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Cover Page, Introduction, Contents, Call for Papers, All in one file

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Modification of nano alginate-chitosan matrix for oral delivery of insulin

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Abstract: The objective of this study is modification of nano chitosan blended alginate matrix (CBAM) by grafting with poly methacrylic acid (PMAA) to utilize for oral delivery of insulin. firstly, chitosan blended alginate matrix converted to nano by freeze drying method and then, free radical graft copolymerizations were carried out at 70 °C, bis-acrylamide as a cross-linking agent and persulfate as an initiator. The cross-linked three-dimensional polymers were characterized by scanning electron microscopy and FT-IR. In the matrices with increase in the content of chitosan had shown increased bioadhesivity. Equilibrium swelling studies were carried out in enzyme-free simulated gastric and intestinal fluids (SGF and SIF, respectively). Insulin was entrapped in these gels and the in vitro release profiles were established separately in both (SGF, pH 1) and (SIF, pH 7.4). Drug release studies showed that the increasing content of MAA in the copolymer enhances hydrolysis in SIF. In these cases, the biological activity of insulin was retained. These results were used to design and improve insulin release behavior from these carriers. [Nature and Science. 2009;7(8):1-7]. (ISSN 1545-0740).

Keywords: Blending, Chitosan, Alginate, Grafting, Oral delivery, Insulin

Introduction

Oral delivery of drugs, especially therapeutic proteins, is the preferred route of administration because it offers advantages over injection, which is the presently accepted route of therapeutic protein administration. The oral delivery route is more natural and less invasive. However, there exist several problems for the development of oral protein delivery systems. One major problem is the degradation of proteins by proteolytic enzymes and the acidic environment of the stomach. Another problem is the low penetration of proteins across the lining of the intestine into the blood stream [1]. Among the various methods that have been developed to assist to these problems [2-5], use of environmentally sensitive hydrogels, especially methacrylic acid (MAA)-based complexation and pH-sensitive hydrogels, is the most promising method.

Natural polymers have potential pharmaceutical applications because of their low toxicity, biocompatibility, and excellent biodegradability. Alginates are natural polymers isolated from several species of brown algae. Alginates are composed of two monomers, α-L guluronic acid and β-D-mannuronic acid. The pH sensitive nature and its ability to control gel permeability means; that these materials have significant potential for drug delivery applications. However the bioadhesive potential of alginate is not sufficient to make suitable for prolonged contact with the intestinal mucosal surface in case of oral drug delivery of poorly absorbable agents. Chitosan, another natural polymer is partially or fully deacetylated derivatives of chitin. The polymer is linear consisting of D-glucosamine residues with a variable number of randomly located N-acetyl glucosamine groups. Chitosan is well known for its bioadhesive nature. Bioadhesion is defined as the ability of a material to adhere to a biological tissue for an extended period of time. In the cationic form, the Dglucosamine residue of chitosan could interact with the sialic acid residues of mucin by electrostatic interaction. The bioadhesive property of chitosan may allow a prolonged interaction of the delivered drug with the membrane epithelia facilitating more efficient absorption. Increased absorption of drugs at mucosal sites by chitosan could be due to prolonged interaction with the membrane epithelia or opening of the tight junctions between cells to facilitate transport [6, 7]. A number of reports suggest the utilization of alginate and chitosan for drug delivery [8, 9]; however, it may need to be further modified for some special applications. Among diverse

approaches that are possible for modifying polysaccharides, grafting of synthetic polymer is a convenient method for adding new properties to a polysaccharide with minimum loss of its initial properties [10]. Graft copolymerization of vinyl monomers onto polysaccharides using free radical initiation, has attracted the interest of many scientists.

In this study our aim was to utilize the pH sensitivity of alginate, (Alginate is stable in acidic pH of stomach, but it swells and starts dissolving slowly in the intestinal alkaline pH) which can be used for protecting insulin in stomach and the bioadhesivity of chitosan to make prolonged contact with the intestinal mucosae, so as to increase the absorption of insulin. The graft copolymerization poly methacrylic acid onto chitosan blended alginate matrix (CBAM) was carried out under free radical polymerization, bis-acrylamide as a cross-linking agent and persulfate as an initiator. Insulin was entrapped in these gels and the in vitro release profiles and stability of insulin in contact with these hydrogels during the release were studied.

