and W_2= Total weight of sample powder in grams taken for extraction.

3.1.2. Serial exhaustive extraction: It is another common method of extraction which involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted. Sometimes Soxhlet extraction has been used for dried plant material using organic solvent. This method cannot be used for thermo labile compounds as prolonged heating may lead to degradation of compounds (Das *et al*., 2010).

3.1.3. Soxhlet extraction: Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermo labile compounds as prolonged heating may lead to degradation of compounds (Nikhal *et al*., 2010).

3.1.4. Maceration: In maceration (for fluid extract), whole or coarsely powdered plant is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved. This method is best suitable for use in case of the thermo labile drugs (Ncube *et al*., 2008).

3.1.5. Decoction: This method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume (Remington and Beringer, 2006).

3.1.6. Infusion: It is a dilute solution of the readily soluble components of the crude drugs. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water (Remington and Beringer, 2006).

3.1.7. Digestion: This is a kind of maceration in which gentle heat is applied during the maceration extraction process. It is used when moderately elevated temperature is not objectionable and the solvent efficiency of the men strum is increased thereby (Remington and Beringer, 2006).

3.1.8. Percolation: This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstrum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstrum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting (Handa *et al*., 2008).

4. Phytochemical screening

Primary phytochemical screening was carried out for all the extracts as per the standard methods.

4.1. Detection of alkaloids: Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate indicates the presence of alkaloids.

Wagner's Test: Filtrates were treated with Wagner's reagent (iodine in potassium iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

4.2. Detection of carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Fehling's Test: Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

4.3 Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test: Extracts were treated with ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

4.4 Detection of saponins

Froth Test: Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

4.5. Detection of phytosterols

Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of conc. sulphuric acid, shaken and allowed to stand.

Appearance of golden yellow colour indicates the presence of triterpenes.

Libermann Burchard's test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

4.6 Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

4.7. Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

4.8. Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

4.9. Detection of proteins and aminoacids

Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

4.10 Detection of diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes (Roopashree *et al*., 2008; Obasi *et al*., 2010; Audu *et al*., 2007).

Test for phlobatannins: Plant powder sample was mixed with distill water in a test tube, then shaked it well, and filtered to take plant extract. Then to each plant extract, 1% aqueous hydrochloric acid was added and each plant sample was then boiled with the help of Hot plate stirrer. Formation of red colored precipitate confirmed a positive result.

Conclusion

Non standardized procedures of extraction may lead to the degradation of the phytochemicals present in the plants and may lead to the variations thus leading to the lack of reproducibility. Efforts should be made to produce batches with quality as consistent as possible and to develop and follow the best extraction processes.

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In-Vivo Cytogenetic Damage in Freshwater Cyprinid Crucian Carp (*Carassius carassius* L.) upon Endosulfan Exposure

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Abstract
The *in vivo* cytogenetic damage in crucian carp, experimentally exposed to sub-lethal concentrations of endosulfan, was evaluated. The $LC_{50\text{-}96h}$ (95% confidence limits) value of endosulfan was 0.070 (0.046 -0.093) ppm; on its basis three test concentrations (sub-lethal-SL-I: 0.052, II: 0.035 and III: 0.017 ppm) were selected for *in vivo* exposure. Autopsy was done on 24, 48, 72 and 96 h post exposure for assessment of chromosomal aberrations (CA) and micronuclei formation (MN). Peripheral blood samples, withdrawn by caudal puncture, were used for micro-nuclei assay and chromosome preparations were made from highly mitotic anterior kidney cells. All the tested concentrations of endosulfan showed increased frequencies of CA and MN in a concentration-dependent manner, with induction of maximum genotoxic effects at highest concentration (SL-I; $p < 0.05$). The group exposed to positive genotoxin, cyclophosphamide also showed significant induction ($p < 0.05$) of CA and MN. The results of the present investigation indicated that endosulfan could potentially induce genotoxic effects in fish, even at sub-lethal concentrations and among the numerous bioindicators used in the context of water monitoring, genotoxicity assessment is one of the most important tools. The potential role of these parameters as bioindicators of aquatic pollutants is discussed.
Keywords: Endosulfan, fish, micronucleus, chromosomal aberration, environmental monitoring.

Introduction

Although modern agriculture seeks to achieve a sustainable use of agro chemicals, the amount of pesticides applied in pest control still represents one of the major burdens to the environment. Environmental pollution caused by pesticides, especially in aquatic ecosystems, poses a potential threat for aquatic organisms, and ultimately the entire food chain (Velisek *et al*., 2010; Ondarza *et al*., 2014). Contamination of aquatic bodies has been well-documented worldwide and constitutes a major issue at local, regional, national, and global levels (Angélique *et al*., 2013).

Some Persistent Organic Pollutants (POPs) are found in the environment and can bioaccumulate in an organism along the food chain. Endosulfan, a cyclodiene insecticide, is one such organochlorine compound that has been classified as highly toxic by the majority of environmental protection agencies (Dar *et al*., 2015). In India, endosulfan is classified as an ''extremely hazardous'' pesticide (Ganeshwade *et al*., 2012). Although its use is restricted to certain crops, residues of this compound have been detected in aquatic environment, and it

consequently affects the fish that play a highly important role at the tropic level. Therefore, there is an urgent demand for studies that can correlate the effects of this pesticide in fish (Hoang *et al*., 2011).

It has been suggested that the progress of environmental toxicology requires the development of a battery of bioindicators to evaluate chemical hazards (Brain and Cedergreen, 2008). In the biomarker selection sensitivity is the key factor because greater the biomarker sensitivity to the xenobiotic exposure the earlier will be its response, avoiding occurrence of deleterious effects on the organism or the population (Den Besten and Munawar, 2005).

Toxic effects of pesticides have been studied in several fish species (Monteiro *et al*., 2006; Toni *et al*., 2010). The use of fish as a bio-indicator of pollutant effects is being more and more used since fish are very sensitive to changes in their environment and play significant roles in assessing potential risks associated with contamination of new chemicals in aquatic environment (Lakra and Nagpure, 2009). Ecotoxicological characteristics of freshwater fish, *Carassius carassius* L., such as its wide distribution, availability throughout the year, easy maintenance and commercial importance makes it an excellent model for toxicity studies. Since there is growing concern over the presence of pesticide residues in the aquatic environment, it is important to develop or standardize existing methods for assessing the deleterious effects of xenobiotics in aquatic organisms (Dar *et al*., 2014).

Negative effects of endosulfan on fish have been well documented including histological (Ballesteros *et al*., 2007), physiological (Dorval *et al*., 2003), hematological (Rehman, 2006), neurological (Dutta and Arends, 2003), behavioural (Rehman, 2006), immunological (Ganeshwade *et al*., 2012) and endocrine-disrupting potential (Bisson and Hontela, 2002), but there is a dearth of data on the cytogenetic damage induced by endosulfan at environmentally realistic concentrations. This is the aim of the present study.

Materials and Methods

Experimental fish and chemicals

Healthy fish specimen of *C. carassius* L. (Family: Cyprinidae and Order: Cypriniformes) of a length of 12.5 ± 1.6 cm and weight of 33 ± 5 g were procured with the help of a local fisherman from the Dal Lake (34°07′N 74°52′E) in the vicinity of the University of Kashmir, Srinagar, India, transported live to the laboratory, and subjected to a prophylactic treatment by bathing in a 0.05 % aqueous solution of potassium permanganate for 2 min to avoid dermal infection. The fish stock was then acclimatized for at least 3 weeks to a 1:1 diurnal photoperiod in well aerated 60 L glass aquaria with 24 h aged dechlorinated tap water (pH 7.6 – 8.4) and fed *ad libitum* daily with commercially available fish food (Feed Royal®, Maa Agro Foods, Visakhapatnam, Andhra Pradesh, India). Waste products were siphoned off daily to prevent increase of ammonia in the water. Every effort as suggested by Bennett and Dooley (1982) was taken to maintain optimal conditions during acclimatization: no fish died during this period. Endosulfan and cyclophosphamide were purchased from the Sigma Aldrich, Bengaluru, India.

Determination of acute toxicity

Determination of the $LC_{50\text{-}96h}$ of endosulfan to *C. carassius* was conducted in a semi-static system with 60 L glass aquaria, changing the endosulfan solution (99.5 % pure) every alternate day to maintain its similar concentration. Briefly, triplicate sets of 10 fish each were exposed to endosulfan at concentrations of 0.01, 0.03, 0.05, 0.07, 0.09, 0.1, 0.5 and 1 ppm derived from a range finding test. Fish were not fed throughout the experiment and lethality was the toxicity end-point. Fish were visually examined daily and considered dead when no respiratory movements or no sudden swimming in response to gentle touching were observed. The $LC_{50\text{-}96h}$ of endosulfan was determined by probit analysis (Finney and Stevens, 1948).

In- vivo exposure

The experiments consisted of five treatments each with 4 replicates, in total 20 aquaria, containing 60 L dechlorinated and well-aerated tap water with 10 fish in each aquarium. Fish maintained in dechlorinated tap water

served as negative (treatment 1) and those exposed to cyclophosphamide at a concentration of 4 ppm (Özkan *et al*., 2011) as positive control (treatment 2). In treatments 3 – 5, the fish were kept in water containing endosulfan at concentrations of 0.052 (SL-I; 3/4 of $LC_{50\text{-}96h}$), 0.035 (SL-II; 1/2 of $LC_{50\text{-}96h}$) and 0.017 ppm (SL-III; 1/4th of $LC_{50\text{-}96h}$) and autopsy was done at 24, 48, 72 and 96 h. On each sampling interval, ten fish were sacrificed; five fish were processed for the chromosomal aberration test (0.05% colchicine treatment was given prior to 3 h of autopsy) and the micronucleus assay was carried out from the blood of the rest five fish as per standard protocols (Dar *et al*., 2014, 2015). Some important physico-chemical properties of test water like temperature 18.2–23.3°C, pH 7.5–8.4, dissolved oxygen 7.9–8.4 mgL^{-1}, total alkalinity 69 –73 mgL^{-1} and ammonical nitrogen 25–29 μgL^{-1} were analyzed throughout the study by standard methods (APHA, AWWA and WPCF, 2005).

Micronucleus test

Blood samples were withdrawn by caudal puncture and peripheral blood smears were immediately made by applying two micro drops of blood on precleaned slides using the standard method of Al-Sabti and Metcalfe (1995). For every sampling time, replicate slides per specimen were prepared and a minimum of 10,000 erythrocytes scored in each treatment group, were examined for the presence of MN. The frequency of MN/fish was calculated per 1000 cells (Raisuddin and Jha, 2004), and was evaluated by scoring the slides under oil immersion at 1000x magnification using Olympus BX 50 microscope (Tokyo, Japan). Coded and randomized slides were scored using blind review by a single observer to avoid any technical variation. The criteria for the identification of micronuclei were according to standard procedures (Fenech *et al*., 2003).

Chromosomal aberration test

Chromosome preparations were made from the highly mitotic head kidney cells, following the techniques of Cucchi and Baruffaldi (1990), and observed with light microscope (100x) for chromosomal aberrations. Replicate slides were selected per fish and a minimum of 25 metaphases were scored from each slide in each group including control (since n = 5 per group/exposure time, minimum 250 metaphases). Notwithstanding the conventional method of scoring, the CA was recorded under two broad categories i.e. classical aberration and non classical aberration. In the classical aberrations, both chromosome and chromatid type breaks, including acentric fragments, sister chromatid union and multiple aberrations (polyploidy, aneuploidy, rings etc) were counted and non-classical aberration comprised of stickiness, pulverization and c-metaphases.

Statistical analysis

Probit analysis was performed with the SPSS (version 16.0) computer program (SPSS Inc. Chicago, IL, USA), with the consultation of Finney's table (Finney and Stevens, 1948). Data was compared for statistically significant difference between control and treatment groups using one-way analysis of variance (ANOVA). Significant difference in ANOVA were further analyzed by post-hoc Bonferroni's, Newman-Keuls and Dunnett's multiple comparison test using Graph Pad Prism 5 software (Graph Pad Software, Inc. San Diego, CA). The p-values less than 0.05 were considered statistically significant.

Results

Acute toxicity

In acute toxicity bioassay, the LC_{50} values (with 95% confidence limits) of different concentration of endosulfan in *C. carassius* (Fig. 1) were found to be 0.215 (0.158-0.272), 0.15 (0.112-0.191), 0.095 (0.075-0.114) and 0.070 (0.046-0.093) ppm for 24, 48, 72 and 96 h, respectively. A dose dependent increase and time dependent decrease were observed in mortality rate such that as the exposure time increases from 24 to 96 h, the median concentration

required to kill thefish was reduced. Based on the $LC_{50\text{-}96h}$ value, the SL-I, II and III were determined as 0.052, 0.035 and 0.017 ppm, which were further used for *in vivo* exposure.

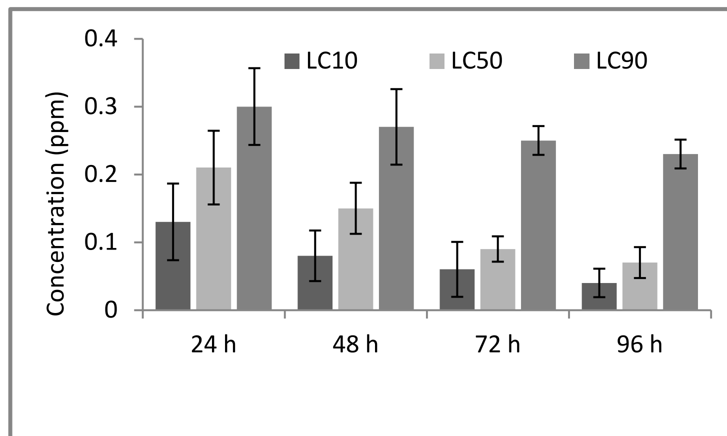

Fig. 1. Lethal concentration (LC) of endosulfan depending on exposure time for *C. carassius*.

Micronuclei Induction

The results of MN induction in peripheral blood erythrocytes of *C. carassius* after exposure to different concentrations of endosulfan are presented in Table 1. It caused one, two and three micronucleated cells but single MN was predominant in the erythrocytes analyzed (Fig. 2). The induction was signficantly ($P \leq 0.05$) hi gher in all the treatment groups compared to the control at all the exposure durations. The maximum MN frequency was observed on day 4 (6.071%; $p < 0.001$) at the highest concentration (SL-I), followed by SL-II and SL-III concentrations. Treatment with genotoxic agent (cyclophosphamide; positive control) also resulted in an extremely significant increase ($p < 0.001$) in the MN frequencies at all the sampling intervals. A concentration-dependent response in MN induction was observed.

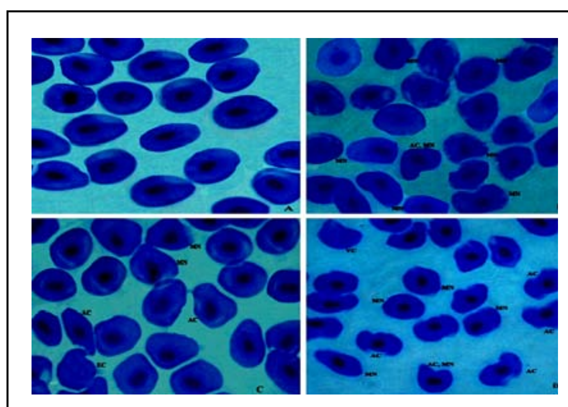

Fig. 2. Some features of erythrocyte alterations and micronuclei induction by endosulfan in *C. carassius* (96 h). A: normal control, B: SL-I (0.017 ppm), C: SL-II (0.035ppm), D: SL-III (0.052 ppm), single micronucleus (MN), two micronuclei (TM), altered cell (AC) and enucleated (EN) condition is unique to Endosulfan (magnification 1000×).

Table 1. Mean (S.E.) percentage micronuclei frequency in peripheral erythrocytes of *C. carassius* exposed to sub-lethal concentrations of endosulfan (n =~ 10000 cells/concentration/exposure time).

Chemical	Conc. (ppm)	Exposure time (days)			
		1	2	3	4
NC	----	0.181 (0.007)	0.230 (0.023)	0.191 (0.016)	0.300 0.015)^{3}
PC	4.00	3.483 (0.126)^{C}	4.100 (0.060)^{C3}	4.386 (0.045)^{C3}	7.526 (0.113)^{C3}
Endosulfan					
SL I	0.052	2.590 (0.080)^{C}	3.171 (0.065)^{C3}	3.575 (0.028)^{C2}	6.071(0.058)^{C3}
SL II	0.035	1.270 (0.021)^{C}	1.801 (0.022)^{C3}	2.168 (0.046)^{C2}	2.550(0.033)^{C2}
SL III	0.017	0.671 (0.017)^{A}	0.911 (0.016)^{A3}	1.010 (0.028)^{A2}	1.406 (0.025)^{A3}

NC: Negative control (tap water), PC: Positive control (cyclophosphamide), SL I: Sub lethal I (¾of LC_{50}), SL II: Sub lethal II (½ of LC_{50}), SL III: Sub lethal (¼ of LC_{50}). Values with different letter superscripts ($^{A}p<0.05$: significant. $^{B}p<0.01$: highly significant. $^{C}p<0.001$: extremely significant) differ significantly from the negative control, whereas values with different numeric superscripts ($^{1}p<0.05$: significant. $^{2}p<0.01$: highly significant. $^{3}p<0.001$: extremely significant) differ significantly between exposure times within concentration.

Chromosomal aberrations

The typical diploid metaphase complements ofish, *C. carassius*, were found to consist of 100 chromosomes of four types such as submetacentric, metacentric, subtelocentric and acrocentric. Various forms of chromosome damage recorded were chromosome and chromatid breaks, fragments, sister chromatid union, dicentric, multiple aberrations, stickiness, pulverization and c-metaphases; whereas gaps were excluded. The frequency of CA observed in *C. carassius* after exposure to different concentrations of endosulfan and cyclophosphamide were significantly ($P \leq 0.05$) higher when compared to the control 1 (Table 2, Fig. 3), at all the exposure durations, and the chromatid and chromosome breaks were more frequent than the other types of aberrations. The maximum CA, like MN frequency, was observed on day 4 (12.14%; $p < 0.01$) at the highest concentration (SL-I). In general, a concentration-dependent response was also observed in case of CA.

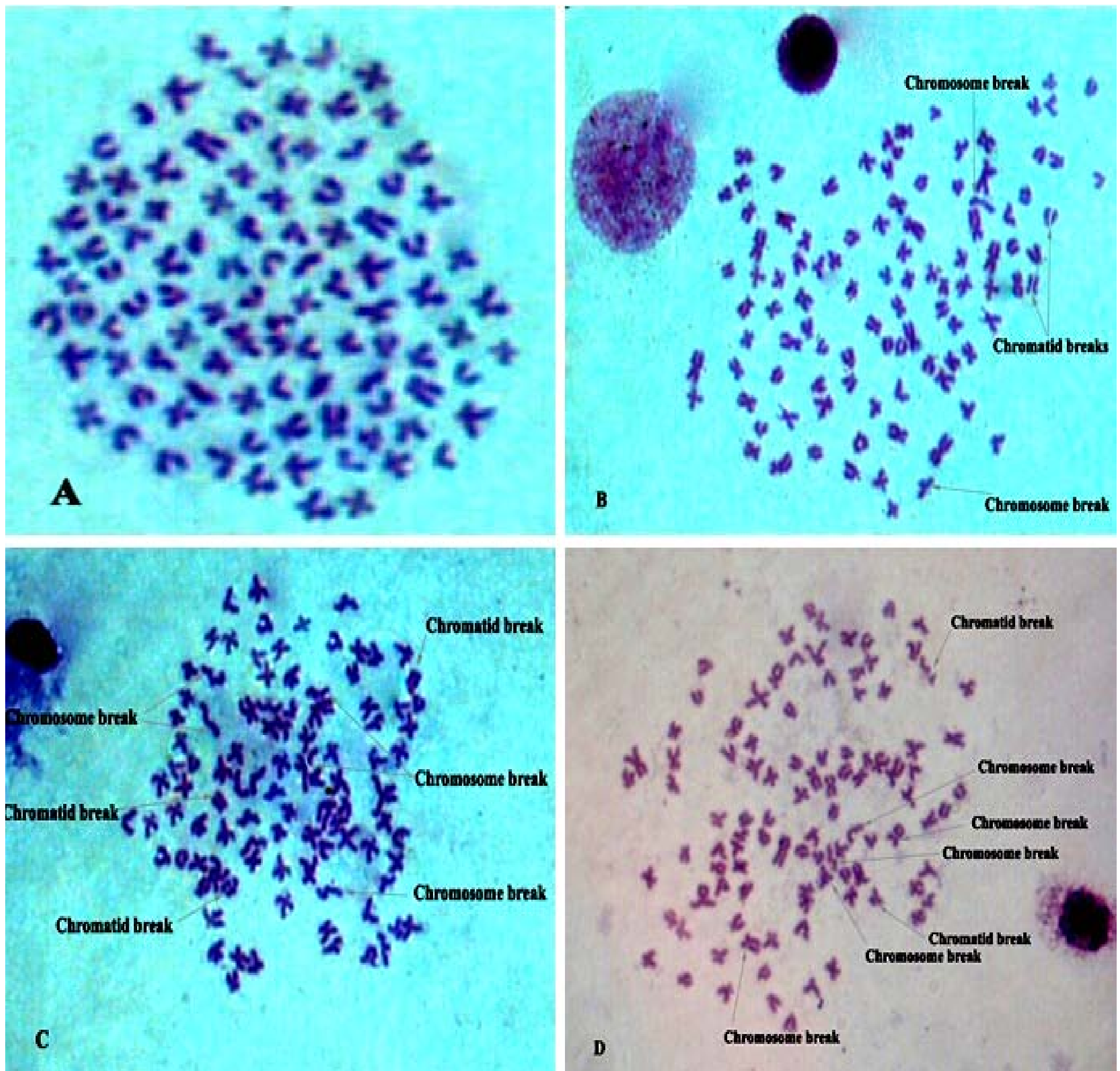

Fig. 3: Metaphase plates prepared from kidney cell of *Carassius carassius* showing (A) normal chromosomes (2n = 100), (B, C and D) chromosomal aberrations from endosulfan exposeflsh (SL I-0.052, SL II-0.035, SL III-0.017 ppm), respectively for 96 h.

Table 2. Chromosomal aberration frequencies induced by endosulfan in *Carassius carassius* kidney cells

Exp. days	Treatment	TMS	Classical aberrations						Non-classical aberr.		TA mean (%) ± S.D.
			Csb	Ctb	Frg	Scu	Dic	Mla	Stp	Cmt	
1	NC	103	1	–	–	–	–	–	1	–	1.94 ± 0.13^{2}
	PC	105	3	1	2	–	–	–	2	1	8.57 ± 0.41^{B}
	SL I	113	2	1	1	–	–	–	1	2	6.19 ± 0.30^{B2}
	SL II	101	2	1	1	–	–	–	1	–	4.95 ± 0.25^{B2}
	SL III	107	1	2	–	–	–	–	–	1	3.73 ± 0.23^{A2}
2	NC	105	2	1	–	–	–	–	–	–	2.85 ± 0.21^{2}
	PC	109	3	2	3	–	–	–	1	1	9.17 ± 0.46^{B}
	SL I	117	2	2	1	1	1	–	2	–	7.69 ± 0.37^{B1}
	SL II	108	1	2	1	–	–	–	1	1	5.55 ± 0.26^{B2}
	SL III	113	1	2	–	–	–	–	1	1	4.42 ± 0.20^{A2}
3	NC	104	1	1	–	–	–	–	1	–	2.88 ± 0.16^{2}
	PC	115	3	2	2	1	1	1	2	–	10.43 ± 0.44^{B}
	SL I	110	2	2	1	1	1	–	2	1	9.09 ± 0.37^{B}
	SL II	106	3	1	1	–	–	1	1	–	6.60 ± 0.34^{B2}
	SL III	109	2	2	1	–	–	1	–	–	5.50 ± 0.29^{B2}
4	NC	119	2	1	–	–	–	–	1	–	3.36 ± 0.22^{2}
	PC	116	3	3	2	2	2	2	2	2	15.51 ± 0.58^{B}
	SL I	107	2	2	1	2	2	2	1	1	12.14 ± 0.47^{B2}
	SL II	102	2	1	1	–	1	1	1	1	7.84 ± 0.30^{B2}
	SL III	112	2	1	1	1	1	–	1	–	6.25 ± 0.27^{B2}

Exp: Exposure time in days. **TMS:** Total metaphasic plates studied. **NC:** Negative control (tap water). **PC**: Positive control (cyclophosphamide: 4 ppm). **SL I:** Sub lethal I (1/25 of LC_{50}: 0.052 ppm). **SL II**: Sub lethal II (1/50 of LC_{50}: 0.035 ppm). **SL III:** Sublethal III (1/75 of LC_{50}: 0.017 ppm). **Csb:** chromosome break**. Ctb:** Chromatid break. **Frg:** fragment. **Scu:** sister chromatid union. **Dic:** dicentric. **Mla:** multiple aberrations. **Stp:** stickiness and pulverization. **Cmt:** c-metaphase. Values with different letter superscripts differ significantly from the negative control (Newman-Keuls and Dunnett's multiple comparison tests), whereas values with different numeric superscripts differ significantly from the positive control (Dunnett's multiple comparison test).

Discussion

At present, more than 1000 chemicals have been classified as pesticides and studies using different models have indicated that some of them have genotoxic properties (Zeljezic and Garaj-Vrhovac, 2002). Fish are often used as sentinel organism for ecotoxicological studies because they play a number of roles in the tropic web, accumulate toxic substances and respond to low concentration of mutagens in a similar way to higher vertebrates (Osman *et al*., 2007).

The LC_{50-96h} value of the endosulfan in the present study was 0.070 ppm which indicated that it is very toxic to fish. Our estimate is higher than the LC_{50-96h} value of 0.0035 ppm for *Channa striatus* (Ganeshwade *et al*., 2012). The variation may be due to the difference and hardiness of the test species and water quality parameters. Micronucleus test, as a genotoxic endpoint, for clastogenic effects of pollutants has been extensively used in fish such as prussian carp (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), tilapias (*Oreochromis mossambicus*) and salmoniform fish (*Umbra pygmea*) (Vernier *et al*., 1997). In the present study, all concentrations of endosulfan induced significantly higher number of MN in ery throcytes and CA in head kidney cells compared to the control and their frequency increased in concentrations and time dependent manner. These results are more environmentally relevant than previous studies, which have typically used injection as the route of exposure, because waterborne exposure is more realistic of what occurs in nature. Presumably, endosulfan has affected the genetic material by absorption through the gill epithelium. Earlier, it has been emphasized that exposure of fish to genotoxic chemicals, for various interval of time, by the respiratory route following the absorption of chemicals through gill epithelium could be occurred (Rishi and Grewal, 1995; Farah *et al*., 2006). Our results are in agreement with some earlier studies (Bahari *et al*., 1994; Ali *et al*., 2009; Dar *et al*., 2015), which have reported the induction of MN from exposure to various xenobiotics present in the aquatic environment. An advantage of chromosomal studies is that they reveal a measure of sub-lethal effects of xenobiotics *in vivo*. The CA were more at higher as compared to lower concentrations tested, throughout the post exposure, except at the termination of the experiment where the CA showed the constancy effect at all the tested concentrations, as reported in case of dichlorvos impacts on *Channa punctatus* (Rishi and Grewal, 1995). The chromatid and chromosome breaks were more frequent than the other types of aberrations at all the exposure durations in our study. An increase in chromatid break and chromosomal exchange due to water pollution has also been reported (Chaurasia *et al*., 2007) Similar results have also been reported (Yadav and Trivedi, 2009) in *Orechromis mosambicus* on exposure to various xenobiotics. The current study, thus, emphasized that the CA and MN assays are sensitive biological makers for evaluating the genotoxic effects of various clastogenic xenobiotics, especially in the aquatic environment.

Conclusions

Considering the mutagenic and genotoxic effects of endosulfan on *C. carassius* obtained in this study by MN and CA assays, there is serious apprehension about the potential danger of this pesticide to aquatic organisms, especially to fish, and indirectly to human beings. Moreover, in the absence of other convenient or practical methods, the MN and CA will continue to play an important role in assessing the genotoxicity induced by pesticides. Information obtained through these integrated studies in fish model may be used as bioindicators for monitoring the genomic damage from environmentally hazardous contaminants in the aquatic environment.

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Alteration in Hematology of *Triplophysa marmorata* Under the Stress of Pollution from Water Bodies of Kashmir Valley

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Abstract
Blood is an indicator of physiological condition of an animal. Therefore, a field study was conducted to investigate the hematological parameters of Kashmir loach, *Triplophysa marmorata* Heckel collected from Sind and Telbal streams. Blood parameters such as hemoglobin, erythrocyte count, total leucocyte count, hematocrit, differential leucocyte count (DLC) of fish were determined. Statistical analysis revealed noticeable differences in RBC and WBC count and PCV values. A significant decrease was marked in RBC count and PCV values ($p<0.001$), whereas an increase in leucocyte count was observed in fish from Telbal stream. The variation in values of different parameters from these water bodies can be attributed to the presence of various toxicants of pollutants in the water body (Telbal stream).
Keywords: *Triplophysa marmorata*, hemoglobin, hematology, RBC, WBC, PCV, pollution.

Introduction

Blood is known to exhibit pathological changes before the onset of any external symptoms of toxicity (Sampath *et al.,* 1998). Since changes in various environmental parameters result in a new steady state of physiological reactions, patterns of these reactions are expected to provide some indication that the organism is faced with. Fishes can be used as a measure of environmental health, as they are in direct contact with their environment and are as such susceptible to any change that may occur in it. It is expected that such changes would be reflected in the physiology of the fish and particularly in the values of hematological parameters (Blaxhall, 1972). Blood is therefore recognized as a potential index of fish response to water quality (Hickey, 1982), as it can be used to ascertain the effect of pollutants in the environment. Blood parameters have been commonly used to observe and follow fish health, since variations in blood tissue of fish are caused by environmental stress (Shah and Altindag, 2005; Bhaskar and Rao, 1985). Blood parameters in fish have been studied to elucidate physiological adaptation and to assess the health of fishes (Vazquez and Guerrero, 2007). Bouck and Ball (1966) stated that hematology may be a useful tool in monitoring stress levels of aquatic pollution on fish. Hematological parameters are increasingly used as indicators of the physiological stress response to endogenous and exogenous changes in fish (Adams, 1990; Santos and Pacheco, 1996; Cataldi *et al.*, 1998).

Hematological abnormalities have also been studied in various toxicants-exposed fish: *Channa punctatus* to lead (Hymavathi and Rao 2000); *Cyprinus carpio* to carbofuran (Chandra *et al.,* 2001); *C. punctatus* to cadmium (Karuppasamy *et al*., 2005) and *Labeo rohita* to synthetic detergents and sublethal levels of nitrite (Chellan *et al.*, 1999; Acharya *et al*., 2005). Changes in hemoglobin content under toxic stress are reflected on the oxygen consumption and metabolism. Since oxygen transport in blood depends upon the hemoglobin contents of erythrocytes in blood of fish, the erythrocyte count (TEC) and hemoglobin (Hb) content are taken as reflection of the pollution stress. Studies have shown that when the water quality is affected by toxicants, any physiological

changes will be reflected in the values of one or more of the hematological parameters (Van Vuren, 1986). McLeavy and Brown (1974) reported leukocytosis in zinc-treated fish, *Oncorhynchus kisutch*, due to tissue damage and subsequent removal of debris.

Interestingly, the hematology of fish continues to offer valuable diagnostic tool and progress in establishing normal range values for blood parameters of different fish species, but the information regarding Triplophysa species is limited.

Kashmir loach (Genus *Triplophysa* of sub family Nemachilinae and Family Balitoridae), locally known as 'Ara gurun', is a small fish having elongated and scale-less body, with eyes high on head, and inferior mouth having two rostral, and one maxillary pair of barbells. The Kashmir loaches normally live among pebble and shingle at the bottom of clear rocky streams but some drift into lakes among the hills and become secondarily modified for life in deeper waters (Hora, 1937). The fish are also considered to be very tasty and are a source of cheap nutrition for the poor. Therefore, considering the importance of hematological parameters as indicators of fish health, the present work dealing with synergetic effect of various pollutants on fish from two water bodies was investigated.

Materials and Methods

Live and Healthy fishes were collected from Telbal stream (Fig. 1) (34° 08' 41" N – 74° 50' 58" E) carrying the wastes from the adjacent agricultural lands in addition to domestic municipal effluents and control Sind stream (34° 16' 59.5" N - 74° 49' 20.2" E) (fig.2). Fish samples were collected during the year 2012.

Fig. 1. A view of Sind stream near study site

Fig. 2. A view of Telbal stream near study site

Physico-chemical features

The physico-chemical characteristics of water were analysed as per the methods described by the CSIR (1974), Mackereth *et al.,* (1978) and APHA (1998). Water temperature and pH were recorded on the spot, whereas for the estimation of dissolved oxygen water samples were fixed at the sampling site in accordance with the azide modification of Winkler method (APHA, 1998). Measurements were made using the following equipment/method(s): water temperature – Celsius mercury thermometer calibrated up to 0.1°C; hydrogen ion concentration - digital pH meter (Microprocessor pH System-1011E); Total alkalinity – Mackereth *et al*., (1978); Ammonical nitrogen-phenate method (APHA, 1998) and Nitrate-nitrogen - Salicylate method (CSIR, 1974).

Fish collection

Live specimens of the Kashmiri Loach *T. marmorata* (Heckel,1838) were captured with the help of local fisherman and transported to the laboratory within 2 hr after capture into tanks containing 20 L of water. Both sexes were used without discrimination. Hematological parameters were investigated in healthy specimens collected from the sampling area. In the laboratory blood was collected within 24 h after the capture by dissection of the caudal peduncle (Roberts., 1981), into a vial containing dried or powdered potassium salt of ethylene diamine tetra acetic acid (EDTA) as anticoagulant to give a concentration of 5mg/ml of blood samples. The blood sample was rocked gently in the vial to allow thorough mixing of its contents. A further 0.5 ml was taken without EDTA and used to prepare blood films. The blood samples were taken in the morning hours. Fishes were physically examined for any sign of infection or diseased condition (Noga, 1993), and only data from fishes with no sign of infection were used.

Hematological analysis

The Haemoglobin (Hb) content of the blood samples was estimated by the cyanomethaemoglobin method (Brown, 1980). The number of red and white blood cells was determined using a Neubauer haemocytometer after the blood has been diluted with Dacie's fluid (Dacie and lewis, 2001).The Haematocrit (PCV) was determined using wintrobes tube method according to Ramnik (1994). Differential counts, Neutrophils, lymphocytes and monocytes were done on blood film stained with Grumwald Giemsa stain as described by Dacie and lewis method (2001).

Results and Discussion

The physico-chemical parameters of water collected from Sind and Telbal streams are presented in Table 1. The water temperature ranged from 15°C to 26°C, pH from 8.0 to 8.3, dissolved oxygen from 7.2 to 8.8 mg/l, free CO_2 from 7 to14 mg/l, Alkalinity from 50 to 175 mg/l, Ammonia from 17 to 97 mg/l, nitrate nitrogen from 119 to 220 µg/l, while as Orthophosphorus from 15 to 123 µg/l and total Phosphorus ranged from 30 to 194 µg/l. A comparison of the two water bodies showed the least oxygen concentration in the telbal water body. Due to continuous fast current, especially in case of Sind Nalla, the running waters contain relatively higher concentration of oxygen than the water of telbal (Vass *et al.*, 1977 and Qadri *et al.*, 1981). The higher value of the free carbon dioxide content has been related to the eutrophication/pollution of water (Todda, 1970 and Coole, 1979). This is also confirmed by our data from Telbal Nalla, which show much higher concentration than the Sind Nalla. Same was true with respect to pH value as well as total alkalinity. Level of phosphorus and inorganic nitrogen in Telbal was much higher than the Sind Nalla. On the basis of physico-chemical limnology it can be said that Sind stream, which is comparatively free from pollution, as compared to the Telbal is best suited for the growth of *Triplophysa marmorata*. Hematological values of the *T .marmorata* from the two habitats are given in Table 2. Definite variations between the study areas were detected in all hematological values. The hemoglobin value of fish decreased at Telbal compared to Sind stream. A similar trend was observed in RBC value. The mean haematocrit values of *T. marmorata* from the two water bodies were within the range of 21 – 26%. PCV decreased significantly ($P < 0.001$) in Telbal stream in comparison to Sind. Study shows an increase in WBC quantity and leukocyte cell proportions (neutrophil, monocyte) in the fish specimens from Telbal stream.

Water parameters are one of the major factors responsible for individual variation in fish hematology. Blood is the most important fluid in the body and its composition often reflects the total physiological condition of an organism. In natural habitat, fish species are pact with different factors such as varied water qualities, pollution, malnutrition, infection and disease, and can adapt themselves such environmental conditions by changing their physiological activities. Although all the above factors are linked to fish health, it is essential to establish and identify the causes of disease in fish which presents as a challenge for the researchers and farmers (Pradhan *et al.*, 2011). Water quality is an important factor, which is responsible for variations in fish hematology, since fishes live

in close association with their environment (Casillas and Smith, 1977). The physico-chemical parameters of the different sites showed that telbal is more polluted than Sind. This different eutrophic status of the water bodies has their bearing on the population and density of the *T. marmorata*. Hemoglobin serves to transport oxygen from gills to different tissues of the fish in the form of oxyhemoglobin and carbon dioxide from tissue to the gills in the form of carboxyhemoglobin. The mean hemoglobin value of *T. marmorata* decreased at Telbal compared to Sind stream. The low Hb value in a fish exposed to pollutant may be related to the inhibitory effect of those substances on the enzyme system responsible for synthesis of hemoglobin (Pamila *et al*., 1991). The pollutants entering into fish system is slowly eliminated (Newman and Mitz, 1988; James and Sampath, 1996 and James *et al*., 1996) and hence the blood parameters get affected on account of pollutant toxicity. The low Hb value in Telbal water body may also be associated with less active fishes. Similar results were reported by Engel and Davis (1964) and Rambhaskar and Srinivasa (1986). Eisler (1965) suggested that there was a correlation between hemoglobin concentration and activity of fish. The more active fishes tend to have higher hemoglobin values than the more sedentary ones (Pradhan *et al*., 2011). Consequently, *Pleuronectes annectens* being a relatively quiet and sedentary species (Okafor, 2006) has a slightly lower hemoglobin concentration than more active African teleost such as *Clarias buthupogon* whose mean hemoglobin concentration is as high as 9.88g/dl (Kori-Siakpero and Egor, 1997). The comparatively high hemoglobin content in *T. marmorata* from Sind stream may be related to its preferred environmental conditions. The count of red blood cells is quite a stable index and the fish body tries to maintain this count within the limits of certain physiological standards using various physiological mechanisms of compensation. Any alteration in the number (quantitative) or morphology (qualitative) of RBCs from normal values can cause various pathological disorders in fish under stressful conditions**.** The high erythrocyte number was associated with fast movement, predaceous nature, and high activity with streamlined body (Rambhaskar and Srinivasa, 1986). The RBC value of *T. marmorata* decreased significantly ($P<0.001$) at Telbal compared to Sind stream. A fall in RBCs count, Hb% and PCV%, in the fishes, due to water pollution, has been reported along with acute anaemia (Singh, 1995). According to Singh *et al*., (2002) the discharge of waste may cause serious problems as they impart odour and can be toxic to aquatic animals. The organic wastes present in Telbal stream seem to cause stress in the fish and as such seem to be responsible for the changes in the hematological parameters. Lowering of TEC count coupled with low Hb content may be due to destructive action of pollutants on erythrocytes as a result of which the viability of the cells may be getting affected (Karuppasamy, 2000 and Zutshi *et al*., 2010). PCV or Haematocrit is an important tool for determining the amount of plasma and corpuscles in the blood (measurement of packed erythrocytes) and is used to determine the oxygen carrying capacity of blood (Larsson *et al*., 1985). Hematocrit or PCV in the present study decreased significantly ($P < 0.001$) in Telbal stream in comparison to Sind stream. Baxhall and Daisely (1973) have reported the possibility of using haematocrit as a tool in aquaculture and fishery management for checking anaemic condition. Reported values for fish haematocrit are usually between 20% and 35% and scarcely attain values greater than 50% (Clark *et al*., 1979). Joshi *et al*., (2002) and Banerjee & Banerjee (1988) have suggested that pollutant exposure decreases the TEC count, Hb content, and PCV value due to impaired intestinal absorption of iron. PCV values always decrease when a fish loses appetite or becomes diseased or stressed (Zutshi *et al.,* 2010). Similar results were obtained by Larsson *et al*., (1985) after exposure of fish to $KMnO_4$, indicative of anaemia and haemodilution, possibly due to gill damage or/and impaired osmoregulation. In fish, as in mammals, blood cells including WBC are frequently used as indicators of health status in fish because WBC are key components of innate immune defense and leukocytes are involved in the regulation of immunological function in the organism (Duthie and Tort 1985; Gallardo *et al*., 2003; Ballarin *et al*., 2004). There was variation in WBC quantity and leukocyte cell proportions (neutrophil, monocyte) in the fish specimens from Telbal stream. The implication of this result is that the fish has been able to defend itself from invading pathogens both by cell-and antibody-mediated responses (Kumar *et al*., 1992). Similar results

were obtained by Sahan and Cengizler (2002) on carp caught from different regions of Seyhan River. Increased levels of TLC have been reported in *Channa punctatus* exposed to lead (Hymavathi and Rao, 2000) and *Clarias batrachus* exposed to mercuric chloride (Joshi *et al*., 2002). Leukocytosis is directly proportional to severity of stress condition in maturing fish and is a result of direct stimulation of immunological defense due to the presence of pollutants in water bodies. This is in conformity with the report by Saravanan and Harikrishnan (1999) in freshwater fish, *Sarotherodon mossambicus*, when exposed to sublethal concentration of copper and endosulfan and by Nanda (1997) in respect of *Heteropneustes fossilis* during nickel intoxication. This may be attributed to alteration in blood parameters and direct effects of various pollutants. These observations are also in good agreement with those of Karuppasamy *et al*., (2005) and Hardikar and Gokhale (2000). In fish, any infestation with any organism activates the cellular and humoral immune system. This is followed by changes in circulating antibodies and percentages and absolute number of the different WBC (Boon *et al*., 1990). Weinreb (1958) used leukocyte count changes as a means of assessing the systematic response of the rainbow trout, *Salmo gairdneri richardsoni* to various injections. Mishra and Srivastava (1980) also reported an increase in leucocytes count of fish due to pollution. Some of the most common causes of pollutant toxicity are inflammatory lesions associated with tissue damage; anemia and neoplasia. The lymphocytes are reported to be responsible for immune response, while neutrophils are reported to show the greatest sensitivity to change in the environment. Their characterization and identification is, therefore, of significance for assessing the changes in the physiological state of fishes. The percentage of these cell types generally decreases during acute exposure to copper (Nussey *et al*., 1995 and Svobodova *et al*., 1994), and in situations of chronic copper exposure, the neutrophil percentage has been reported to increase (Dick and Dixon, 1985). In the present study, the increases in WBC and neutrophil quantities in the samples collected from Telbal seem to be a response of cellular immune system to pollution (Palikova and Navratil, 2001; Şahan and Cengizler, 2002 and Saravanan *et al.,* 2003). The TEC, Hb and PCV and similar other indices based on blood parameters provide vital clues about the overall health of the fish vis-à-vis the condition of the fish environment.