Experimental

Materials

Chitosan blended alginate matrix (CBAM) was prepared by the methods described in the literatures [11-13] and then dried by freeze drying method. The insulin used was recombinant human insulin (AK2U Nobel France; lot # 821156, Batch L-00023822). Sodium alginate of medium viscosity (3500cps for a 2% solution at 25 °C) was obtained from Sigma chemicals Co. Chitosan was obtained from Aldrich (85% deacetylation). Methacrylic acid (MAA) and bis-acrylamide were purchased from Merck Co. All the other chemicals used were of analytical reagent grade. Enzyme-free SGF (pH 1) or SIF (pH 7.4) were prepared according to the method described in the literature [14].

The IR spectra were recorded on a Shimadzu FT IR-408 spectrophotometer. The amount of released drug was analyzed using a high-performance liquid chromatography- ultraviolet (HPLC-UV) Waters bus SAT/IN Module at 210 nm. Isocratic elution was performed using 30% acetonitrile and 70% buffer containing 0.1M KH_2PO_4 and 1% triethylamine adjusted to pH 3.0 with phosphoric acid. The column used was Nulcleosil-C185-m PHASE SEPARATIONS 4.6-250 mm Analytical Cartridge (part no. psl841020) equipped with a precolumn.

Copolymerization: General Procedure

CBAM with different molar ratios of MAA were polymerized at 60-70 °C in a thermostatic water bath, bis-acrylamide as a cross-linking agent (CA), using persulfate as an initiator ([I] = 0.01 M) and water as the solvent (50 mL). After the desired time (48 h) the precipitated network polymers was collected, washed with deionized water for 1 week and the water was changed every 12 hours in order to remove any unreacted monomers. After washing, the samples were dried in air and stored in desiccators until use. The values are given in Table1. IR (KBr): 3380-2500 (broadened, -COOH group), 1720, 1520, 1240, 1225 cm^{-1}.

Table 1. Composition of copolymers

Polymers	Molar composition in the feed			
	CBAM 1:10	CBAM 1:12	MAA	CA (%)
P-1	1	---	3	5
P-2	1	---	3	10
P-3	1	---	5	5
P-4	1	---	5	10
P-5	---	1	3	5
P-6	---	1	3	10
P-7	---	1	5	5
P-8	---	1	5	10

Measurement of swelling ratio

To measure the swelling, preweighed dry drug-free hydrogels were immersed in various buffer solutions (pH 7.4 and pH 1) at 37 °C. After excess water on the surface was removed with the filter paper, the weight of the swollen samples was measured at various time intervals. The procedure was repeated until there was no further weight increase. The degree of swelling was calculated according the relation:

$$SW\ (\%) = [(Ws-Wd)/\ Wd] \times 100$$

Where, Ws and Wd represent the weight of swollen and dry samples, respectively. The study of swelling shows that swelling of hydrogels increases with time, first rapidly and then slowly, reaching maximum constant swelling (mass equilibrium swelling, MES). In all cases, the swelling weight reached its equilibrium after 5 hours. Time-dependent swelling behaviour of cross-linked polymers in pH 1 and pH 7.4 at 37°C are given in Table 2.

Table 2. Percent of swelling and drug loading numbers

polymers	maximum constant swelling (%) pH 1	maximum constant swelling (%) pH 7.4	Percent of insulin-loading
P-1	400	1300	80
P-2	340	1150	72
P-3	310	1550	95
P-4	280	1450	89
P-5	390	1500	89
P-6	340	1450	82
P-7	300	1800	99
P-8	260	1700	93

Insulin Loading in Hydrogels

Insulin can only be dissolved in acidic aqueous solutions of around pH 3.0; insulin was first dissolved at pH 3.0 and then the pH was increased to pH 7.2 using 0.1M KOH. Subsequently, 10 mg of each hydrogel was placed in 3 mL of insulin solution (1.0 mg/mL) to absorb the total amount of the insulin solution. After approximately 60 min, the completely swollen hydrogels loaded with insulin were placed in desiccators and dried under vacuum at room temperature.

Quantitative analysis of insulin

Three milligrams of polymer-drug adduct was dispersed in 3 mL of mobile phase solution. The reaction mixture was maintained at 37 °C. After 4 h the hydrolysis solution filtered and analyzed by HPLC for the determination of total insulin in hydrogels. The results obtained are presented in Table 2.