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Table 1. Some important physico-chemical parameters (Mean ± S.D) of the water bodies

Parameters	Sind	Telbal
Water Temp. ^{0}C	20.66±7.57	17±2.64
pH	8.1±0.1	8.0±0.36
Dissolved Oxygen(mg/l)	8.13±0.83	7.43±0.35
Free Co_2 (mg/l)	8.33±1.52	12.66±1.52
Alkalinity(mg/l)	66±14.42	161±18.52
Ammonical-Nitrogen (µg/l)	22±4.58	60.33±32.51
Nitrate-Nitrogen (µg/l)	153±49.66	167±48.13
Orthophosphorus (µg/l)	26.33±11.50	87±31.32
Total phosphorus (µg/l)	44±14	166.33±28.00

Table 2: Mean values of the Hematological parameters of *T. marmorata* from Sind and Telbal stream

Parameters	Sind	Telbal
Hb(g/dl)	10.43±0.10	9.65±0.91
RBC($10^6/mm^3$)	1.37±0.01***	1.19±0.01***
PCV (%)	26.61±0.55***	21.54±0.79***
WBC($10^4/mm^3$)	40.34±1.07***	40.79±0.56***
DLC Lymphocyte (%)	71±5.86	68±6.54
Monocyte (%)	1.66±0.81	2.5±1.04
Neutrophil (%)	25.33±3.88	26.5±6.59
Basophil (%)	1.83±1.16	2.83±0.75
Eosinophil (%)	1.00±0.89	1.16±0.75

Data is presented as Mean ± S.D. Data was analyzed using one way ANOVA. The values were considered significant for* $p < 0.05$, **$p<0.01$ and ***$p<0.001$

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Morphometric Partioning of the Respiratory Surface Area and Diffusion Capacity of Gills in an Air Breathing Fish *Channa Gachua*

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Abstract
The gills and the respiratory swim bladders of juvenile specimens (mean body mass 100 g) of the teleost *Channa gachua* were evaluated using stereological methods in vertical sections. The surface areas, harmonic mean barrier thicknesses and morphometric diffusing capacities for oxygen and carbon dioxide were estimated. The average respiratory surface area of the swim bladder (2173 cm^2/ kg) exceeded that of the gills (780 cm^2/kg) by a factor of 2.79. Due to the extremely thin air blood barrier in the swim bladder (harmonic mean 0.22 m) and the much thicker water blood barrier of the gills (9.61 m) the morphometric diffusing capacity for oxygen and carbon dioxide was 88 times greater in the swim bladder than in the gills. These data clearly indicate the importance of the swim bladder, even in juvenile *Channa gachua* that still engage in aquatic respiration. Because of the much greater diffusion constant of carbon dioxide than oxygen in water, the gills also remain important for carbon dioxide release.
Keywords: Bimodal respiration, surface area, water air blood barrier, thickness *Channa gachua.*

Introduction

Bimodal breathing involve dual mode of oxygen uptake from the water by gills and from the air by an air breathing organ which has evolved among teleost fishes. Within bimodal breathers, amphibious species which can breathe in or out of water can be distinguished from aquatic species; can breath while remaining in the water (Graham, 1997 and Qaisur, 2011). In addition obligate air breathers who are dependent on aerial gas exchange can be differentiated from facultative air breathers who only supplement their oxygen needs by air breathing (Hughes *et. al.,* 1974; Fernandes, 1996; Perna and Fernandes, 1996; Santos *et. al.,* 1994; Mazon *et. al.,* 1998; Moraes *et. al.,* 2005; Fernandes *et al.,* 2007; Cruz *et al.,* 2009). The accessory air breathing organs are located in the region of the head consisting of buccal and pharynx epithelia, pharyngeal pouches, modified branchial and opercular surfaces (Munshi, 1985) structures localized in the digestive tract (stomach and intestinal tract) (Carter and Beadle, 1931; Gee and Graham, 1978; Silva *et. al.,* 1997) and skin (Banerjee and Mittal, 1976; Bicudo and Johansen, 1979; Moraes *et. al.,* 2005). The teleost *Channa gachua* commonly referred to as the "garai" is an aquatic obligate air breather that uses its swim bladder to breathe atmospheric air. Its gills exhibit pronounced changes as the fish matures. Small fish have gill filaments with well defined lamellae (Brauner *et. al.,* 2004; Costa *et. al.,* 2007), while large fish have column shaped filaments that appear to have a smooth surface (Brauner *et. al.,* 2004). The *Channa gachua* swim bladder is highly vascularized and adapted for aerial respiration (Graham, 1997). Early studies reported that in 1-3 kg fish between 75% and 95% of the total oxygen uptake was derived from the air (Stevens and Holeton, 1978). Brauner and Val (1996) confirmed these values for 1.7 kg fish, showing that 79% of their total excreted CO_2 was processed through the gills. Juvenile (10–100 g) *Channa gachua* are dependent on aerial respiration than the adults (Brauner *et al.,* 2004; Brauner and Val, 2005). Recently oxygen uptake and CO_2 excretion measurements in small (67 g) and large (724 g) showed that the specific oxygen uptake rate (molO_2/ g/ h) of small fish is 0968-4328 matter two thirds that of the large ones, and the water fraction of oxygen uptake and

CO_2 excretion is lower in small individuals, despite the drastic morphological changes in the gills as the fish matures (Gonzalez *et. al.,* 2010). Physiological studies have documented the bimodal gas exchange in *Channa gachua* (Brauner and Val, 1996; Brauner *et. al.,* 2004; Gonzalez *et. al.,* 2010). However, morphological data on respiratory organs are scarce. Recently, the gill surface area of small *Channa gachua* was estimated to be similar to sluggish water breathing and facultative air breathing fish (Costa *et. al.,* 2007), but no data on swim bladder surface area are currently available. The present study delivers morphometrical data for the gas exchange organs of fish at a critical stage in their development where they are obligate air breathers but still engage in aquatic respiration. The main goal of the present study was to quantify the relative morphological adaptation of gill and swim bladder partitioning for gas exchange in juvenile *Channa gachua* .To this end the surface area, diffusion barrier thickness and the morphometric diffusion capacity for oxygen and CO_2 of the gills and the swim bladder were estimated using the same stereological methods in vertical sections, thus ensuring a comparison that is free of methodological differences.

Materials and Methods

Live specimens of *Channa gachua* were procured from local fish dealers at Hazaribag (Latitude 25º 59'N and Longitude 85º 22'E) and maintained in large glass aquaria size (90x60x60cm) with continuous flow of water. The specimens were fed on chopped goat liver daily during a minimum acclimation period of 15 days in the laboratory. Six juvenile *Channa gachua* (body mass (MB) = 85–110 g; mean = 100 ± 9 g; body length (LB) = 20–26 cm; mean = 24 ± 2 cm) were used in the present investigation. The gills were immediately removed and fixed by immersion in 2.5% phosphate buffered glutaraldehyde solution with a pH of 7.8 and an osmolality of approximately 300 mosmol. The specimen was then opened ventrally, and the swim bladder was exposed and fixed with fixative solution. Next, the entire fish was immersed in the above mentioned fixative at 4°C. Sampling and processing for light (LM) and transmission electron (TEM) microscopy. The sampling and embedding procedures were designed to combine the Cavalieri principle for determining the reference volume with the stereological vertical sectioning method for measuring surface area, as described by Moraes *et. al., (*2005) for the swim bladder and by Costa *et al.,* (2007) for gills. Briefly, the rakers and bone of the epibranchial and cerato branchial elements of each gill arch were removed in the way that the gill filaments from anterior and posterior hemibranchs were kept attached in the gill arch tissue. The epi and ceratobranchial portions were separated, and the latter portion was cut in half yielding three samples from each gill arch. The samples were dehydrated by graded ethanol series and embedded in historesin. Random numbers were assigned to the samples and then the samples were placed with the opercular side (horizontal plane) down. The samples were embedded in methacrylate in stacks of 3 one atop the other with each sample rotated sequentially ± 15°C relative to the previous one around the vertical axis. Historesin was used as the embedding methacrylate due to the negligible shrinkage of section and the fish tissue (Cruz *et al.*, 2009 b). The entire gill from one side of the animal was contained in four blocks, properly oriented for stereological vertical sectioning. Ten equidistantly spaced vertical sections of a 3 µm thickness that had been stained with toluidine blue and acid fuchsin were used to estimate the gill volume using the Cavalieri method as well as the surface area in vertical uniform random (VUR) sections (Michel and Cruz Orive, 1988). Surface area and volume of the gills were determined using stereological point and intersection counting methods (Howard and Reed, 2007; Costa *et. al.,* 2007) under a BX51 Olympus microscope with a 20×/0.80 oil immersion lens. A new angle was selected at random for each histological section. The harmonic mean thickness of the air blood barrier was then measured as 2/3 the harmonic mean intercept length (Weibel and Knight, 1964). The fixed swim bladder was removed and transected by 10 equidistantly spaced slices, beginning at a random starting point within the first interval (Moraes *et al.,* 2005). A square lattice grid was placed on the anterior surface of each section and point counts of the projected surfaces of the parenchyma, central lumen and ventral membrane

were performed. The volume of these three components was estimated using the Cavalieri method (Michel and Cruz-Orive, 1988), and 10 tissue samples from the parenchyma of each fish were then taken by systematic random sampling and processed for light microcopy to estimate the differential tissue volume. The samples were rotated ± 18^0 C relative to the previous one and embedded in historesin (Leica) with the adventitial side down, defining the horizontal plane. Tissue was sectioned vertically (3µm thickness) and stained with toluidine blue and acid fuchsin. To measure differential tissue volumes and the respiratory surface area, stereological point and intersection counting methods were employed using the CAST System software. To measure the air blood distance, 10 systematic random samples were taken from the posterior surface of swim bladder slices of each fish and processed for transmission electron microscopy. The samples were embedded in epon 812 (EMS, Hatfield, USA) sectioned at 60 nm in thickness and contrasted on 300 mesh grids using standard uranyl acetate and lead citrate procedures. The exact magnification was calculated for each series of electron micrographs with the aid of a calibration grid. The intersections were used as starting points to estimate the air blood diffusion distance (Fig. 4A). The harmonic mean thickness of the air blood barrier was measured as 2/3 the harmonic mean intercept length (Weibel and Knight, 1964). Anatomical diffusion factor and the morphometric diffusion capacity of the water blood or air blood barrier. The morphometric diffusion capacity (Dm) was calculated as the product of the ADF and the weighted mean of Krogh's diffusion coefficient (K) for the respective cell layers (epithelium/endothelium/pillar cells) or basal membrane/connective tissue (for gills and swim bladder) of the diffusion barrier. The volume proportion of the different components was estimated separately by point counting (Fig. 3B). Each gill or swim bladder element was multiplied by the appropriate K value and this weighed numerical ratio yielded a K value for oxygen (KO_2) or CO_2 (KCO_2) in the water (air) blood barrier corrected to 25 ^oC. The relative and absolute variables were first calculated for each animal (Howard and Reed, 2007) to determine the precision of the estimates of volume, area and barrier thickness. To evaluate the variability between animals, the mean values were accompanied by the respective standard errors (SEM) for the gills and swim bladder (Howard and Reed, 2007).

Results

The gills of *Channa gachua* had the same basic structure as those seen in most teleost fish four gill arches, each bearing two rows of filaments. Lamellae, the gas exchange units of the gills, projected from both sides of the filaments. The lamellae consisted of the pillar cell system covered by basement membrane and two or three epithelial cell layers. In general, the cells of the inner most epithelial cell layer were flat and those of the outermost cell layer were cuboidal. Mucous and chloride cells were distributed throughout the filament epithelium, which was stratified and contained in 5–7 cell layers. The swim bladder lay ventral to the vertebral column and was fused to dorsal muscles of the body wall and ribs. It extended the full length of the body cavity from the posterior part of the head and kidneys to the posterior end of the intestine. The trunk kidneys projected dorsally as a median ridge into the lumen of the swim bladder. The highly vascularized, dorsolateral wall of *Channa gachua* swim bladder constituted the parenchymal layer that also enveloped the kidneys. The parenchyma was contiguous with a tough, translucent membrane that delimited the central lumen of the organ ventrally. This membrane contained some capillaries close to the mucosal surface, and their potential respiratory function could not be completely disregarded. The parenchyma was irregularly subdivided by numerous septa to form compartments (ediculae) of highly variable size that were supported by smooth muscular trabeculae, which consisted of a connective tissue matrix with numerous large and small blood vessels. The parenchyma tissue consisted of numerous small vessels and a dense capillary layer just below the respiratory epithelium. The respiratory epithelium covered all surfaces of the ediculae and consisted of pavement and columnar epithelial cells. The pavement cells had a low number of mitochondria very small lamellar bodies and, in general were underlined by the capillaries and separated from

them by the basal lamina. The apical surface of pavement cells was smooth with short microvilli distributed at the cell border. Columnar cells were distributed among the pavement cells. They were characterized by microvilli at the cell surface and numerous mitochondria and lamellar bodies of different sizes. The lamellar bodies reached up to 1.5 um in diameter. Tight junctions characterized the junction complex between pavement and columnar cells. Numerous neutrophils were found in the capillaries and blood vessels. The parenchyma tissue was rich in collagen fibers consisting of a layer between the capillaries and the small and large blood vessels in the inner tissue. The air blood barrier was composed of the endothelial cells of capillaries (23%), basal lamina of the swim bladder epithelium (24%) and the pavement epithelial cells themselves (57%) respectively.The gills of juvenile *Channa gachua* had a volume of 7.5 ± 0.3 cm^3 including rakers, epi and ceratobranchial bones, filaments and lamella. Most of total gill volume (80%) consisted of the long rakers and branchial bones. The gill filaments made up only 15% of the total gill volume, while 5% was ascribed to the lamellae (3.6% epithelium, 1.1% blood spaces and 0.3% pillar cells. The swim bladder length was approximately 0.7 of the total body length with a total volume of 9.5 ± 0.3 cm^3. Most of this volume (7.5 cm^3) represented the air in the central lumen and correspond to 78.6% of total swim bladder volume. The volume of ventral membrane and the parenchyma of swim bladder were 0.5 cm^3 and 1.6 cm^3 which corresponded to 5.4% and 16% of the total swim bladder volume, respectively. The tissue of the respiratory portion of the swim bladder (parenchyma excluding the trabeculae and trabecula air) consisted of 0.1% epithelium, 1.7% capillaries and 1.8% large vessels and approximately 4.4% of other tissues, such as connective tissue and nerves. The mean respiratory surface area of the protruded lamella (portion of the lamellae that contacts water) was 780.21 ± 31.23 cm^2 kg^{-1} ranging from 690.06 to 880.96 cm^2 kg^{-1}. This represented approximately 70% of filament and lamellar surface areas taken together. The potential respiratory surface area of the lamellae was 5.1 times greater than the filament surface area. The surface area of the ediculae tissue of swim bladder was 2173.05 ± 118.89 cm^2 kg−1. Of this area, 1912.39 cm^2 kg^{-1} (94%) was potentially respiratory, while 167.97 cm^2 kg^{-1} (6%) was not potentially respiratory. The latter consisted of large trabeculae that lacked capillaries under the epithelium. Thus, the potentially respiratory surface area of parenchyma of swim bladder was 2.8 times greater than that of gill lamellae in these juvenile specimens. The gill lamellar epithelium had an arithmetic mean thickness of 18.15 ± 1.34 um and ranged from 14.76 to 22.72 µm. The harmonic mean was 9.61 (range 8.1–12.2 µm). The gas exchange barrier of the swim bladder had an arithmetic mean of 1.56 ± 0.44 µm with a range of 1.08–2.71 µm, and its harmonic mean was 0.22 um, ranging from 0.16 to 0.43 µm. In the large trabeculae regions the harmonic mean is up to 5–7 um higher as there are not capillaries just under the epithelium. The anatomical diffusion factor (ADF) of the swim bladder (8650.74 ± 752.16 cm^2 µm−1 kg^{-1}) was thus 107.3 times greater than that of the gills (80.65 cm^2 um^{-1} kg^{-1}. calculated as the mean of the ADF values for the individual fish. The morphometric diffusion capacity for oxygen of gills was 0.021 ± 0.002 cm3 min^{-1} $mmHg^{-1}$ kg^{-1}, while that of the swim bladder was 1.86 ± 0.19 (cm^3 min^{-}1 $mmHg^{-1}$ kg^{-1}). The morphometric diffusion capacity for CO_2 was 0.403 ± 0.035 and 35.545 ± 3.69 cm^3 min^{-1} $mmHg^{-1}$ kg^{-1} for the gill lamella and the swim bladder, respectively. The morphometric DO_2 and DCO_2 values of the swim bladder were approximately 88 times greater than those for the gills.

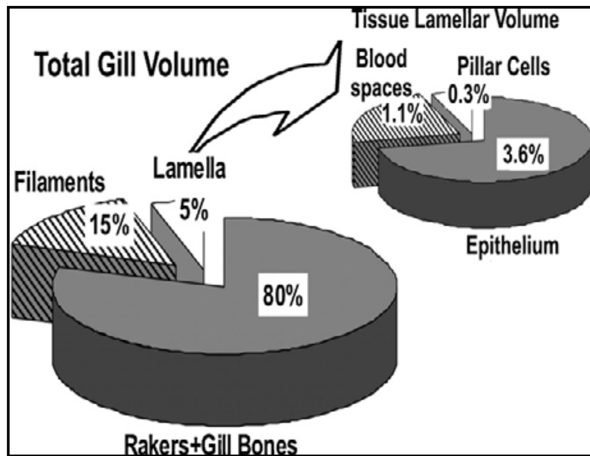

Fig. 1. ***Channa gachua*** **gills, Percentage volumes of the gill structural elements including rakers, gill arch bones, filaments and lamellae.**

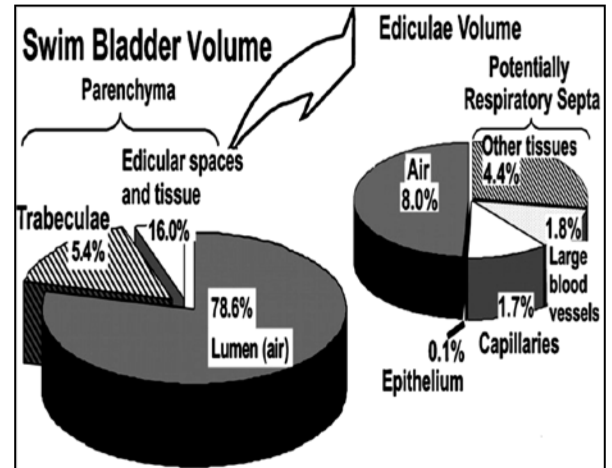

Fig. 2. ***Channa gachua*** **swim bladder, Volume percentages of elements in the entire swim bladder.**

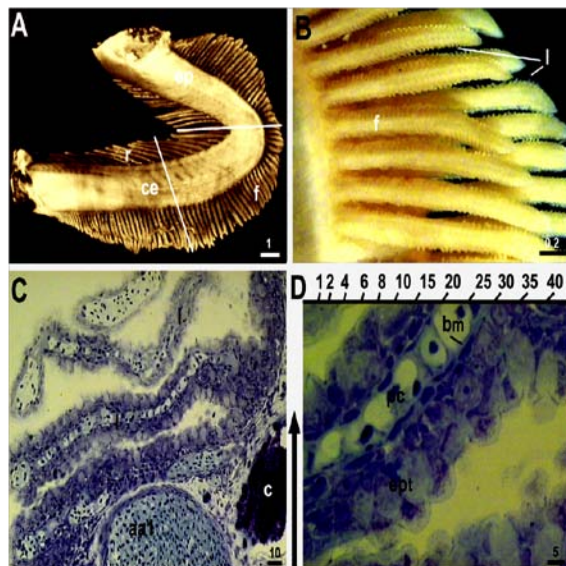

Fig. 3. ***Channa gachua*** **representation of gill arches**

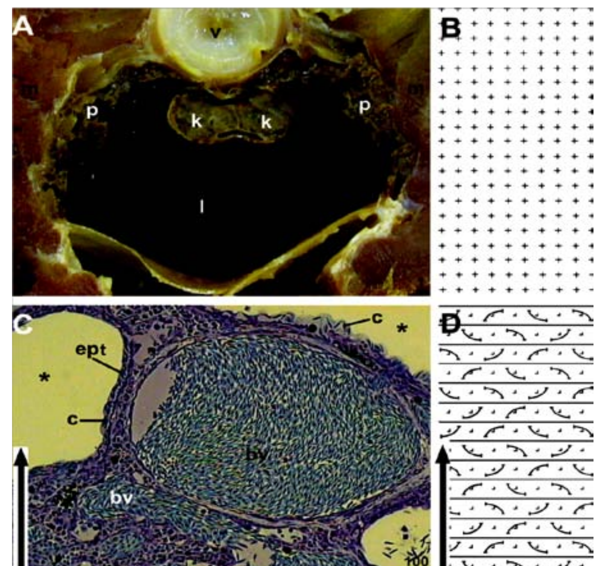

Fig. 4. ***Channa gachua*** **swim bladder Cross section of swim bladder showing the parenchyma**