Insulin stability during release studies from hydrogels

In order to study the stability of insulin in contact with hydrogels, two different conditions were chosen: 37 °C and darkness, 37 °C and light. Insulin was loaded in hydrogels as described and then the peptide stability was investigated during release under the above mentioned conditions at two different pH values of 1 and 7.4. Samples were analyzed under each condition after 24 and 48 h. In this condition insulin remained fairly stable at both pH values during the course of experiments, indicating that adsorption of the peptide to the hydrogels and their release afterwards did not substantially influence the stability of this peptide drug. To investigate the protective ability of the hydrogel for insulin in the harsh environment of the stomach, insulin and insulin-incorporated were treated with a simulated gastric solution that contained endopesidase pepsin. After the treatment in gastric solution, the biological activity of insulin was determined with HPLC. These results indicated that all insulin was degraded immediately after insulin was in contact with gastric fluid and the main cause of degradation was the proteolytic enzyme, pepsin. After being treated with gastric fluid, all of hydrogels demonstrated a protective effect on insulin and the biological activity remained after the treatment with gastric fluid of hydrogels. Studies of hydrogel showed that when the MAA content increased, degradation of insulin decreased.

Insulin release from hydrogels

Insulin release from the delivery systems was tested in the pyrex glasses. The powdered hydrogel (10mg) was poured in 5ml of aqueous buffer solution (pH=7.4 & pH=1) at 37 °C. The rotation speed was adjusted with stirrer. Samples were measured using HPLC-UV at 210 nm. The flow-rate and injection volume were 1 ml/min and 60 μL, respectively. Insulin was detected at a retention time of 5.5 min and the detection limit

was 0.3 µg/mL. Triplicate samples were used. The amounts of insulin released from hydrogels was collected by taking 60-µL samples at predetermined time intervals and analyzed by HPLC.

In situ Bioadhesivity Studies

Bioadhesivity testing was done by a novel in situ method as described by Ranga Rao and Buri [15]. A freshly cut 5-6 cm long piece of small intestine of rat was obtained and cleaned by washing with isotonic saline. The piece was cut open and the mucosal surface was exposed. Known weights of hydrogels were added evenly on the mucosal surface. The intestinal piece was maintained at 80% relative humidity for 30 mts in a desiccator. The piece was taken out and phosphate buffer pH 6 was allowed to flow over the intestinal piece for about 2 mts at a rate of 20 ml/min. The perfusate was collected and dried to get the particles not adhered. The percent of bioadhesion was estimated by the ratio of amount applied to adhere hydrogels. The values are given in Table 3.

Table 3. Particles adhered onto rat intestine (%)

polymers	Percentage adherence
P-1	**76**
P-2	**75**
P-3	**73**
P-4	**71**
P-5	**73**
P-6	**71**
P-7	**69**
P-8	**69**

Results and discussions:

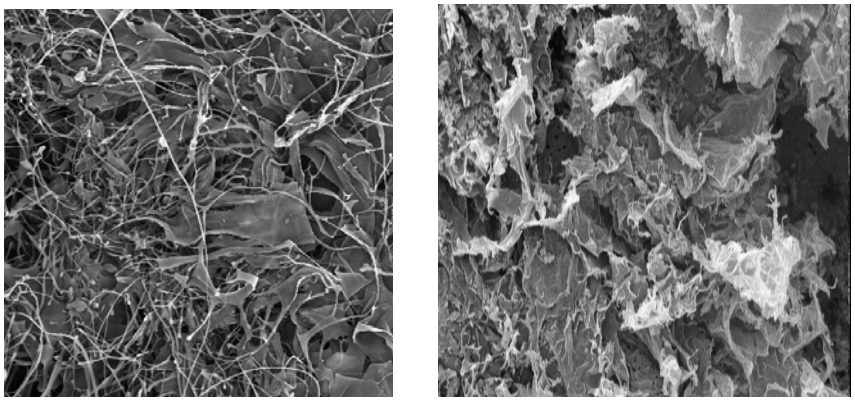
Fourier transform infrared (FT-IR) spectroscopy of matrix was carried out in order to detect any peak shift that could be attributed to interactions between the two polymers, such as hydrogen bonding or complexation. In general, the FTIR spectrum of blank chitosan-alginate particles showed a broad band around 3500-3100 cm-1, indicating enhanced hydrogen bonding compared to that of chitosan or sodium alginate alone [16]. Moreover, the N-H bending vibration of nonacrylated nonacrylated 2-aminoglucose primary amines of chitosan (1570 cm^{-1}) and asymmetric and symmetric –C-O stretching at 1407 cm^{-1} of sodium alginate disappeared, indicating that the ($-NH_3^+$) of chitosan reacted with the ($–COO^-$) of alginate [17]. Absence of these bonds in the FTIR spectra of chitosan/sodium alginate matrix indicated the formation of a strong electrostatic bond between them.