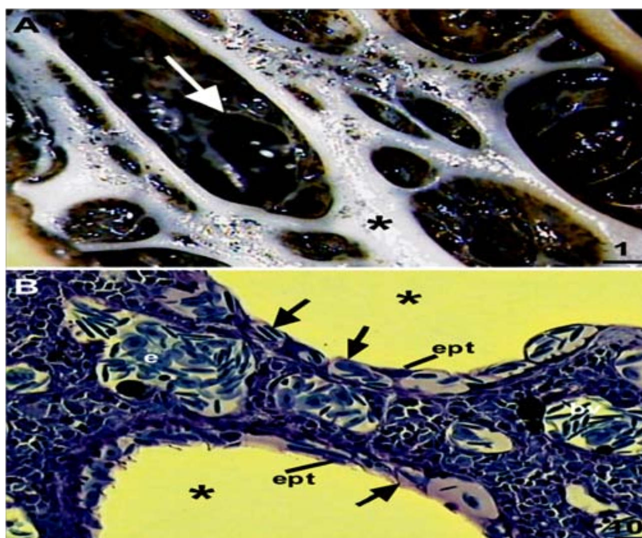

Fig. 5. ***Channa gachua*** **swim bladder (A) Respiratory region (parenchyma) of the swim bladder ing trabecula (B) Respiratory tissue of an inter radicular septum, showing capillaries (arrow) underlying the epithelium.**

Table 1. Dimension (means ± SEM) of the respiratory organs of the *Channa gachua.* Body weight (100 ± 9 g)

Parameter	**Gills**	**Swim bladder**
Total respiratory surface area (cm^2)	76.36 ± 2.27	157.01
Respiratory surface area ($cm^2\ kg^{-1}$)	780.21 ± 31.23	2173.05 ± 118.89
Arithmetic mean of water/blood or air/blood barrier (µm)	18.15 ± 1.34	1.56 ± 0.44
Harmonic mean of water/blood or air/blood barrier (µm)	9.61 Range 8.10–2.16	0.22 Range 0.16–0.43
ADF (cm2 µm)−1 k	80.62 ± 5.38	8424.68 ± 752.16
DmO_2 (cm3 min−1 mmHg−1 kg−1)	0.021 ± 0.002	1.86 ± 0.19
$DmCO_2$ (cm3 min−1 mmHg−1 kg−1)	0.403 ± 0.035	35.545 ± 3.69

Discussion

This study clearly demonstrates the importance of the swim bladder for gas exchange in *Channa gachua* even at a size where branchial water breathing is still possible. Although the basic gill structure of *Channa gachua* did not differ from that of other species, its respiratory surface area is lower, and its water blood diffusion barrier is very thick compared to some water breathing species and similar to those of air breathing fish (excepting the lungfish which has a non respiratory functional gills. The extensive surface area and the very thin air blood diffusion barrier

of its modified swim bladder shows 2.8 times greater surface area and 43 times thinner gas diffusion distance than in the gills, respectively. Based on these values alone, it is clear whether the swim bladder is the major respiratory organ. Compared to the accessory organs for respiration of other air breathing fish, the *Channa gachua* swim bladder has a larger surface area and a similar air blood diffusion barrier that favor air respiration. Physiological data have shown that in general three quarters of all oxygen needs are taken up by the swim bladder (Brauner *et al.,* 2004; Gonzalez *et al.,* 2010). Assuming that oxygen is 30 times less soluble than carbon dioxide in water, and that approximately equal molar equivalents of oxygen and CO_2 are taken up and given off, the gills would be able to excrete 30 mol. of CO_2 for each mole of oxygen taken up in the swim bladder. Additionally, the diffusion capacity expresses the rate of gas exchange per unit of driving pressure. As the driving pressure for oxygen falls between breaths, the rate of oxygen uptake in the swim bladder will fall accordingly, while it will remain at constant, albeit low, levels in the gills. Earlier physiological studies suggested that a spatial uncoupling between oxygen uptake in the swim bladder and CO_2 excretion in the gills might occur (Brauner and Val, 1996). In 2-3 kg fish, at least 78% of total oxygen uptake is from air, but only 37% of total CO_2 is excreted into the swim bladder. Slightly different data were found by Brauner and Val (1996), reporting an oxygen uptake of 78% by the swim bladder with only a 15% CO_2 excretion into this organ. Thus, 79% of CO_2 must have been excreted by the gills with an additional 6% via the kidney in the urine. Recently, Gonzalez *et al.*, (2010) showed that gill morphology changes do not place limitations on oxygen uptake in large fish and that the CO_2 excretion through the gills is similar (85–90%) in small (67 g) and large (724 g) fishes. They also showed that at lower blood pH, PCO_2 and concentrations of HCO_3^- in small fish suggest a possible diffusion limitation for CO_2. Ammonia excretion is mainly conducted through the gills. Our morphometric data support this functional partitioning, which may be less pronounced in our juvenile specimens than in large fish. The respiratory surface area of gills (78 cm^2) of *Channa gachua* is smaller than in typical water breathing fish and the facultative air breathers such as *Lepisosteus osseus* (83 cm^2) (Landolt and Hill, 1975), *Amia calva* 191 cm^2 (Crawford, 1971) and *Hoplerythrinus unitaeniatus* (125 cm^2) (Fernandes *et al.,* 1994). However, compared with the obligate air breathers such as *Anabas testudineus (*94 cm^2) (Hughes *et al.,* 1973) or the American lungfish *Lepidosiren paradoxa* 0.38 cm^2 (Moraes *et al.,* 2005), the gill surface areas of small *A. gigas* are relatively well developed (Costa *et al*., 2007). Low gill surface area in air-breathing fish has been considered an adaptation to reduce the loss of oxygen in hypoxic waters (Graham, 1997). Low surface area and a large water blood distance reduce the gill diffusion capacity for oxygen uptake, but it may favor the air-breathing fish living in hypoxic and stagnant waters by reducing the oxygen loss into water through the air breathing organ via the gills. It may also solve problems related to osmoregulation by reducing water influx and ion losses. The reversible protrusion of lamellae in the water breathing fish (*Carassius carassius*) when the oxygen supplying meets the species needs (Sollid *et al*., 2003; Sollid and Nilsson, 2006) supports the hypothesis that the low gill surface area of *Channa gachua* is able to support its ability to live in the hypoxic and ion poor waters of the rivers because the swim bladder is the main respiratory organ in this species. The compromise between gas exchange and other gill functions such as osmoregulation, acid base balance and nitrogen excretion also have to be considered. In general, both swim bladder types have low blood supply, excepting in air breathing fish that has a modified swim bladder for atmospheric air respiration. In this case, the swim bladder has a highly vascularized extense trabeculae region which varies among species. In *A. gigas* volume percentage of respiratory region of swim bladder in relation to body mass is the smallest among fish that use the swim bladder as both a respiratory organ and a hydrostatic organ (Graham, 1997). Just the reverse is seen in the parenchyma of *L. paradoxa* weighing approximately 600 g (Moraes *et al.,* 2005), where the lung volume (25 ml kg^{-1}) is too small to achieve neutral buoyancy, but the parenchyma makes up 43% of the total lung volume and has a surface to volume ratio of only 129 cm^{-1} (Moraes *et al.,* 2005). The swim bladder surface area of *Channa gachua* is between 5-33 fold that of the accessory organs of several air breathing fish taking oxygen directly from the air.

Conversely, the thin air blood barrier of the swim bladder, with a harmonic mean ranging from 0.16 to 0.43 um, is lower than that of other air breathing organs. The air breathing *A. gigas* exhale first and, then inhale the atmospheric air. The air inhalation is the result of the action of a buccal pump combined with the swim bladder aspiration by Farrell and Randall (1978) and Qaisur Rahman (2011) in *Channa gachua* reported that the ventral membrane of the swim bladder may act as a diaphragm like septum that stretches down wards between the body flanks, creating suction and filling the air breathing organ. It needs to rise in the water surface to gulp air. Its gills surface, although similar to some facultative air breathing fish, and function efficiently enough to fulfill all oxygen requirements. The lower morphometric and DO_2 of the gills in addition to low oxygen solubility in the natural environment of *Channa gachua* restricts oxygen uptake by the gills compared to swim bladder oxygen uptake from the air. Energy requiring physiological mechanisms however results in greater CO_2 excretion through the gills despite a larger morphometric DCO_2 for the air breathing organ.

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Studies on the Seasonal Variation in Total Lipid and Carbohydrate Content of Muscle Tissue of *Schizothorax labiatus*

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Abstract.
The study was conducted to determine the lipid and carbohydrate content in muscle tissue of fresh water teleost *Schizothorax labiatus* from the Jhelum river of Kashmir valley. *Schizothorax labiatus* was sampled from January 2012 to December 2012. Lipid and carbohydrate content was determined in muscle tissue in four different seasons of the year (Spring, Summer, Autumn and Winter). It was observed that the Lipid and carbohydrate content varied by months and seasons ($P<0.05$). The lipid content in *Schizothorax labiatus* observed was 0.032, 0.026, 0.031, 0.033, 0. 029, 0.037, 0.039, 0.036, 0.033, 0.034, 0.030, 0.027 g/g of tissue in January, February, March, April, May, June, July, August, September, October, November and December. The carbohydrate content ranged from 0.00093 ± 0.00011 - 0.00183 ± 0.00015. The percentage of lipid was maximum in the month of July and lowest in the February. Seasonally the highest carbohydrate content in the muscle tissue was observed in summer season (0.158%) followed by winter (0.123%) and spring (0.119%) and lowest in autumn season (0.114%).
Keywords: Muscle tissue, lipid content, carbohydrate content, *Schizothorax labiatus.*

Introduction

Fish are quite different from the other animal food sources, because they provide low energy and have high-level proteins, which contain all essential amino acids. So they are beneficial nutrition sources (Weatherley and Gill, 1998). The lipids are the important biochemical compounds of fish. Fish store the lipids in various organs; particularly in muscles and liver. The carbohydrate content in fish muscle is very low, usually below 0.5 % and do not show much variation. The carbohydrate occurs in glycogen and as part of the chemical constituents of nucleotides. Carbohydrates are not stored in large quantities by fish as energy-rich compounds unlike mammals. These values also give estimation of food composition of fish, its physiological
condition (Salam and Davies, 1994). Therefore the present study has been undertaken to evaluate the lipid and carbohydrate content in the cold freshwater teleost of Kashmir valley. *Schizothorax labiatus* locally known as *Chhush* is a very valuable fish of Kashmir valley. This fish inhabits the streams, rivers and lakes. It differs in tooth shape, gill-racker counts, morphometry, scale counts, pharyngeal bone and color pattern from the other species of genus Schizothorax.

Materials and Methods

The study was conducted from January 2012 to December 2012 and was carried out in the Department of Zoology University of Kashmir Hazratbal Srinagar. Five to six samples of fishes were collected in each month. Samples of *Schizothorax labiatus* were bought from their natural habitat, i.e, Jehlum River Srinagar Kashmir. Fishing was performed with the help of professional local fishermen.The fish samples were immediately transported to the Ichthyology laboratory and were washed with tap water. After that the fish was weighed and total length, standard length and width of each of these fish were determined. The fish were dissected to collect the muscle tissues. The muscle sample were then weighed and homogenized in a homogenizer, before the analysis of biochemical components.

Lipid and carbohydrate Estimation

Total lipid content of fish was estimated by Folch's method (Folch *et al*., 1957) and carbohydrate content was determined using Phenol Sulphuric acid method (Dubois *et al*., 1956).

Statistical Analysis

Statistical analysis revealed that lipid contents are significantly different in different seasons. The data was evaluated by one-way ANOVA followed by Tukys test to detect inter seasonal differences. Differences were considered to be statistically significant if $P < 0.05$.

Results

The proximate composition of lipid and carbohydrate in the experimental fish for different months is presented in Tables below. Lipid content of *Schizothorax labiatus* showed fluctuating trend and ranged from 0.26g/g in February - 0.39 g/g in the month of July (Table 1). There was increase in lipid content from winter, spring, summer and then gradual decrease was seen in Autumn. Seasonally the higher lipid content was observed in summer i.e 3.733% and lowest in winter i.e. 2.833% (Fig 1). The trend of Carbohydrate content decreases from January to February and then shows slight increase in March and again decreases in April and May (Table. 2). The carbohydrate content again fluctuates and showed increase in summer season where highest was seen in the month of June (0.00183 ± 0.00015). In September the value decreases (0.00093 ± 0.00011) followed by increase in October and November and again values decreases in December (0.00110 ± 0.000200). Seasonal variations showed highest values of carbohydrate percentage in summer followed by winter season in *Schizothorax labiatus* i.e., 0.158% and 0.123% respectively, whereas, the lowest carbohydrate percentage was recorded in autumn season (0.114%).

Table 1. Monthly variation in total lipid content of *S. labiatus* (g/g of tissue).

Month	**Species S. labiatus**
January	0.032 ± 0.010
February	0.026 ± 0.017
March	0.031 ± 0.005
April	0.033 ± 0.001
May	0.029 ± 0.010
June	0.037 ± 0.020
July	0.039 ± 0.010
August	0.036 ±0.001
September	0.033 ±0.013
October	0.034 ±0.014
November	0.030 ±0.010
December	0.027 ±0.002

Data is expressed as mean ± SD of three separated experiments

Table 2. Monthly variation in muscle carbohydrate content of *Schizothorax labiatus*. (g/g of tissue).

Month	Species S. *labiatus*
January	0.00150 ± 0.00161
February	0.00109 ± 0.00011
March	0.00140 ± 0.00036
April	0.00118 ± 0.00020
May	0.00101 ± 0.00011
June	0.00183 ± 0.00015
July	0.00153 ± 0.00010
August	0.00138 ± 0.00013
September	0.00093 ± 0.00011
October	0.00111 ± 0.00011
November	0.00139 ± 0.0001
December	0.00110 ± 0.00020

Data is expressed as mean ± SD of three separated experiments.

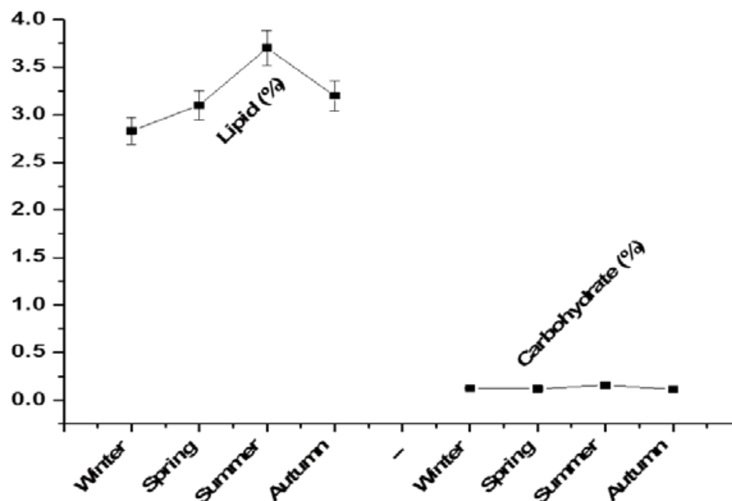

Fig. 1. Graph showing seasonal variation in lipid and carbohydrate % age of muscle content.

Discussion

Fish oil is one of the most important natural sources of polyunsaturated fatty acids which have been proven to have useful effects on human health (Saoud *et al*., 2008). The quantity and composition of fatty acids from lipids are not only associated with the species, but also depend on diet, temperature, seasonality, age and gender (Ackman, 1989). The monthly and seasonal variation in lipid and carbohydrate content in muscle tissues of *Schizothorax labiatus* was monitored from January 2012 to December 2012. The carbohydrate content of the sampled fish in winter, spring, summer and autumn season was estimated as 0.123%, 0.119%, 0.158% and 0.114% respectively. Highest lipid content is observed in summer and lowest in winter season in the species.

The highest lipid content seen in summer season is due to the fact that summer is the nutrition period and intensity of feeding is high therefore maximum amount of lipid is observed in this season and fishes have to store extra lipid for use in winter. According to (Robards *et al*., (1999) and Henderson *et al*., (2000) large amount of the energy that is stored for wintering and spawning is accumulated during periods of intensive feeding. Maximum amount of lipid content in summer season in our results is in agreement with the Bumb (1992) who also analyzed the variation of fat content with feed intake and found that intensive feeding of *Ambassis commersoni* coincides with the occurrence of high fat content in the muscle of fish.

During autumn season the lipid content decreases as in this period the temperature and food availability also changes which leads to decrease in lipid content in the species. Also this period is the period for development of gonads and hence low content of lipid in muscle of fish is observed. In winter season there is scarcity of food and temperature is not feasible for different algae to grow which leads to low availability of food during this season. So low amount of lipid is observed in winter season and fish also can use the stored fat to cope up with the unfavorable conditions occurring during this season. This is in agreement with the work done by Salihoglu and Mutlu (2000) on garfish in which the decline in fat and protein might be because of a potential starvation due to the sharp decrease in the amount of plankton in January. The spring season is the breeding season of the fish. According to Ayas *et al.,* (2005) the variation in fat content is related to feeding and reproduction, so decrease in lipid content is observed in the breeding season of the studied fish. Also during spring season the food that is taken is utilized for the process of spawning.

In the present study the low value of carbohydrates indicates that glycogen in these fishes does not contribute significantly to the total reserves in the body. In some marine fishes same results were observed by (Jayasree, 1994). Carbohydrate content of fish is affected by some environmental and physiological factors like seasons, spawning and feed intake. In present study the low value of carbohydrate could be due to the fact that a carbohydrate does not contribute much to the reserves in the body. This is in agreement with Mathana *et al.,* 2012.We know that winter season is resting phase and in this season energy is reserved so carbohydrate content is more as compared to autumn season. Spring seasons is spawning season of fish hence in this season the carbohydrate is low because the food eaten by fish is utilized for gonadal maturation. Same result was found by Chellappa *et al.,* (1989) in male three-spined stickle backs and stated that energy reserves drops during growth and gonadal maturation. The high content of carbohydrate was found to be in the summer season due to the fact that summer season is the Post spawning season which results in high feeding activity and hence increase in carbohydrate content. Bumb (1992) also analyzed the variation of carbohydrate content with feed intake and found that intensive feeding in *Ambassis commersoni* coincides with the occurrence of high carbohydrate content in the muscle of fish and is in agreement with our observed result.

Conclusions

The results reveals that the carbohydrate and lipid composition of the experimental fish varies with the change in season i.e winter, spring, summer and autumn seasons and with the availability of food during these seasons. Thus

the present study provides valuable information about the biochemical composition of fish studied in order to make best use of the fish species as food.

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Mutagenic, Antimutagenic and Anticarcinogenic Properties of Medicinal Plants with Special Reference to *Melissa officinalis*: A Review

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Abstract
Mutations are the cause of innate metabolic defects in cellular systems, triggering morbidity and mortality in living organisms. Mutagens are not only involved in genotoxicity and carcinogenicity but also involved in the inception of several chronic diseases. One of the best ways to minimize the detrimental effects of mutagens is by use of natural antimutagens. Medicinal herbs have been on the forefront whenever we talk about antimutagenic and anticancer remedies. Medicinal plants have a vital role in the prevention and treatment of cancer. Present review attempts to furnish a brief overview on mutagenic, antimutagenic and anticarcinogenic properties of medicinal plants.
Keywords: Mutagenic, anticarcinogenic, medicinal herbs, *Melissa officinalis.*

Introduction
For a long time plants have been providing essential nutritional values, medicinal properties and notable physiological effect to life and are a good source of food. Traditional medicine refers to the application, approach, knowledge and belief in incorporating plant or animal based properties in remedies, singularly or in combination, for the purpose of treating or preventing disease as well as to maintain the well-being of an individual. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. As such herbal remedies have been used to cure a variety of disorders or conditions such as diabetes, cardiovascular problems, weight control, dermal infirmities, sexual malfunctions and of course cancer. According to World Health Organization, more than 70% of the world's population uses traditional medicine in order to fulfill their health necessities. The principles underlying herbal medicines are relatively simple, although they are quite distinct from conventional medicine and herbal medicine. India in general and Jammu and Kashmir in particular is a rich source of medicinal plants and a number of plant extracts are used against diseases in various systems of medicine like ayurvedic, unani and siddha (Table 1). Only a few of them have been scientifically explored. Plant derived natural products such as flavinoids, terepenes and alkaloids (Osawa *et al*., 1990) has soon received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemo preventive effects.

Medicinal plants as mutagens
Mutations are the cause of innate metabolic defects in cellular systems, triggering morbidity and mortality in living organisms. The mutagens are involved in the initiation and promotion of several human diseases, including cancer (Bhagavathy *et al*., 2011).

Mechanism of mutagenesis is complex however many mutagens and carcinogens may act through the generation of Reactive Oxygen Species (ROS). ROS may play a major role as endogenous initiators of degenerative processes, such as DNA damage and mutation, which may be related to cancer, heart disease and ageing (Maryam *et al*., 2010).

Somatic gene mutations are the basic events for the conversion of a normal cell to a mutant cell. This mutant cell is then converted to malignant cell through several genetic changes. Several chemicals have been implicated in cancer causation. Some of these are sodium azide, ethidium bromide, hydroxyl amine, ethylnitronitrosoguanidine (MNNG), N, N' bis-(1-naphthyl) N, N'-diphenyl -1, 1 biphenyl-4, 4'-diamine (α-NPD), etc. (Prabhu *et al*., 2010).

Since plants are used in many areas for a number of purposes, there are many studies on plant extracts that examine their mutagenic properties for safe consumption. Because many plants synthesize toxic substances for defense against organisms including viruses, bacteria, and fungi, these compounds could have potentially deleterious effects in humans. Although there are a number of studies on the mutagenic effects of plant extracts, e.g., positive results have been reported from the Ames test using extracts of *Crinum macowanii*, *Catharanthus roseus*, *Combretum mkhzense*, *Diospyros whyteana*, *Plumbago auriculata*, *Ziziphus mucronata*, and *Chaetacme aristata*

A hydroalcoholic extract of *Ocotea duckei* leaves was found to be mutagenic for the *Salmonella typhimurium* TA97a, TA100, and TA102 strains, with or without S9 mix (Marques *et al*., 2003). Deciga-Campos *et al.*, (2006) found that *Gnaphalium* sp. and *Valeriana procera* extracts induced mutations of *S. typhimurium* TA98 with or without S9 mix and of TA100 with S9 mix, respectively. The tubers of *Gloriosa superba* were found to contain potent mutagenic properties in an Ames mutagenicity test on *Salmonella* (Hemaiswarya *et al.,* 2009). It was also shown that compounds present in the methanolic extracts of the leaves of *Alchornea castaneaefolia* and *Alchornea glandulosa* were mutagenic in an Ames test (dos Santos *et al*., 2010).

Medicinal plants as anticancer drugs

The natural world has been providing life saving antibiotics, nutritive supplements and our most potent anti-cancer drugs. Natural products especially those from plants have been a valuable source of new cancer drugs for many decades. Medicinal plants are the most exclusive sources of life saving drugs for the majority of the world's population. The use of plant products in the treatment of cancer has been of recent interest.

From thousands of years, plants have been utilized as medicines (Ruffa *et al*., 2002). Major constituents of more than 50% of all the drugs in clinical use are natural products and their derivatives. One of the potential uses of plant-derived compounds is as antimutagenic agents (Calomme *et al*., 1996; Ammar *et al*., 2007). As a result of human civilization and global environmental pollution, the rate of mutations has increased and one of the ways to neutralize the effect of such mutagenic agents is to identify those substances that can antagonize their effect. Plants are the promising source of antimutagens which occur in them as secondary metabolites. These antimutagens may help in strengthening the cell defences against environmental mutagens/stress. Nowadays, there is an increasing interest in natural compounds that can act as protectors against diseases (Aydin *et al*., 2004). Several therapeutic properties of medicinal plants are known in obstetrics and gynecology (Abo *et al*., 2000), respiratory disorders (Neto *et al*., 2002), skin disorders (Graf, 2000), cardiac diseases (Ankli *et al*., 2002), and mental health (Ahmad *et al*., 1998). It has been suggested that halving the rate of mutations would delay the onset of most cancers and might be adequate in the lifetime of many individuals (Loeb *et al.,* 2003). There are different classes of secondary metabolites present in the plants exhibiting antimutagenic activities.

From the earliest times, herbs have been prized for their pain-relieving and healing abilities and today we still rely largely on the curative properties of plants. According to World Health Organization, 80% of the people living in rural areas depend on medicinal herbs as primary healthcare system. The synthetic anticancer remedies are beyond the reach of common man because of cost factor. Herbal medicines have a vital role in the prevention and treatment of cancer and medicinal herbs are commonly available and comparatively economical. A great deal of

pharmaceutical research done in technologically advanced countries like USA, Germany, France, Japan and China has considerably improved quality of the herbal medicines used in the treatment of cancer. Some herbs protect the body from cancer by enhancing detoxification functions of the body. Certain biological response modifiers derived from herbs are known to inhibit growth of cancer by modulating the activity of specific hormones and enzymes. Some herbs reduce toxic side effects of chemotherapy and radiotherapy.

Scientists all over the world are concentrating on the herbal medicines to boost immune cells of the body against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body (Larkin, 1983; Saxe, 1987).

Most plants synthesize toxic substances which act as a defensive mechanism against insects and herbivores. In addition to that, the poisonous substances may also affect organisms that feed on them including humans. Therefore, it is reasonable that while some medicinal plants may suppress the effects of mutagens, others may have toxic or mutagenic effects (Vicentini *et al.*, 2001). Thus, studies of their mutagenic as well as antimutagenic potential are necessary to establish the safe use of these medicinal plants.

Apart that, the plant has the potential to treat Herpes Simplex Virus infection, minimize inflammations and to reduce *in vitro* carcinogenic effects. *A. vasica*, also known as Malabar Nut Tree is part of Acanthaceae plant family which is used widely among Indians for the treatment of inflammation (Chakraborty and Brantner, 2001), cold, cough, chronic bronchitis (Amin & Mehta, 1959), cataract (Patel *et al.*, 2012), asthma, piles, glandular tumor & to cure fresh wounds (Ayyanar and Ignacimuthu, 2008; Dhuley, 1999; Palasuwan *et al.*, 2005). The pharmacological activities may be due to the presence of vasicine, vasicinone and vasicinol, which are the major alkaloids found in *A. vasica* (Padmaja *et al.*, 2011). *Carica papaya* (papaya) belongs to the family Caricaceae, native to all tropical countries. The plant is well known for its fruits & its parts are used for microbial infections (Sharmeen *et al.*, 2012), treating burns and wounds, fever, intestinal nematode infection, asthma, and gastric (Runnie *et al.*, 2004; Starley *et al.*, 1999; Stepek *et al.*, 2004). Moreover, according to Mazzio and Soliman (2009), the extract of papaya leave showed antitumor activity when tested in Neuro-2A cell lines.

***Melissa officinalis* used as antimutagenic and anticarcinogenic drug**

Lemon balm (*Melissa officinalis* L.) belonging to the Lamiaceae family is a perennial herb. It grows wildly in Europe and the Middle Asia and is used as aromatic, culinary and medical herb (Kato- Noguchi, 2003). This plant is successfully cultivated and popularized by the agricultural research organizations of Taiwan. It is also applied for tea and steeped wine manufactures in Taiwan due to its health profit. Reports indicated that lemon balm had many beneficial effects such as anti-bacterial, sedative, spasmolytic, mnemonic improvement, and could reduce excitability, anxiety, stress, gastrointestinal disorders and sleep disturbance (Mentle *et al.*, 2000; Perry *et al.,* 1999).

Many reports indicated that low polar extract of *Melissa officinalis* (lemon balm) leaves, especially its essential oil, had good antioxidant and antitumoral activities (DeSousa *et al.*, 2004; Marongiu *et al.,* 2004; Mimica-Dukic *et al.,* 2004). Dastmalchi *et al.,* (2008) demonstrated that ethanolic extract of lemon balm cultivated in Iran could present good antioxidant activity. Capecka *et al.,* (2005) also found that methanolic extracts of lemon balms cultivated in Poland had good radical scavenging ability. There is no thorough report concerning anti-proliferative activity of the polar extract from lemon balm leaves for cancer cells. Marnett and DuBois (2002) indicated that cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are rate-limiting enzymes in the biosynthesis of prostaglandins. COX-1 is constitutively expressed in most mammalian tissues and plays a role in tissue homeostasis. COX-2, an inducible isoform, could be stimulated by carcinogens, growth factors, inflammatory cytokines and tumor promoters (Shen *et al.*, 2008). Abnormal or excessive COX-2 expression has been suggested in many pathological conditions such as angiogenesis, inflammation and tumor promotion (Rao *et al.*, 2001). An inhibition of the activity or expression of COX-2 was an important target for antiinflammation or cancer chemoprevention (Shen *et al.*, 2008). However, the suppressing activity of the extract of lemon balm leaves is still unavailable.

Table 1. A list of some of the important medicinal plants of Jammu and Kashmir used in treatment of Cancer and various other diseases.

S. No.	Botanical Name	Common Name	Family	Chemical Constituents	Uses
1.	*Artemesia annua L.*	Sweet Wormwood	Asteraceae	Artemisinin, Camphor	Cancer treatment, Parasite, Malaria.
2.	*Aconitum heterophyllum*	Atis root, Atis	Ranunculaceae	Alkaloids, Atisin	Analgesic, anti-inflammatory, anti-pyretic, aphrodisiac, induce appetite.
3.	*Bergenia ciliate*	Zakhm-e-hayat	Saxifragaceae	Bergenin, Catechin, Gallic acid	To dissolve kidney stones and as a diuretic.
4.	*Chrysanthemum cinerariifolium* L.	Pyrethrum, Insect Plant	Asteraceae	Yejuhua lactone, Asteglasine, Sesquiterp, Amyrins, Lupeol, Arteglasin, Acacetin, Flavinoids, Linalool, Thymol	Wide variety of medicinal properties, including anti-HIV-1, antibacterial and antimycotic, acts as natural source of insecticide.
5.	*Bupleurum falcatum*	Sickle leaf hare's ear	Apiaceae	Tripenoid Saponins, Saikosaponin a, Saikosaponin b4, Saikosaponin c, Olysaccharides, Bupleurans	Anti-inflammatory, Antitussive, Diaphoretic, Hepatoprotective.
6.	*Ferula jaeschkeana* L.	Heeng	Apiaceae	Resin, Endogenous gum, Volatile oil, Asaresinotannols, Ferulic acid, Umbelliferone	To treat tumors, chronic wounds.
7.	*Juglans regia*	Walnut	Juglandaceae	Nucin, mucilage, albumin, mineral matter, cellulose and water.	Treatment of skin troubles, anti-scorbutic pickle, sore and slightly ulcerated throats.
8.	*Melissa officinalis*	Lemon balm	Lamiaceae	Trans-ocimene, cis-ocimene, cis-3-hexenol, citronellal, linalool, geraniol.	Antibacterial activity, Antioxidant
9.	*Origanum vulgare*	Oregano	Limaceae	Carvacrol, thymol, limonene, pinene, ocimene, caryophyllene	Antioxidant, Antimicrobial activity.
10.	*Podophyllum hexandrum* Royle	Indian Mayapple	Berberidaceae	Podophyllin	Anticancerous
11.	*Rumax nepalensis* L.	Jangli Palak	Polygnaceae	Anthraquinone, Nepthaline, Chrysophanol, Glucopyranoside, Nepodin.	Applied to skin sores, Syphilitic ulcers
12.	*Scutellaria species*	Blue Skullcap, Hoodwort, Mad dog Skullcap	Limiaceae	Flavinoids, diterpenes, tannin, beta elemane, calamenene	Antitumor, hepatoprotective, antioxidant, anticonvulsant, antibacterial, antiviral.
13.	*Taxus wallichiana Zucc.*	Himalayan Yew	Taxaceae	Abeo-baccatin, bujanone, bujanol.	Anticancer drug, paclitaxel.

Conclusion

Untoward mutations are associated with a number of serious diseases for which useful medications are few and treatment is often limited to deal with symptomatologies, many of the environmental pollutants, residues of pesticides and toxins present in food and drugs are common agents of mutagenic damage in human population. Science has long acknowledged the value of healing substances found in nature, such as digitalis, aspirin, penicillin, insulin, steroids, etc. There has been a resurgence of interest, both scientifically and popularly, in the utilization of natural approaches. Experiments on cell lines and in animals demonstrated that herbal drugs having anticancer role by inducing apoptosis and differentiation, enhancing the immune system, inhibiting angiogenesis and reversing multidrug resistance. However, the mechanism of the anticancer role has not yet been fully elucidated. Further research is needed to explore the molecular mechanism of herbal drugs.

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CT Guided Biopsy, Fine-Needle Aspiration (FNAC) Result Analysis and Various Symptoms for the Diagnosis of Lung Cancer in Kashmiri Population

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Abstract
Lung cancer is most common cancer worldwide representing approximately 12.7% of all new cancers and 18.2% of all cancer related deaths, throughout the world. In India, it is the commonest and the leading cause of cancer related mortality in both men and women. Most patients have been found to be in the advanced stage of the disease. It is the most lethal cancer among males accounting for 10.9% of all cancer cases and 13% of cancer related mortality. Human pulmonary neoplasms can be subdivided into two major forms: non-small cell cancers and small cell cancers. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases. The non-small cell cancers include adenocarcinoma, squamous cell carcinomas, large cell carcinomas, and adenosquamous cell carcinomas. Adenocarcinoma has become the most important form of lung cancer over the past 20 years with both a relative and an absolute increase in incidence rates. In most of the developed countries, it has become the dominant histological type of lung cancer. It has also overtaken squamous cell carcinoma as the most common form of lung cancer among males in some countries while it has continued to be the commonest type among females. This histological shift has been linked to changes in the smoking habits of the population in these regions as well as in the design and composition of cigarette being marketed therein. Specific signs, symptoms and radiological criteria for diagnosis of the patients who were suspected for Lung cancer and all patients were diagnosed by CT- guided biopsy, branchioscopy and FNAC. In this hospital based analytical study an attempt was made to correlate clinical and radiological profile of suspected case of lung cancer with CT-Guided FNAC/biopsy. Though the clinical symptoms were not specific, CT-guided FNAC/biopsy proved to be valuable in confirmation of diagnosis of lung cancer and histopathology proved to be a diagnostic tool for almost all suspected.
Keywords: CT-Guided FNAC, branchioscopy, FNAC, adenocarcinoma.

Introduction

Lung cancer is one of the commonest cancers and the leading cause of cancer related mortality worldwide (Jemal *et al*., 2011). In the beginning of the century, lung cancer was considered to be rare (Nath *et al.,* 1935). But now, it has reached epidemic proportions. This is the leading cause of cancer death in developed countries and is rising at alarming rates in developing countries (Khuri *et al.,* 2001). It accounts for 12.7% of all new cancer cases and 18.2% of all cancer related deaths, throughout the world. In India, it is the commonest and the leading cause of cancer related mortality in both men and women (Brambilla *et al.,* 2001; Hussain *et al.,* 2010). It is the most lethal cancer among males accounting for 10.9% of all cancer cases and 13% of cancer related mortality (Parkin., 2008). According to National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) 2010 report, lung cancer is the second most common cancer worldwide, in both males (accounting 15% of all cancer) and females (accounting for 14% of all cancer) and it is the most common cause of cancer death worldwide (Long., 2012).

Compared to western population, epidemiological study shows there is an increased prevalence of lung cancer in the Indian population (Rawat *et al*., 2009). In India, approximately 63,000 new lung cancer cases are reported each year (Ganesh *et al.,* 2011).

Lung cancer was reported to be the second most common malignancy in an earlier hospital based study from Kashmir valley (Shah *et al.,* 1990), the first being cancer of the upper gastrointestinal tract (Shah *et al.,* 1990; Dhar *et al.,* 1993). However, a recent study shows that Srinagar, the summer capital of Jammu & Kashmir has the highest incidence of lung cancer among males in India (Koul *et al*., 2010).

Human pulmonary neoplasms can be subdivided into two major forms: non-small cell cancers and small cell cancers. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases. The non-small cell cancers include adenocarcinoma, squamous cell carcinomas, large cell carcinomas, and adenosquamous carcinomas. Adenocarcinoma has become the most important form of lung cancer over the past 20 years with both a relative and an absolute increase in incidence rates. In most of the developed countries, it has become the dominant histological type of lung cancer (Jindal *et al.,* 1990). It has also overtaken squamous cell carcinoma as the most common form of lung cancer among males in some countries while it has continued to be the commonest type among females (Little *et al.,* 2007). This histological shift has been linked to changes in the smoking habits of the population in these regions as well as in the design and composition of cigarette being marketed therein (Alberg *et al*., 2007). We take only specific signs, symptoms and radiological criteria for patients who were suspected for Lung cancer and all patients were diagnosed by CECT- guided biopsy, branchioscopy and FNAC. The aim of the peresent study is to diagnose lung cancer through the CT-Guided FNAC.

Materials and Methods

Study design and method

Patients enrolled: 50 patients were enrolled in the study and most of them were above 30 years age with clinical symptoms of cough since 20 days, hemoptysis, fever, weight loss, dyspnea and hoarseness of voice and/or with any radiological features like space occupying lesion, hilar prominence, mediastinal widening and collapse with consolidation. These cases with above criteria were subjected to CT guided biopsy /FNAC and branchioscopy.

Results

The demographic profile revealed that in our study 50 patients who were suspected for lung malignancy due their clinical signs and symptoms with positive radiological findings in favour of lung cancer, all the 50 patients were diagnosed histopathologically as lung cancer by CT-guided biopsy/ FNAC. Most of the malignant patients were above age of 40 years and patients with malignancy were presented with average age 58.94 years, most of the patients were within range of 50 to 84 years.

In malignant cases (50), 40 patients were male and remaining 10 patients were females (Table. 1). Most of the patients with lung cancer 17 were farmer, 10 were house wives, 13 were from service class and rests of the patients were drivers, shopkeepers, labours.

Most of the patients who were suspected for lung cancer in study were smokers 35; only small number of patients non-smokers.

The patients which were suspected of having lung cancer presented with cough which was the major symptom (42 out of 50) and all patients diagnosed as having lung cancer were presented with cough. Other major symptoms beside cough were dyspnea and weight loss 74%, 44% were having Haemoptysis and 40% were having chest pain. 38% patients were having fever and 20% patients complain of Hoarseness of voice.

We took only specific signs, symptoms and radiological criteria for patients who were suspected for Lung cancer and all patients were diagnosed by CT- guided FNAC/biopsy. Among all suspected cases, all the 50 cases were diagnosed histopathologicaly as malignant and no cases has been found non – malignant. In diagnosed malignant

cases non small cell lung carcinoma was almost found in all cases. In NSCLC type Lung cancer adeniocarcinoma was most common (80%) and squamous cell carcinoma second most common type of non small cell Lungs cancer (20%). FNAC/biopsy taken in all suspected patients with CT-guided. It was conclusive in 49 patients and inconclusive in one patient. FNAC/Biopsy was conclusive in 98% of patients, so CT-guided FNAC is valuable procedure regarding diagnosis of lung cancer though lung biopsy remain gold standard procedure for definitive diagnosis.

Table 1. Demographic characteristics of lung cancer patients

S.No	Variables	Year Nov 2013 To June 2014
1.	Total Cases	50
2.	Mean Age	58.94
3.	Sex	
	1. Male	40
	2.Female	10
4.	Male :Female	4
5.	Rural : Urban	1.5
6.	Occupation	
	1.Farmers	17
	2. Service class	13
	3. House wife	10
	4. Others	10
7.	Diagnosis	
	1. CT Guided Biopsy/FNAC	47
	2. Branchioscopy	03
	3. Both	06
8.	Histological type	
	1. Adenocarcinoma.	42
	a)Male	33
	b) Female	09
	2. Squamous Cell Carcinoma	08
	a)Male	07
	b) Female	01
9.	Symptoms:	
	a) cough	42
	b) cough+ dyspnea +weight loss	37
	c) Haemoptysis.	22
	d) Chest pain.	20
	e) Fever.	19
	f) Hoarseness of voice.	10
10.	Smoking:	
	a) Smokers	35
	b) Non smokers	15

Discussion

Lung cancer is the most frequent malignant disease and the most common cause of cancer death in the world. However, the clinical profile of lung cancer in India was ifferent from the west, in that Indian patients present almost 15-20 years earlier in the 5th or 6th decades of life (Parkin *et al*., 2005). However, a recent study shows that Srinagar, the summer capital of Jammu & Kashmir has the highest incidence of lung cancer among males in India (Koul *et al*., 2010).

Adenocarcinoma has become the most important form of lung cancer over the past 20 years with both a relative and an absolute increase in incidence rates. In most of the developed countries, it has become the dominant histological type of lung cancer (Jindal *et al., 1990)*. It has also overtaken squamous cell carcinoma as the most common form of lung cancer among males in some countries, while it has continued to be the commonest type among females. In present study we found that adenocarcinoma is gradually becoming prominent subtype among males in kashmir valley. Majority of lung cancer cases have been convincingly proven to be associated with smoking habits. Of all lung cancer deaths in world 85% are attributed to tobacco smoking, which contain harmful carcinogens. This study shows the clinicoradiological profile of suspected cases and views the most common clinical, radiological and pathological profile of lung cancer in SKIMS Soura.

The lung cancer was predominantly seen in males, who were accounted for 80%. The male female ratio was 4 in this study. This finding has been consistent with the other studies in India, that the lung cancer is predominantly seen in male. Jindal & Behera., (1990) reported the sex ratio as 4.5 whereas Kashyap *et al.,* (2001) reported sex ratio in lung cancer was 6.17. It has been reported as low as 2.9 by Jha *et al.,* (1972).

As in this study we found high male female ratio showing less awareness about the health in females. This study also shows that there is less awareness in farmers about health and major reason of malignancy in the farmers was higher smoking habit of cigarette and Hukka than the urban one.