The structure of polymers can be analyzed via using scanning electron microscope. As a sample, Figure 1 showed SEM image of chitosan blended alginate matrix of P-1.

The SEM studies show that due to the strong interaction from the intermolecular hydrogen bonds and electrostatic interactions between carboxyl groups on alginic acid and amino groups on chitosan, not only creates good miscibility between chitosan and alginate but also the prepared polymer adapts fibrous structure. In the grafting, by development of intermolecular hydrogen bonding between its amino groups and the carboxyl groups of PMAA, interaction of alginate/chitosan reduced and ruined fiber structures.

To achieve successful colonic delivery, a drug needs to be protected from absorption of the environment of the upper gastrointestinal tract (GIT) and then be abruptly released into the proximal colon, which is considered the optimum site for colon-targeted delivery of drugs. These requirements have prompted the development of polymeric systems that swell minimally under acidic conditions but extensively in basic intestinal medium. The composition of the polymer defines its nature as a neutral or ionic network and furthermore, its hydrophilic/hydrophobic characteristics. Ionic hydrogels, which could be cationic, containing basic functional groups or anionic, containing acidic functional groups, have been reported to be very sensitive to changes in the environmental pH. The swelling properties of the ionic hydrogels are unique due to the ionization of their pendent functional groups.

Hydrogels containing basic functional groups is found increased swelling activity in acidic conditions and reduced in basic conditions but on the other hand pH sensitive anionic hydrogels shows low swelling activity in acidic medium but very high activity in basic medium.



A B

Figure 1. SEM images of chitosan /alginate matrix before (A) and after (B) the grafting

The equilibrium swelling ratio of the hydrogels was a function of the network structure, crosslinking ratio, hydrophilicity and degree of ionization of the functional groups. With increased cross-linking and an increase in the reticulated degree of the polymer, diffusion of the water in the network's polymer is reduced and the swelling is slower. The existence of hydrogen-bonding interactions between –COOH groups in the polymer matrix results in a complex structure within the network, and so the movement of polymeric segments is restricted. This also accounts for minimum swelling of the gel in a medium of pH 1. However, when the sample is placed in a medium of pH 7.4, the almost complete ionization of –COOH groups present within the polymer network not only increases the ion osmotic swelling pressure to a great extent but also enhances the relaxation of macromolecular chains because of repulsion among similarly charged $–COO^-$ groups. These two factors ultimately result in a greater increase in the water uptake.

The loading numbers in Table 2 shows existence of polar functionally groups as carboxylic acid need not only for loading insulin on the polymer but also for pH-sensitive properties of polymer. Hydrogen bonding is a key contributor to the specificity of intermolecular interactions in polymer bonded drugs systems. The hydrogen-bonding and electrostatic interactions increased with MAA content in the copolymer networks. The hydrogen-bonding and electrostatic interactions increased with MAA content in the copolymer networks.

The preparation of CBAM was difficult to achieve with higher ratio of chitosan to alginate. Since due to ionic interaction immediately after mixing of chitosan with alginate rigid gel formation will occur, this makes the particle formation difficult. In our study we tried 1:10, 1:12 ratio of chitosan to alginate for preparation of matrices. The chitosan blending was done to provide additional bioadhesivity to alginate matrices. Among diverse approaches that are possible for modifying natural polymers, grafting of synthetic polymer is a convenient method for adding new properties to a natural polymer with minimum loss of its initial properties. All the matrices with the presence of chitosan had shown increased bioadhesivity (Table 3). The binding of chitosan residues with sialic acid residues make prolonged contact of the drug with the epithelium, also it was assumed that opening of the intercellular junctions by chitosan could lead to the enhancement of peptide absorption across the mucosa.

Insulin Release

To develop potential applications of polymer-bonded drugs (PBDs) containing insulin as the pharmaceutically active compound, we studied the hydrolysis behavior of hydrogel polymers under physiological conditions. Although the polymers were not soluble in water, they were dispersed in a buffer solution, and the hydrolysis was evaluated as a heterogeneous system. The degree of hydrolysis of the hydrogels containing insulin as a function of time is shown in figures 2 and 3.