A significant proportion of the cases in the study were within range of 50-70 years (68%) the mean age was 58.9 years, and one patient with minimum age (28 yrs.) Who was diagnosed as adenocarcinoma, but he was associated with neck pain and Gout. Jindal and Behera., (1990) reported mean age 54.3 years whereas Gupta *et al*., (2001) and Kashyap *et al*.,(2003) reported mean age at presentation as 60 and 54.6 years respectively. This observation reconfirmed the established fact of increasing incidence of lung cancer as the age advances and need of detailed evaluation of elderly patients who present features suggestive of lung cancer.

In this study we include all suspected cases with clinical and radiological specified criteria's which include smoking. We found that patients who have history of smoking 35patients (70%) were malignant and 15 cases were malignant form without history of smoking (30%). This is showing increasing malignancy in non smoker patients also. It indicates increase in air pollutions, either by motor vehicle or by factories or by aerosolized fumes in kitchen particularly in females.

In the patients who were diagnosed as malignt (50), the maximum patients were smokers with smoker to nonsmoker ratio 2.3. Jindal and Behera., (1990) reported ratio as 2.7. While Arora *et al*., (1990) reported the ratio was 1.2, nonetheless lung cancer has been prominently seen in smokers in each of the previous and in this study also.

There are important differences in the clinical spectrum of lung cancer patients in India compare to those in the west Jindal and Behera., (1990) . Most of the patients have advanced disease at the time of diagnosis.

Most common symptom experienced by our patients was cough associated with 42 patients who were suspected for lung cancer. The next most common symptom reported were dyspnea and weight loss (74%). 44% were having blood in sputum and 40% were having chest pain, 38% were having fever and 20%. patients were complaining of hoarseness of voice.

Most common radiological finding in lung cancer patients of this analytical study was space occupying lesion (mass) which was found in 65% of all malignant patients (33). It was right side in 25 patients (75.75% and left side

in remaining 8 patients (24.25%). The space occupying lesion was more commonly seen in right lung and in upper zone. Others major radiological finding was Pleural effusion in 12 patients (24%).

In the same study of 336 patients with bronchogenic carcinoma carried out in Chandigarh by Jindal *et al.,* (1990), commonest finding was opacity with or without collapse (64%) and pleural effusion (23%). In a study by Jagdish *et al.,* (2009) mass lesion was reported in 46.31% cases, collapse-consolidation 40.89 and pleural effusion in 4.43% cases. There is wide variability in these observations in different studies; however the finding of a mass lesion at the time of diagnosis of lung cancer is high.

This shows how a lung cancer lesion grows to such extent and cause symptoms when it is of significant size and probably has metastasized already by the time of diagnosis.

Finally out of all NSCLC cases 20% patients had squamous cell carcinoma (10), 80% had adenocarcinoma (40). Thus Adenocarcinoma was more frequently diagnosed than any other form of lung cancer. But seconds most common was Squamous cell carcinoma. This is showing the increasing of adenocarcinoma subtype of lung cancer in Kashmir as in rest of the India as well as in Western countries.

In other Indian studies Jagdish Rawat *et al.,* (2009) studied 203 cases of lung cancer. They reported Squamous cell carcinoma in 91(44.83%), Adenocarcinoma in 40(9.70%), Large cell carcinoma in 17(8.37%), Undifferentiated carcinoma in 21(10.34%) and small cell carcinoma in 34 (16.75%) cases. Jindal and Behera., (1990) in their study reported incidence of squamous cell carcinoma 34.3%, Adenocarcinoma 25.9%, small cell carcinoma 20.3% and large cell carcinoma in 7.3%. In another study by Navneet *et al.,* (2010) reported incidence of Squamous cell carcinoma 34.8%, Adenocarcinoma 26%, small cell carcinoma 18.4% and other in 20.8%. This study in Kashmir by Khan *et al.,* (2006) found incidence of Squamous cell carcinoma to 48 (77.3%), Small cell carcinoma 55 (17.1%), Adenocarcinoma 17(5.3%) and Large cell carcinoma 1(0.31%).

There is a variation in histological diagnosis in these previous studies; however squamous cell cancer has been the most common histological type of lung cancer in India as shown by these studies.

In present study the most histological diagnosis came out to be Adenocarcinoma in 84% of the patients.

The same variation in present and previous studies may be due to including of all suspected cases than others in which studies were done in already diagnosed cases.

The most histological type in smoker and non smoker was adenocarcinoma, which is 83.33% and 16.66% respectively. In male patients adenocarcinoma was the most common diagnosis in which majority was smokers.

In female most common type was adenocarcinoma (21.42%). Navneet *et al*.,(2010) reported the incidence of squamous cell carcinoma in smokers as 38.5% and adenocarcinoma as a most common histological type in non smokers was 46.3% cases.

Conclusions

In this hospital based analytical study an attempt was made to correlate clinical and radiological profile of suspected case of lung cancer with CT-Guided FNAC/biopsy. Though the clinical symptoms were not specific, CT-guided FNAC/biopsy proved to be valuable in confirmation of diagnosis of lung cancer and histopathology proved to be a diagnostic tool for almost all suspected. The Radiological guided FNAC made the histology an easier procedure. The result in this study is close to earlier studies in terms of clinical presentation and features. Majority of patients having adenocarcinoma, were male and smokers. Squamous Cell Carcinoma was also diagnosed in higher percentage of patients.

The main limitation of this study was small size of study population, In view of this the result may not be a true presentation of the trends in general population so more studies are required to confirm the result.

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Antibiotic Resistance of Microbes Obtained from Sindh, a Glacier Fed River of Sonamarg Kashmir

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Abstract
Antibiotic-resistant microbes find their way into aquatic ecosystems from human and animal sources. These microbes have potential to spread their genes into water-indigenous microbes, which also contain resistance genes. Resistance to several different antibiotics at the same time is even more significant problem. It is because of the acquired resistance that microbe's particularly bacterial isolates must be subjected to antibiotic susceptibility testing. Awareness of antibiotic resistant microbes in aquatic ecosystems is growing. In the present study bacteria cultured from the river waters were tested for resistance against eight antibiotics [Cephalothin (Ch), Clindamycin (Cd), Trimaxozole (Co), Erythromycin (E), Gentamycin (G), Ofloxacin (Of), Penicillin (G), Vancomycin (Va)] and Amphotericin-B was used for fungi. The results reveal that, all the strains showed high resistance to almost all the drugs tested against except Gentamycin and Ofloxacin that showed 100% susceptibility. For fungal species it was found that *Asperigillus* spp. and *Candida* spp. were susceptible while *Pencillium* spp. was found to be resistant. Our findings from this preliminary research have indicated that microbes in our aquatic systems are resistant to the antibiotics and hence pose a potential threat to ecosystem function and potentially human health.
Keywords: Aquatic ecosystem, bacteria, antibiotics, pathogens.

Introduction

The microbial quality of aquatic ecosystems particularly of those which are important for drinking purposes, have focused on the occurrence of pathogens in drinking water distribution systems (Berry *et al*., 2006; Szewzyk *et al*., 2000). Environmental contamination affects microbial communities in a myriad of ways. Source and amount of pollutants together with ecosystem dynamics modulate the responses of microorganisms to anthropogenic impacts (Cardoso *et al*., 2012). Microbe communities react with drastic changes in ecosystem functioning, species composition and abundance (Nogales *et al*., 2011; Vieira *et al*., 2008). Several consequences may arise from aquatic pollution. The connection between these impacts and potentially pathogenic bacteria is of particular relevance for human welfare (Nogales *et al*., 2011). There are various ways to estimate the anthropogenic load on an ecosystem, including chemical analysis, bioindication, bioassays, etc. (Doust *et al*., 1994; Ostroumov, 2000, Lindstron-Seppa *et al*., 2001; Samecka-Cymerman and Kempers, 2001). Bacteria are often used to monitor the state of the environment (Jacobs *et al*., 1995; Schwedt *et al*., 1997; Backhaus and Grimme, 1999), as their evolution rate is very high, and under the effect of environmental factors they can acquire various specific features, and thus, serve as good indicators for the presence of pollutants. One of the properties of bacteria used to assess the anthropogenic load is their antibiotic resistance (Hagedorn *et al*., 1999; Goni-Urriza *et al*., 2000; Mary *et al*.,

2000; McArthur and Tuckfield, 2000). Thus, the presence of antibiotic resistant microbes in aquatic ecosystems can greatly affect public health and hence is an emerging issue for the public in general and the drinking water industry in particular (Armstrong *et al*., 1981; Schwartz *et al*., 2003). Although several studies have detected antibiotic resistant bacteria in drinking water systems (Armstrong, 1982; Armstrong *et al*., 1981; Pavlov *et al*., 2004; Schwartz, 2003; Zhang *et al*., 2009), most previous studies focused on cultivable bacteria and/or indicator organisms. The aim of this work was to investigate the dynamics of antibiotic resistance of microbes in aquatic system against the selected antibiotics.

Materials and Methods

Sampling site

Sonamarg – the Meadow of Gold is situated at an altitude of 2730m. The Sindh River that meanders through the valley is locally known as "SENDH" originates from the Panjtarni glacial fields at an altitude of 4,250 m (a.s.l) at the base of Saskut, a peak (4,693 m a.s.l) in the Ogput Range running parallel to the North-West to South-East. On its descend, the Sindh receives glacial melt waters from the glaciers like Nicchang, Mashram Bal and Kolhai (The largest glacier of Kashmir) in addition to the glaciers of the Nilgrar region, Thajwas glaciers and Harmukh glaciers. From Saskut, River Sindh drops steeply north-westward to reach the main strike valley. Gathering momentum, the river runs towards Sonamarg between steeply towering mountain areas, over a boulder streambed, emerging into the pleasant upland serenity of the Sonamarg, as if to rest before it plunges roaring headlong torrent sharply to the Southwest through the Gangagir gorge, 4000 ft (1,230 m) deep. The climate of Sonamarg is very bracing, with average temperature around 14°C. Winters (November to April) are chilly with temperature goes down to subzero levels. Two sites selected for the present study were Yousmarg (Site I) renowned for its green pastures, pines and fir with geographical co-ordinates lying between 34^{0} 17′ 0″N and 75^{0} 19′ 0″E and an elevation of 2,712 m (a.s.l) and Thajwas Grar (Site II) known for the glaciers, the miniature plateaus, snowfields, pines and islets with geographical co-ordinates between 34^{0} 17′ 50″N and 75^{0} 12′ 52″ E and an elevation of 2,617 m (a.s.l).

Sample collection

Samples of water were collected from the selected sites for six months from July 2010 to December 2010 in suitable plastic bottles, which were previously carefully cleaned, rinsed three to four times with distilled water (A.P.H.A, 1998). During collection of samples, extreme care was exercised to avoid contamination. The collected samples were later processed for microbial analysis. Water samples obtained from different sites were serially diluted five folds and then spread plate technique was followed for isolation of bacteria and fungi, spreading 0.1ml inoculum from the serial dilution tubes on the Petri dishes containing nutrient agar and Rose- Bengal, Streptomycin, Agar medium for bacteria and fungi respectively. In case of bacterial isolation inoculum from the serial dilution tubes was spread onto the Petri dishes containing Nutrient agar medium by two different techniques which are Serial dilution (Clesceri *et al*., 1998) and Spread plate (Sharp and Lyles, 1969) and were incubated at a temperature of 37 °C for 24-48 hours. For provisional identification of bacteria important Gram staining were performed and then Antibiotic sensitivity tests were done.

Antibiotic sensitivity test of the isolated strains was carried out using Kirby-Bauer Method (Bauer *et al*., 1966) using antibiotic octa-combi discs from Hi-media. The media used for test are Sabouraud dextrose agar and Mueller Hinton Hi-Veg Broth. In this method the standardized bacterial isolate is spread on an agar plate and then paper disc containing specific concentration of antibiotics are placed and incubated at 37°C overnight. The bacterial strains were tested for sensitivity against eight antibiotics namely Cephalothin (Ch), Clindamycin (Cd), Trimaxozole (Co), Erythromycin (E), Gentamycin (G), Ofloxacin (Of), Penicillin (G), Vancomycin (Va). Fungal strains were tested against amphotericin-B Isolates susceptible to the antibiotic, does not grow around the disk thus

forming a zone of inhibition. Strains resistant to an antibiotic grow up to the margin of disk. The diameter of zone of inhibition was measured and results were compared from the Kirby Bauer chart as sensitive, intermediate or resistant.

Results and Discussion

During the study period four strains of bacteria were isolated from Site I and six strains were from Site II and were identified on the basis of macro-morphological characters and Gram staining. The strains isolated were given codes ranking from B1 to B7. Most of the colonies were circular, entire and flat in appearance, margin, and elevation respectively and the majority of the strains isolated were Gram negative. About 60% of strains isolated were observed as Gram-negative cocci. Presences of gram negative cocci are of much concern because of their pathogencity resulting in diseases in humans. Wernar *et al*., 1969 has reported that outbreak of gastro enteritis is because of sewage-polluted water containing *E. coli* Pathogenic bacteria has also been isolated from River Tawi in Jammu (Gandotra *et al*., 2009). Similarly *Asperigillus* spp*., Candida* spp. and *Pencillium* spp. were found at Site I while at Site II only two *Asperigillus* spp. and *Pencillium* spp. were found. The relevance of fungi and their activities in water is emphasized by increasing knowledge of their pathogenicity for humans, animals and plants, their role as food for energy, their activity in natural purification processes, their exploitation for science and technological use (Cooke,1954; Castellani, 1963; Curtis,1972; Kishimoto and Baker, 1969; Suzuki, 1962). The comparative study of observation of investigators indicates that some species of fungi especially water moulds show variation in their ecological requirements (Mer *et al*., 1980).

The isolated strains were then tested for sensitivity against eight antibiotics namely Cephalothin (Ch), Clindamycin (Cd), Trimaxozole (Co), Erythromycin (E), Gentamycin (G), Ofloxacin (Of), Penicillin (P), Vancomycin (Va). The results of antibiotic sensitivity test for bacteria (Table 1) reveal that, in general 46.42% of strains were resistant, 35.7% of strains were susceptible, and 17.8% of strains showed intermediate sensitivity. In addition, all the strains showed high resistance to almost all the drugs tested against except Gentamycin and Ofloxacin that showed 100% susceptibility. Resistance of a single bacterial isolates to more than one antimicrobial drug has also been reported (Norelli *et al*., 1991; Sayah *et al*., 2005). The increasing spread of antibiotic resistance among environmental bacteria has led some authors to consider antibiotic resistant bacteria and antibiotic resistance genes (ARG) as emerging pollutants (Kümmerer, 2009; Wright, 2010: Pruden *et al*., 2006). The high level of drug resistance in tested bacterial strains could be related to the production of antimicrobial compounds found in some fish-farm isolates belonging to the genus Bacillus (Sarker *et al*., 2010). Further, the antibiotic sensitivity was also carried out for fungi. The antibiotic used was Amphotericin-B. The results (Table 2) reveal that *Asperigillus* spp. and *Candida* spp. were susceptible while *Pencillium* spp. was found to be resistance.

In conclusion, significant levels of antibiotic re sistance were found among isolated species. The development of new antibiotics as well as their increasing and, occasionally, indiscriminate and irrational use provokes the release of antibiotic and antibiotic-resistant bacteria via sewage or treated wastewater to the environment. The anthropogenic impact on the aquatic system is likely to have been associated with the higher levels of resistance. Unless preventive measures are taken to combat the spread of antibiotic resistance, by reducing pollution and making conscious use of these drugs, humanity may be faced with the end of the antibiotic era.

Table 1. Antibiotic sensitivity behaviour of bacterial isolates.

Bacterial Strain	Antibiotic agent							
	Clindamycin	Trimaxozole	Erythromycin	Gentamicin	Oflaxacin	Pencillin	Vancomycin	Cephalothin
I3	R	R	I	S	S	R	R	R
I4	S	S	R	S	S	S	I	R
I2	S	S	I	S	S	S	R	R
I6	R	R	R	S	S	R	I	I
I1	R	R	I	S	S	I	R	R
I5	R	R	R	S	S	I	R	R
I7	R	I	R	S	S	I	R	R

R-Resistant; I-Intermediate; S- Susceptible. (46.42% resistant, 35.7% susceptible, and 17.8% intermediate).

Table 2. Antibiotic sensitivity behaviour of isolates for fungi.

Fungal strain	Antibiotic Agent (AmphotericinB)
Asperigillus spp.	Susceptible
Candida spp.	Susceptible
Pencillum spp.	Resistant

Fig. 1. Antibiotic test (octacombi-discs) for bacteria

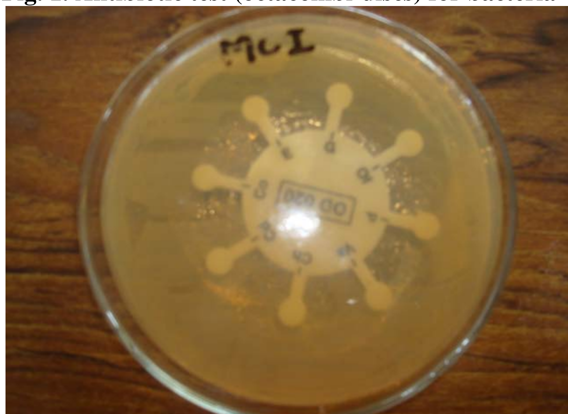

Fig. 2. Antibiotc test (ampicillin-A) for fungi

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Morphology, Physiology and Taxonomy of *Streptomyces verticillatus*

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Abstract
The antibiotic research from the discovery of Fleming to present time has been an exciting, fascinating, continuously changing and developing adventure. Antibiotic drug discovery is an indispensable process to combat aggressive ability of pathogenic microorganisms and emerging infectious diseases against health and well-being of people through-out the world. The search for new antibiotics has stimulated a variety of different approaches for identification of novel producers throughout the world. Actinomycetes, as potential candidates, continue to be isolated by mix of traditional and modern methods from a wide variety of soils and substrates. The lists of new antibiotics and new actinomycetes species suggest that the careful exploration of new soils and habitats might continue to be useful. Therefore, in this regard, distribution, isolation, identification and detection of active *Streptomycetes* microflora of Kashmir, India were investigated. In preliminary screening of soil inhabitant Actinomycetes, we isolated (*Streptomyces verticillatus*) from soils of an apple orchard in Budgam District, J&K India. It revealed high antagonistic activity against wide range of pathogenic bacteria. Identification to the genus level was based mainly on morphological characters. Microscopy and direct observations revealed the morphological criteria as: color of aerial mycelia was grey, reverse color was Yellow and spore chains were spiral with smooth spore surface. Physiological characterizations indicated that it can utilize inositol, glucose, fructose, rhamnose and raffinose but not arabinose, xylose and sucrose and without melanoid or soluble pigments.

Keywords: Garden soil, actinobacteria, antimicrobial activity, fermentation, *Streptomyces plicatus,* melanoid pigments

Introduction

Actinomycetes are the most economical and biotechnologically valuable class of prokaryotes producing bioactive secondary metabolites notably antibiotics (Blunt and Prinsep, 2006) anti tumor agents, immunosuppressive agents (Mann, 2001) and enzymes (Berdy, 2005; Cragg and Newman, 2005; Strohl, 2004). Goodfellow and Haynes (2005) reviewed the literature on isolation of actinomycetes and suggested that only 10 % of actinomycetes are isolated from nature. Most of the antibiotics in use today are derivatives of natural products of actinomycetes and fungi. Although soils have been screened by pharmaceutical industry for about 50 years, only a small fraction of actinomycetes taxa have been discovered.

The actinomycetes are gram positive, high G+C (>55%) organisms that tend to grow slowly as branching filaments. Actinomycetes encompass a wide range of bacteria. They have universal occurrence and play an active part in the cycle of nature. The class Actinobacteria holds some of the resilient species, capable of growing in extreme, hostile and polluted environments. Their adaptation has been the outcome of several chemical entities which are answers to a number of medicinal and industrial questions of today.

Kashmir offers a favorable environment as well as rich soil diversity for microbial research studies. Very little research has been carried out in Kashmir regarding Actinomycetes except some stray reports. But the studies on soil actinobacteria in Kashmir are largely unexplored. Hence we have taken an initiative to isolate and identify soil actinomycetes from different soil types and to characterize them.

The isolation and identification of novel Streptomycetes can be a laborious process and can best be justified by subjecting the newly isolated test organism to as many meaningful test systems as is feasible (Nolan and Cross,

1988). The Waksman in 1940s showed that actinomycetes are not only capable of producing medically useful antibiotics, but also stimulated the intensive search for new active strains. Since the systematic screening programs in the search for antimicrobial metabolites were first performed by Waksman, microbial cultures have been a major source of antibiotic substances (Okami and Hotta, 1988). Actinomycetes have been screened widely, but it would be no gross oversimplification to state that their world-wide economic importance centers around their pharmacologic activities; especially their bioactivity against infectious agents.

The Streptomycetes have been the source of the majority of antibiotics, but in recent years, interesting products have been also isolated from species belonging to non Streptomycetes genera. Consequently, there has been a growing trend from actively pursuing research centre's that have made their screening programmes more intensive, resulting in a growing number of reports of novel active compounds (Suzuki *et al.*, 1994). Their characterizations are based on morphological and physiological criteria revealed by many laboratory tests. In this research streptomyces strain was isolated from agricultural soils (Apple Garden) of Budgam District and showed prominent biological activity against wide range of pathogens was identified as (*Streptomyces verticilatus*).

Materials and Methods

Sample collection: Soil samples (approx. 500 g) were collected by using clean, dry and sterile polythene bags along with sterile spatula, marking pen and other accessories. The site selection was done by taking care of the point where widely varying characteristics as possible with regard to the organic matter, moisture content, and particle size and colour of soil and to avoid contamination as far as possible. Samples were stored in sterile polybags and transported to the laboratory where they were kept in refrigerator until analysis.

Isolation: Soil sampling was employed as used by Valan *et al*., (2009) using standard dilution plate technique. The samples were taken for the serial dilution upto the 10^7 dilution, 0.1 ml of each dilution was inoculated in duplicate plates of the Starch casein Agar media for the isolation of actinomycetes by the spread plate technique. After incubation all plates incubated at 30^oC in the incubator for 3 weeks. Nystatin and chloramphenicol were used as antifungal and antimicrobial agent in media. Pure strains of actinomycetes were isolated by streak plate method. Strains were identified on the basis of their phenotypic, physiological and biochemical characteristics.

After proper incubation period, most of the Streptomycetes produced colonies visible to the naked eye (Nolan and Cross, 1988). Selection of candidate colonies was performed by using a stereomicroscope. Streptomycetes colonies were picked on the basis of some morphological features.

Culture Media for Isolation, Screening and Identification: Different bacteriological media were employed for isolation and identification of isolates according to International Streptomyces Project. Oatmeal agar (ISP-medium No. 3) is a standard medium for morphological studies and color determination of all cultures (Shirling and Gottlieb, 1966). Trace salts solution was used in Oatmeal agar medium. Its application is complementary of other media wherever be necessary. Starch Casein glycerol agar was used for isolation, identification and supporting Streptomycetes strains (Kuster and Williams, 1964). Pepton-yeast extract iron agar (ISP- medium No. 6) is standard medium for physiological studies such as determination of melanin production (melanoid pigments) in cultures (Shirling and Gottlieb, 1966). Carbon utilization medium (ISP-medium No. 9) is a standard medium for physiological studies such as determination of ability of Streptomycetes to use different carbon sources in cultures (Shirling and Gottlieb, 1966 and Dietz and Thayer, 1980).

Morphological Characters: The morphological characteristics of Streptomycetes as described by Cross and Goodfellow (1973), were determined as follows:

Morphology of spore bearing hyphae: Characteristics of spore-bearing hyphae were determined by direct examination of the culture surface (21 days old) on opened dishes of the crosshatched cultures under light microscope using 100 x magnification. Using these criteria, species were divided into sections as Rectus or straight, flexible or flexuous, Retinaculum-Apertum and spiral.

Color determination: Observation was made after 15 days and was limited to mature cultures with heavy spore mass surface using code for determining the color of aerial mycelium of Streptomycetes composed by Prauser (1964) for color tabs of Baumann Farbtonkarte Atlas. The color of substrate mycelium was viewed from the reverse side.

Morphology and Ultrastructure of Spores: Spore morphology was determined using slide culture Technique. Thin blocks of agar were cut and placed on sterile glasses and inoculated with isolates all over the agar block surface. A cover slip was then placed over each inoculated agar block and the slides were placed in moist chamber and incubated until good growth of isolate observed. The coverslips were removed from agar blocks, mounted on glass slide, stained properly and observed under oil immersion to study arrangement of spores.

Physiological Characters: The most important physiological criteria used for taxonomical characterizations were: Melanin pigment-production, chitinase degradation activity and use of carbon sources as described by Korn-Wendisch and Kutzner, 1992 as follows: **1- Melanin Production:** Peptone iron agar was used for the detection of deep brown to black diffusible pigment (+). Absence of the color was recorded as negative (-). **2- Carbon Utilization:** Utilization of sugars as L-arabinose, D-xylose, meso- inositol, D-mannitol, D-fructose, rhamnose, raffinose and sucrose was investigated as described in the ISP (Shirling and Gottlieb, 1966).

Results

Taxonomy: Based on morphological, physiological and biochemical characterization, the active isolate was identified as (*Streptomyces verticilatus*). Table 1 shows identification criteria of this strain. It can utilize Xylose, Inositol, Mannitol, Fructose and Rhamnose but not Arabinose, Sucrose and Raffinose.

Table 1. Biochemical and physiological features of strain

S. No.	Test	Result
1	Gelatin liquefaction	+
2	Citrate Utilization	+
3	Nitrate reduction	-
4	Gram staining	+
5	Indole production	-
6	Methyl red	+
7	Voges prauskouer	+
8	Urease production	-
9	**Sugar utilization**	
	Xylose	+
	Arabinose	-
	Sucrose	-
	Fructose	+
	Mannitol	+
	Raffinose	-
	Inositol	+

Fig. 1. Streptomyces species on starch casein agar

Discussion:

For decades, microbial natural products have been one of the major sources of novel drugs for pharmaceutical companies, and today all evidence suggests that novel molecules with potential therapeutic applications are still waiting to be discovered from these natural sources, especially from actinomycetes. Any appropriate exploitation of the chemical diversity of these microbial sources relies on proper understanding of their biological diversity and other related key factors that maximize the possibility of successful identification of novel molecules. The strain was identified as Streptomyces verticilatus. Our study correlates with the results found by Anderson *et al.,* 2001. Arunachalam *et al.,* (2010) identified streptomyces using cellulose production. The morphological and biochemical characteristics of 71 *Streptomyces* spp. isolated from soil samples collected at different places of Venezuela were studied by Taddei *et al.,* (2006). Our results are confirmed by studies carried out by Kalyani *et al*., 2012. The future investigation of this strain should be focused on its capability in gene transfers, use as biofungicide or biofertilizer, determination of its spectrum of activity on human and animal pathogens and its chitinase activity on insect's body wall in biological control of insect pests.

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Development of Agro-Technique for some Important Maps of Kashmir Himalaya

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Abstract

Development of agro-technique is one of the basic conservation strategies for sustainable development and used of medicinal plants. Keeping in view the immense importance of development of agro-techniques the present study was carried out. Agro-techniques have been standardized for various Medicinal and Aromatic Plants (MAPs). Present study revealed that sandy soil was the suitable for successful establishment of most of the transplants. Organic manure, timing of transplantation and proper irrigation prove crucial for establishment of these prized plants upon transplantation. Most of the transplants thrive well in plain beds with raised bunds. The present communication will prove helpful for mass cultivation of these MAPs under *ex-situ* conditions.

Keywords: Agro-technique, medicinal plants, sandy soil

Introduction

Medicinal plants constitute a considerably large component of natural vegetation. Several of these species are in great demand for domestic consumption as well as for commercial use by the herbal industry (Somashekhar and Sharma, 2002). About 800 species are estimated to be in trade with a turnover of Rs.4000 crores per year. This high demand by the herbal industry has put enormous pressure on the wild populations leading to destructive collection of the produce. Absence of commercial cultivation of these species, results in increased dependence on the wild collections, which has further aggravated the situation . Today, a large number of medicinal plants species are considered threatened due to such high demand and destructive collection practices. Thus, the conservation efforts are of immediate need to save these species in the wild by maintaining their wild populations, without which the species may be wiped out (Somashekhar and Sharma, 2002).

Conservation of species diversity is one of the main goals of the 2010 biodiversity target. *Ex-situ* conservation is an integral part of conservational strategies and is becoming more important as a backup technology (Lozoya, 1994; Nautiyal and Nautiyal, 2004).Information on the propagation of medicinal plants is available for less than 10% and agro-technology is available only for 1% of the total known plants globally. This trend shows that developing agro-technology should be one of the thrust areas for research (Kuniyal *et al*., 2005). Furthermore, in order to meet the escalating demand of medicinal plants, farming of these plant species is imperative. Apart from meeting the present demand, farming may conserve the wild genetic diversity of medicinal plants. Farming permits the production of uniform material, from which standardized products can be consistently obtained. Cultivation also permits better species identification, improved quality control, and increased prospects for genetic improvements (Khan and Khanum, 2000). Selection of planting material for large-scale farming is also an important task. The planting material therefore should be of good quality, rich in active ingredients, pest- and disease-resistant and environmental tolerant (Kala *et al.,* 2006). Keeping in view the rising demands of MAPs and increasing threats in their natural habitats, the present study was carried out to develop the *ex-situ* conservation protocols for their sustainable development and use.

Materials and Methods

During the present investigation extensive surveys were conducted in Kashmir Himalaya for the collection of Medicinal and Aromatic Plants (MAPs) from their natural habitats. Different vegetative propagules or sexual seeds and in some cases sapling were collected and were transplanted at Kashmir University Botanical Garden (KUBG). The plants were transplanted in the beds with different dimensions, topology, soil texture, nutrient concentration, moisture content, etc.

Results

The work on development of agro-techniques for the successful survival and mass cultivation of selected MAPS at KUBG was carried out (Plate 1). During the present study different agro-techniques such as soil: sand combinations, nutrient requirements, type of bed, irrigation practice, time of collection and sowing of propagules, etc. were employed. During the course of present investigation rhizome, bulb, corm and saplings of different species were used for the successful survival and mass multiplication of the selected species in Kashmir University Botanical Garden. Some of the representative species are as:

1. *Colchicum luteum* Baker

Mode of propagation: The plant species was successfully propagated through corms.
Soil requirements: Sandy soil was found to be the most efficient for successful cultivation of the species.
Nutrient requirements: Humus rich soil was found to be effective.
Time of sowing of corms: Corms were sown in the month of November- December and March- April. However, pre winter sowing showed better results.
Type of bed: Sloppy bed.
Irrigation practice: The crop is very sensitive to water logging, so field should be free from excess water.
The corms of the species were collected from the natural habitats and were sown in sloppy beds with sandy soil. The beds should be well drained in order to protect the corm from rotting.

2. *Inula royleana* C.B.Clarke

Mode of propagation: Both vegetative (Rhizome) and sexual means (Seeds)

a. Vegetative propagation

Young saplings or rhizomes of the species were collected from the natural habitats and were transplanted in the KUBG.
Soil requirements: Sandy soil was found to be the most suitable for the successful survival of the saplings and also for the sprouting of the rhizomes.
Nutrient requirements: A high quantity of organic manure is required for its cultivation at low altitudes.
Time of sowing of saplings and rhizome: Saplings in the month of June and rhizomes in the month of October.
Type of bed: Plain beds with raised bunds.
Irrigation practice: The saplings or rhizomes should be irrigated immediately after transplantation. Irrigation should be done twice a week for a month; afterwards frequency of irrigation should be reduced to once a month.

b. Through sexual seeds

Seeds collected from the natural habitats were sown in the KUBG. It was found that both seed germination and percentage seedling survival was higher when sown at 0.5-0.7cm depth.
Soil requirements: Loamy soil was found to be most effective for seed germination and seedling survival.
Nutrient requirements: A high quantity of organic manure is required.
Time of collection of seeds: Seeds were collected in the month of September- October.

Type of Bed: Plain beds with raised bunds.
Irrigation practice: Beds should be irrigated immediately after seeds were sown and this practice should be continued for first 3-4 days and after wards twice a week.

3. *Dioscorea deltoidea* Wall. ex Kunth
Mode of propagation: The plant species was successfully propagated through the use of tubers.
Young saplings or tubers of the species were collected from the natural habitats and were transplanted in the KUBG. The established seedling were supported in order to avoid the lodging and subsequent death.
Soil requirements: Sandy loam soil is most suitable for its cultivation.
Nutrient requirements: Farmyard manure is added to the soil at the time of pre-planting.
Time of sowing of saplings and tuber: Saplings in the month of May and rhizomes in the month of August.
Type of Bed: Plain beds with raised bunds.
Irrigation practice: The tuber is very sensitive to water logging, so field should be free from excess water.

4. *Inula racemosa* **Hook. F.**
Mode of propagation: Both vegetative (Rhizome) and sexual means (Seeds)
a. Vegetative propagation
Young saplings or rhizomes of the species were collected from the natural habitats and were transplanted in the KUBG.
Soil requirements: Well-drained, clay-loam soils are ideal for the rhizome sprouting and seedling survival
Nutrient requirements: The farmyard manure is added to the soil before sowing of saplings or rhizome.
Time of sowing of saplings and rhizome: Saplings in the month of April and rhizomes in the month of August.
Type of bed: Plain beds with raised bunds.
Irrigation practice: The saplings or rhizomes should be irrigated immediately after transplantation. Irrigation should be once, twice a week for a month; afterwards frequency of irrigation should be reduced to once a month.
b. Through sexual seeds
Seeds collected from wild were sown in the KUBG. It was observed that both seed germination and percentage seedling survival was higher when sown at 0.7-1.0 cm depth.
Soil requirements: Loamy soil was found to be most effective for seed germination and seedling survival.
Nutrient requirements: A high quantity of farmyard manure was found to be effective.
Time of collection of seeds: Seeds were collected in the month of September.
Type of Bed: Plain beds with raised bunds.
Irrigation practice: Light irrigation at an interval of three to four weeks.

5. ***Picrorhiza kurroa*** **Royle ex Benth.**
Mode of propagation: The plant species was propagated through the use of rhizome (Stolons).
Young saplings or rhizomes of the species were collected from the natural habitats and were transplanted in the KUBG.
Soil requirements: Sandy textured loam soil is the best suitable for its cultivation.
Nutrient requirements: Farmyard manure or high quantity of forest leaf litter is mixed with the soil before transplantation.
Time of sowing of saplings and rhizome: Saplings in the month of July and rhizomes in the month of October. However, it should be noted that minimum rhizome length should be 2-3cm.
Type of Bed: Plain beds with raised bunds.

Irrigation practice: Irrigation should be on alternate days.

Note: Although the plantlets were raised from the rhizomes and also the saplings collected from the natural habitats were successfully grown but the plant species do not survive for more than 2 years in the KUBG. In the future course special agro-techniques will be employed for long term survival of the species.

6. *Arnebia benthamii* (Wallich ex G. Don and I. M. Johnston)

Mode of propagation: The plant species was successfully propagated through the use of rhizome.

Young saplings or rhizomes of the species were collected from the natural habitats and were transplanted in the KUBG.

Soil requirements: Sandy soil is the best suitable for its cultivation.

Nutrient requirements: Farmyard manure was found effective in propagation of the species.

Time of sowing of saplings and rhizome: Saplings in the month of July and rhizomes in the month of October. However, it should be noted that each rhizome cutting have minimum of 3-4 buds.

Type of bed: Raised beds.

Irrigation practice: Light irrigation at an interval of three to four weeks.

7. *Rheum webbianum* Royle.

Mode of propagation: Both vegetative (Rhizome) and sexual means (Seeds)

a. Vegetative propagation

Young saplings or rhizomes of the species collected from the natural habitats were transplanted in the KUBG.

Soil requirements: Well-drained, sandy porous soil was found to be ideal for the rhizome sprouting and seedling survival.

Nutrient requirements: The farmyard manure or litter is added to the soil before sowing of saplings or rhizome.

Time of sowing of saplings and rhizome: Saplings in the month of July and rhizomes in the month of October.

Type of bed: Plain beds with raised bunds.

Irrigation practice: The saplings or rhizomes should be irrigated immediately after transplantation. Irrigation should be twice a month

b. Through sexual seeds

Seeds collected from wild were sown in the KUBG. It was observed that both seed germination and percentage seedling survival was higher when sown at humus rich soil.

Soil requirements: Loamy soil was found to be most effective for seed germination and percentage seedling survival.

Nutrient requirements: A high quantity of farmyard manure was found to be effective.

Time of collection of seeds: Seeds were collected in the month of September- October.

Type of Bed: Plain beds with raised bunds.

Irrigation practice: Light irrigation at an interval of three to four weeks.

8. *Atropa acuminata* Royle.

Mode of propagation: Both vegetative (Rhizome) and sexual means (Seeds)

a. Vegetative propagation

Rhizomes or young saplings of the species were collected from the natural habitats and were transplanted in the KUBG.

Soil requirements: Clay-loamy soils are ideal for the rhizome sprouting and seedling survival.

Nutrient requirements: The litter is added to the soil before sowing of saplings or rhizome.

Time of sowing of saplings and rhizome: Saplings in the month of May and rhizomes in the month of August.
Type of bed: Plain beds with raised bunds.
Irrigation practice: The saplings or rhizomes should be irrigated immediately after transplantation. Irrigation should be twice a week.

b. Through sexual seeds

Seeds collected from wild were sown in the KUBG.
Soil requirements: Loamy porous soil was found to be most effective for seed germination and percentage seedling survival.
Nutrient requirements: Manure and litter was found to be effective.
Time of collection of seeds: Seeds were collected in the month of September.
Type of bed: Plain beds with raised bunds.
Irrigation practice: Irrigation at an interval of three to four days a week.

9. *Ajuga bracteosa* Wallich ex Benth.

Mode of propagation: Both vegetative (stoloniferous branches of rhizome) and sexually (Seeds)

a. Vegetative propagation

Rhizomes or young plants of the species were collected from the natural habitats and were transplanted in the KUBG.
Soil requirements: Coarse sandy soil is ideal for the rhizome sprouting and seedling survival.
Nutrient requirements: Low to moderate manure is added to the soil before sowing of saplings or rhizome.
Time of sowing of saplings and rhizome: Saplings in the month of May and rhizomes in the month of August.
Type of bed: Raised sloppy beds
Irrigation practice: The saplings or rhizomes should be irrigated immediately after transplantation. Afterwards the irrigation should be reduced as the plant is very sensitive to the excessive water.

b. Through sexual seeds

Seeds collected from wild were sown in the KUBG.
Soil requirements: Sand and soil in the ratio of 2:1 was found to be most effective for seed germination and percentage seedling survival.
Nutrient requirements: Litter was found to be effective for seed germination.
Time of collection of seeds: Seeds were collected in the month of July- August.
Type of bed: Raised sloppy beds.
Irrigation practice: Irrigation should be 2 days a week.
Note: It was found that species thrive well when raised through seeds than rhizome in KUBG.

10. *Fritillaria roylei* Hook.

Mode of propagation: Vegetatively (Bulb)
Bulb or young saplings of the species were transplanted in the KUBG.
Soil requirements: Sandy and porous soil was found to be the most suitable for the successful sprouting of the bulbs and also for seedling survival.
Nutrient requirements: A high quantity of organic manure is required for its cultivation at low altitudes.
Time of sowing of saplings and rhizome: Saplings in the month of May and bulb in the month of September-October.
Type of bed: Raised beds.

Irrigation practice: The saplings or bulbs should be irrigated immediately after transplantation. Irrigation should be done thrice a week for a first 15 days; afterwards frequency of irrigation should be reduced to once a month.

11. *Skimmia anquetillia* Taylor and Shaw.
Mode of propagation: Vegetatively (Root)
Roots or young saplings of the species were transplanted in the coniferatum section of KUBG.
Soil requirements: Loamy soil was found to be the most suitable for the successful seedling survival.
Nutrient requirements: A high quantity litter isrequired for its cultivation at low altitudes.
Time of sowing of saplings and rhizome: Saplings in the month of May- June.
Type of bed: Raised sloppy beds around conifers.
Irrigation practice: The saplings should be irrigated immediately after transplantation. Irrigation should be done twice a week for a month; afterwards frequency of irrigation should be reduced to thrice a month.
Note: The plant species form an intimate association with conifers and should be transplanted around the conifers.

12. *Aconitum heterophyllum* Wallich ex Royle.
Mode of propagation: Vegetatively (Rhizome)
Vegetative propagation
Rhizomes of the species were collected from the natural habitats and were transplanted in the KUBG.
Soil requirements: Sandy loam and porous soil was found to be the most suitable for the sprouting of the rhizomes and establishment of saplings.
Nutrient requirements: A high quantity of organic manure and litter is required for its cultivation at low altitudes.
Time of sowing of rhizome: Rhizomes were sown in the month of October.
Type of bed: Plain beds with raised bunds.
Irrigation practice: The saplings or rhizomes should be irrigated immediately after transplantation. Irrigation should be done thrice a week for a month; afterwards frequency of irrigation should be reduced to twice a month.
Note: The established plants were provided with support so as to prevent it from the lodging.

13. *Jurinea macrocephala* (Royle) C.B. Clarke.
Mode of propagation:Vegetative (Rhizome) and sexual seeds (seeds)
a. Vegetative propagation
Rhizomes and young saplings of the species were collected from the natural habitats and were transplanted in the KUBG.
Soil requirements: Sandy soil was found to be the most suitable for the sprouting of the rhizomes and establishment of saplings.
Nutrient requirements: A high quantity of farmyard manure and/ or vermicompost is required for its cultivation at low altitudes.
Time of sowing of rhizome: Rhizomes were sown in the month of October.
Type of bed: Plain beds with raised bunds.
Irrigation practice: The rhizomes and / or saplingsshould be irrigated immediately after transplantation. Irrigation should be done continuously a week for a month; afterwards irrigation should be reduced to twice a month.
b. Through sexual seeds
Seeds collected from the natural habitats were sown in the KUBG. It was found that both seed germination and percentage seedling survival was higher when sown at 0.5cm depth.

Soil requirements: Sand,soil and manure in the ratio of 1:2:1 was found to be most effective for seed germination and percentage seedling survival.
Nutrient requirements: Litter and manure was found to be effective for seed germination.
Time of collection of seeds: Seeds were collected in the month of October
Type of bed: Plain beds with raised beds.
Irrigation practice: Irrigation should be 2 days a week.

14. *Bergenia ciliata* (Haw.) Sternb.
Mode of propagation: Vegetative means (Rhizome)
Vegetative propagation
Rhizomes and young saplings of the species were collected from the natural habitats and were transplanted in the rocky section of KUBG.
Soil requirements: Rocky soil was found to be the most suitable for the sprouting of the rhizomes and survival of the saplings.
Nutrient requirements: Low manure application is required.
Time of sowing of rhizome: Rhizomes were sown in the month of August, September and October.
Type of bed: Sloppy rocky beds.
Irrigation practice: Should be irrigated immediately after transplantation. Irrigation should be done manually for a month; afterwards irrigation should be reduced.
The present study revealed that on transplantation maximum number of MAPs can be raised by vegetative propagules or by the saplings (Fig.1). Sandy soil was the suitable soil for successful establishment of the studied MAPs transplanted at KUBG (Fig.2). Organic manure was the nutrient requirement in most of the cases; our results are in conformity with that of Nautiyal and Nautiyal, 2004. Most of the transplants thrive well in plain beds with raised bunds; however, some plants grow well in slopy, raised and slopy rocky beds (Fig. 3). The suitable time for transplantation is September- October and April- May.

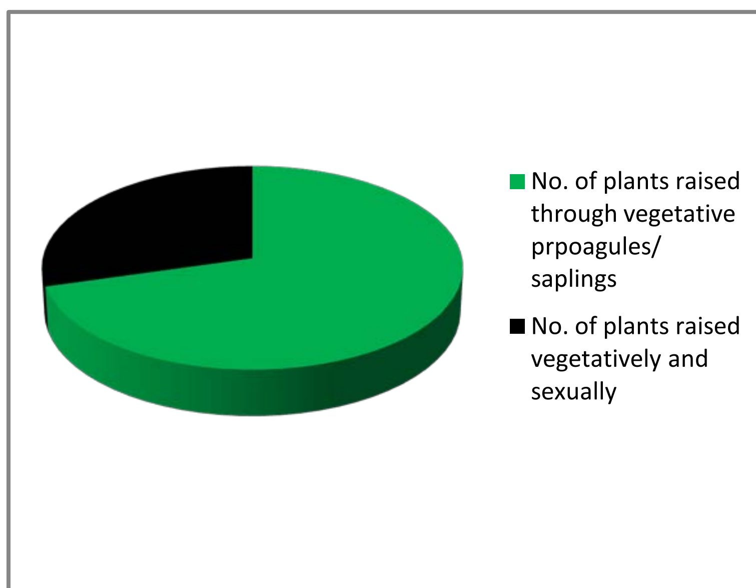

Fig. 1. Proportion of plants raised by means of vegetative and sexual propagules

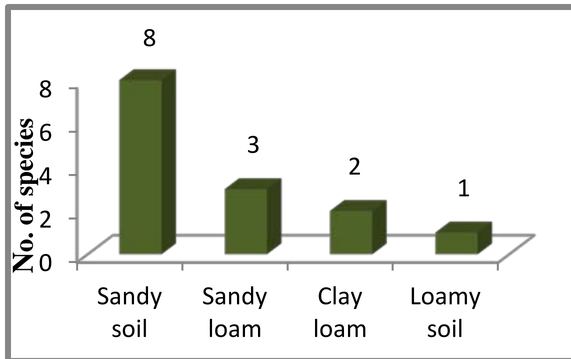

Fig. 2. Requirements of different soils for successful establishment upon transplantation

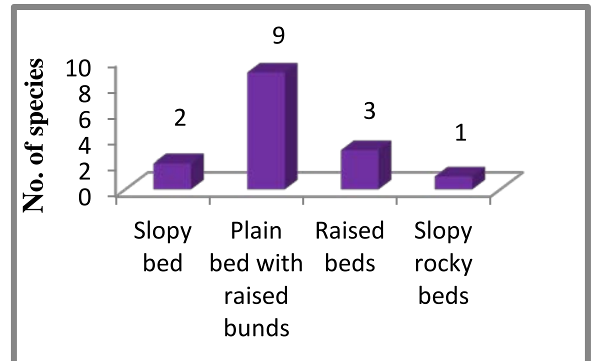

Fig. 3. Different types of beds required by various MAPs for successful establishment upon transplantation

Plate 1. Development of different agro-techniques

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Antimicrobial Activity and Phyto-Chemical Analysis of an Alcoholic Extract of *Rumex dentatus* L.

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Abstract
Rumex dentatus- a medicinally important plant, belonging to family *Polygonaceae,* found throughout temperate western Himalayas, from Kashmir to Kumaon; contain a large number of chemically complex and biologically active compounds. The antimicrobial activity of various concentrations ranging from 150-500µg/ml of alcoholic (butanol) extract of *Rumex dentatus* L. was analyzed using Agar disk diffusion method on different clinical bacterial strains (*Shigella flexneri*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) and fungal strains (*Aspergillus versicolor*, *A. flavus*, *Acremonium* spp., *Candida albicans*, and *C. kruesie*). The tested extract showed maximum antibacterial effect against *K. pneumoniae*. While as in case of the fungal strains the maximum antifungal activity was observed against *Candida albicans*. The phytochemical tests carried out on this crude extract showed the presence of Flavonoids, Terpenoids, Alkaloids, Saponins, Tannins, Anthraquinones and Cardiac glycosides. Total phenolic content of this extract; estimated quantitatively from standard calibration curve of Gallic acid showed a maximum yield of 145µg/mg. Thus, it can be concluded that the alcoholic extract has got a broad spectrum antimicrobial activity and could be a potential alternative for treating various diseases.
Keywords: Antimicrobial activity, phyto-chemical analysis, *Rumex dentatus*, extracts.

Introduction

Medicinal plants have been used for centuries as remedies for human diseases and offer a new source of biologically active chemical compounds as antimicrobial agents. Medicinal plants are the richest bio-resources of drugs of traditional medicinal systems, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, and intermediate chemicals entitled for synthetic drugs (Hammer *et al*., 1999; Das *et al*., 2010). A whole range of plant derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating diseases are now being described as functional ingredients and neutraceuticals. The potential of higher plants as source for new drugs is still largely unexplored and among the estimated 250,000 - 500,000 plant species, only a small percentage has been investigated phyto-chemically, with their fraction submitted to biological or pharmacological screening even smaller (Gerhartz *et al*., 1985; Kroschwitz *et al*., 1992). Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava *et al*., 1996). The WHO estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicine (Tripathi and Tripathi, 2003; Steenkamp *et al*., 2004). Therefore, attention to traditional medicine and the use of medicinal plants is being widespread and plants still represent a largest source of natural antioxidants and antimicrobial components (Sokmen *et al*., 1999; Conforti *et al*., 2008). Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led scientists to investigate the antimicrobial activity of medicinal plants (Bisignano *et al*., 1996; Maoz and Neeman, 1998; Hammer *et al*., 1999; Das *et al*., 2010).

Nowadays, multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. In addition to this, problems are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reaction. This situation forced scientists to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need to look for new and effective therapeutic agents which could be available from various medicinal plants (Khanahmadi *et al*., 2010). Reactive Oxygen Species (ROS) including free radicals such as (O_2- •, OH•) and non free radicals (H_2O_2, $^1O_2$) along with different forms of active oxygen are involved in diverse physico-chemical processes in the body (Qureshi *et al*., 2009) which have main role in the pathogenesis of different diseases, such as neurodegenerative disorders (Knight, 1997), diabetes, cancer (Dreher and Junod, 1996), cardiovascular diseases, atherosclerosis (Halliwell and Gutterridge, 1985), liver cirrhosis (Slater, 1987), cataracts and inflammation (Turkoglu *et al*., 2007; Conforti *et al*., 2008). The antioxidants prevent diseases by various mechanisms; by scavenging free radicals against oxidative stress and inhibiting lipid peroxidation (Miller and Rice-Evans, 1997) thus, their use in the form of herbal antioxidants in food and drug industries in the world is spreading widely (Kirca and Arslan, 2008). The protective effect of plant products in disease prevention are due to the presence of several components such as enzymes, proteins, vitamins (Halliwell, 1996), carotenoids (Edge *et al*., 1997), flavonoids (Zhang and Wang, 2002) and other phenolic compounds (Argolo *et al*., 2004). Plants offer a large range of natural compounds belonging to different molecular families, called Phytochemicals. These phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties, thus attracting several researchers to their elucidation to provide knowledge that will lead to advancement of medicine. There are more than thousand known phytochemicals produced from plants which help them to protect from various stresses and recent researches demonstrate, that they can protect humans against diseases as well.

Material and Methods

1.1 *Plant material*

R. dentatus L., a perennial or less commonly annual plant was collected as a whole plant locally from Srinagar and identified at Kashmir University Herbarium (KASH), Centre of Plant Taxonomy, Department of Botany, University of Kashmir, Srinagar.

1.2 *Extraction of plant material*

The dried parts of the plant (50grams) were powdered and macerated. Crude extraction with butanol was carried out in a soxhlet extractor to get the respective extract which were later dried, weighed and kept for further usage in sterilized caped vials at 4 °C.

1.3 *Test organisms*

The test microorganisms used in this study [bacteria: *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Shigella flexneri* (*S. flexneri*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Staphylococcus aureus* (*S. aureus*); fungi: *Aspergillus versicolor* (*A. versicolor*), *Aspergillus flavus* (*A. flavus*), *Penicillium dimorphosporum* (*P. dimorphosporum*), *Acremonium* spp., *Candida albicans* (*C. albicans*) and *Candida kruesie* (*C. kruesie*) were obtained from Bacteriological and Mycological section, Department of Microbiology, SKIMS, Soura, Srinagar.

1.4 *Antimicrobial activity*

The *in vitro* antibacterial activity test was carried out using the disk diffusion method (Bauer *et al*., 1966).

1.5 *Phytochemical analysis*

Phytochemical analysis for major phytoconstituents of the plant extracts was undertaken using standard qualitative methods as described by various authors (Das *et al*., 2010; Vogel 1958; Rizk and Bashir, 1980; Tiwari *et al*., 2011;

Eleazu *et al*., 2012). The plant extracts were screened for the presence of biologically active compounds like glycosides, phenolics, alkaloids, tannins, flavonoids, saponins and steroids.

Results and Discussion

Several bench top assays such as antimicrobial assays including antibacterial and antifungal and phyto-chemical tests for alkaloids, terpenoids, flavonoids, saponins, tannins, anthraquinones, cardiac glycosides and total phenols of *R. dentatus* were carried out in the study. The Total phenolic content of all the extracts of the plant was determined according to Folin-Ciocalteu procedure (Padmaja *et al*., 2011). The observations were recorded and enlisted under following headings.

3.1 ***Total yield of plant extracts***

The %age yield of crude extracts of *Rumex dentatus* as obtained by soxhlet extraction process and their morphological characteristics is depicted in Table 1. Under present study, butanol extract of whole plant showed a yield of 4.8G.

Table 1. Extraction yield and macroscopic characteristics of the crude extract of *R. dentatus*

Solvent	Plant part used	Colour	Odour	Consistency	Extracted yield (G)
Butanol	Whole plant	Dark green	Characteristic	Thick viscous fluid	4.8

3.2. ***Qualitative analysis of phyto-chemical constituents***

The phytochemical tests were carried out with crude extract of *R. dentatus* to indicate the presence or absence of Flavonoids, Terpenoids, Alkaloids, Saponins, Tannins, Anthraquinones and Cardiac glycosides in it (Table 2). This phytochemical screening indicated that the extract tested positive for alkaloids, terpenoids, flavonoids, tannins and total phenols. The extract was negative for saponins, anthraquinones and cardiac glycosides.

Table 2. Qualitative analysis for various secondary metabolites in extract of *R. dentatus*

Phytochemicals		Butanol
Alkaloids	Dragendorff's Test	+ve
Terpenoids	Salkowski test	+ve
Flavonoids	Alkaline Reagent Test	+ve
Saponins	Froth Test	-ve
Tannins	Ferric Chloride Test	+ve
Anthraquinones		-ve
Cardiac glycosides	Keller-Killiani Test	-ve
Total Phenols	Ferric Chloride Test	+ve

'+ve' = presence; '-ve'= absence

3.3 ***Quantitative estimation of phenolic compounds***

The total phenolic content of crude extract of *R. dentatus* (Table 3) was estimated quantitatively from standard calibration curve (Fig. 1). For butanol extract the total phenolic content was found to be 145µg/mg.

Table 3. Total phenolic content of extract of *R. dentatus*

Plant Extracts	Concentration (µg/mg GAEq)
Butanol	145

***GAEq – Gallic Acid Equivalent**

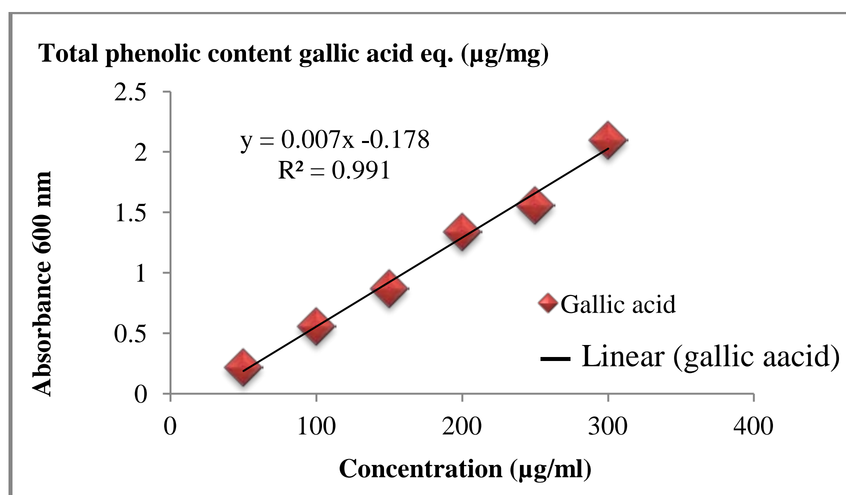

Fig. 1. Standard calibration curve for total phenolic content expressed as gallic acid equivalent

3.4 ***Antimicrobial Activity***

The antimicrobial activities of different concentrations (ranging from 150 µg/mL to 500 µg/mL) of crude extract of *R. dentatus* viz., butanol was determined against different bacterial and fungal strains and recorded as inhibition zone diameter (IZD), measured in "mm" with 10% aqueous DMSO as negative control, gentamycin as positive control for bacteria and nystatin for fungi (Tables 4 and 5). The butanol extract of *R. dentatus* displayed promising antimicrobial activity against a wide range of bacteria. Of all the tested fungal strains, only *C. albicans* was found to be inhibited by butanol extract in comparison to the positive control nystatin.

Table 4. Antibacterial activity of alcoholic extract of *R. dentatus*

Test Organisms	**Butanol**			**Gentamycin**
	150µg/ml	250 µg/ml	500 µg/ml	
S. flexneri	11±1.0	18±1.73	18±0.57	37±1.0
K. pneumoniae	15±1.0	19±0.57	20±0.57	35±1.0
E. coli	15±0.57	17±0.57	18±1.0	30±1.15
P. aeruginosa	-	17±0.57	19±0.57	25±1.52
S. aureus	-	8±1.0	11±0.57	32±1.0

Table 5. Antifungal activity of alcoholic extract of *R. dentatus*

Test Organisms	**Butanol**			**Nystatin**
	150µg/ml	**250µg/ml**	**500µg/ml**	
A. versicolor	-	-	-	10±2.51
A. flavus	-	-	-	14±1.52
Acremonium sp.	-	-	-	15±1.52
C. albicans	14±1.0	15±2.64	17±1.52	12±0.57
C. kruesie	-	-	-	21±1.73

The history of medicine includes many ludicrous therapies. Nevertheless, ancient wisdom has been the basis of modern medicine and will remain as one important source of future medicine and therapeutics. The future of natural products drug discovery will be more holistic, personalized and involve wise use of ancient and modern therapeutic skills in a complementary manner so that maximum benefits can be accrued in the management of plant and human disease management. Plants provide a large range of natural compounds belonging to different molecular families which offer various medicinal properties to humans. These molecules possess interesting biological activities which attracted several researchers to their elucidation to provide knowledge that will lead to advancement in medicine. In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents (Krishnaraju *et al*., 2005). Thus, it is anticipated that phytochemicals with adequate antibacterial and antioxidant efficacies will be used for the treatment of bacterial infections and other diseases in man (Balandrin *et al*., 1985). Ethno-botanical information revealed that the plant selected in this study is traditionally used for various medicinal purposes (Hussain *et al*., 1997; Liu *et al*., 1997; Yildirim *et al*., 2001; Manandhar, 2002; Hussain *et al*., 2006; Islam *et al*., 2006).

Phytochemical screening of different extracts of *R. dentatus* done as described in literature (Ayoola *et al*., 2008) revealed the presence of various biologically active compounds like flavonoids, terpenoids, saponins, alkaloids, tannins, cardiac glycosides, phenols and anthraquinones in the crude extract. The total phenols extracted and quantified according to Folin-Ciocalteu method (Padmaja *et al*., 2011), in terms of Gallic acid equivalents (GAE) showed highest concentration of phenols (145µg/mg of GAE) for butanol extract. It is well-known that phenolic

compounds contribute to quality and nutritional value in terms of modifying colour, taste, aroma, and flavor and also in providing health beneficial effects. They also serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to prevent molecular damage and damage by microorganisms, insects, and herbivores (Vaya *et al*., 1997). Secondary metabolites of plant origin appear to be one of the alternatives for the control of antibiotic resistant human pathogens. The most important bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds. Thus, antibacterial activity may be due to the presence of secondary metabolites (Arokiyaraj *et al*., 2009). The results of the phytochemical analysis in butanol extract as recorded in Table 2. Anthraquinones were absent in both this extract of *R. dentatus*, which is in accordance with the study conducted by Hariprasad and Ramakrishnan, (2011). They reported complete absence of alkaloids and anthraquinones in all the extracts of *Rumex vesicarius* L. The presence of these biologically active phytochemicals in extract of *R. dentatus* is also confirmed by the study conducted by Fatima *et al*., (2009) showing that alkaloids and saponins were only present in methanol extracts of leaves, shoots and roots of *R. dentatus*; anthraquinones and tannins were present only in alcoholic extract.

The butanol extract of *R. dentatus* displayed promising antimicrobial activity against a wide range of bacteria and fungi that were tested. Butanol extract inhibited maximum number of bacterial strains. The results indicate that butanol yielded more potent extract with higher antimicrobial activity thus inhibiting the highest number of bacterial strains, as in consonance with the study of Humeera *et al*., (2013). Rabe and Van Staden, (1997) and Vlachos *et al*., (1996) reported similar findings on the high antibacterial activity. This may also be attributed to the presence of soluble phenolic and polyphenolic compounds (Kowalski and Kedzia, 2007). The results are also in confirmation with a recent study of Bandh *et al*., (2011). George *et al*., (2002) explained that the observed differences to be due to the fact that while synthetic antibiotics are in a pure form, crude plant extracts contains some impure substances that may be inert and do not have any antibacterial activities. This extract of *R. dentatus*; namely butanol inhibited clinical isolate of *C. albicans* only. The inhibition zones produced by this extract for *C. albicans* was significantly higher as compared to the standard antibiotic used. Several studies have attributed the antifungal activity of plant extract to the presence of saponins (Aboaba and Efuwape, 2001; Mohanta *et al*., 2007).

Owing to the widespread ability of flavonoids to inhibit spore germination of plant pathogens, they have been proposed for use against fungal pathogens of man (Cushnie and Lamb, 2005). Several studies have been conducted to understand the mechanism of action of plant extracts, but it is still unclear (Hadizadeh *et al*., 2009). However, some researchers attributed the antifungal activity to the phenolic compounds. The amphipathicity of these compounds can explain their interactions with bio-membranes causing the inhibitory effect (Veldhuizen *et al*., 2006). Omidbeygi *et al*., (2007) suggested that extract components cross the cell membrane, interacting with enzymes and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and, ultimately their death. Sharma and Tripathi, (2006) concluded that plant extracts may act on the hyphae of the mycelium, provoking exit of components from the cytoplasm, the loss of rigidity and integrity of the hyphal cell wall, resulting in its collapse and death of the mycelium. It is evident from the results of the current study that susceptibility of pathogens to plant extracts depends upon solvent used for extraction and extract concentration (Abou-Jawdah *et al*., 2002), as well as the organism tested (Kumaran *et al*., 2003; Hadizadeh *et al*., 2009). It is quite possible that the extract that was ineffective in some cases do not possess antibiotic properties, or they may have contained active constituents, just not in sufficient concentrations so as to be effective. It is also possible that some of the active chemical constituents were not soluble in this plant extract. The drying process may have caused conformational changes in some of the chemical constituents found in this plant. So, it is not surprising that there are differences in the antimicrobial effects of the extracts of the medicinal plants, due to the phytochemical properties (Stainer *et al*., 1986).

The antibacterial activity against a wide range of pathogens in this study makes *R. dentatus* a promising plant for further pharmacological investigations. The aim of this study was achieved by proving the *in-vitro* antimicrobial activity of alcoholic extract concentrations of this plant used traditionally as a folklore medicine. This study may contribute to the increased scientific investigation done on indigenous medicinal plants, including *R. dentatus* used traditionally in different parts of Kashmir valley.

Conclusions

It can be concluded that the alcoholic extract of plant has got a broad spectrum antimicrobial activity which is shown by the presence of different phytochemicals in this extract and thus, the plant could be used as a potential alternative for treating various diseases.

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