The order of hydrolysis in this series was significantly affected by polymer composition. The increase of CBAM content resulted in less collapsed networks at low pH. This led to a relatively large pore size of the networks. Thus, insulin could diffuse readily from the gel at low pH. In the other hand, with increases of percentage of chitosan in matrix, the pH sensitivity of alginate is altered or reduced. But, as the content of MAA in the feed monomers increased, hydrolysis rate decreased at pH 1 but increased at pH 7.4. This was because a higher MAA content in the polymer networks led to higher carboxylate anion concentration at high pH. In other words, the existence of hydrogen-bonding interactions between –COOH groups in the polymer matrix results in a complex structure within the network, and so the movement of polymeric segments is restricted.

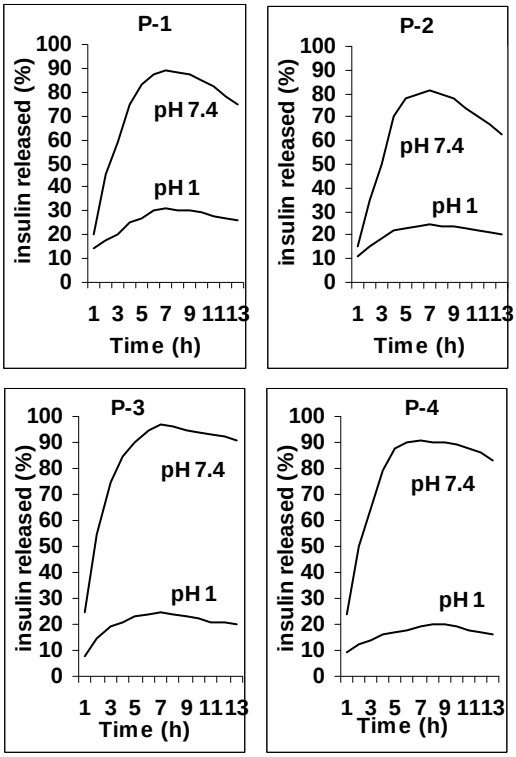


Figure 2. Release of insulin from polymeric carriers containing (CBAM 1:10) as a function of time at 37 °C.

This also accounts for minimum hydrolyzing of the gel in a medium of pH 1. However, when the sample is placed in a medium of pH 7.4, the almost complete ionization of –COOH groups present within the polymer network not only increases the ion osmotic swelling pressure to a great extent but also enhances the relaxation of macromolecular chains because of repulsion among similarly charged $–COO^-$ groups. These two factors ultimately result in a greater increase in the water uptake. In pH 7.4 with completed ionization and an increase in the hydrophilicity of the polymer, diffusion of the hydrolyzing agents on polymer is increased and the hydrolysis rate increased [18]. In the other hand, At an incorporation pH 7.4, the carboxylic acid groups in hydrogels as well as the insulin, which has pI of 5, were negatively charged resulting in repelling each other. Because the increase of MAA content in the hydrogels provides more hydrogen bonds at low pH and more electrostatic repulsion at high pH. This effect response to decrease or increase insulin release in pHs 1 and 7.4, respectively.

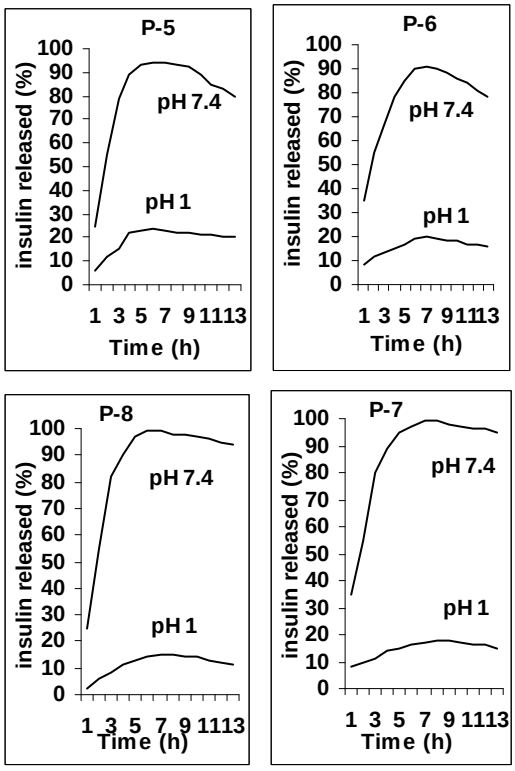


Figure 3. Release of insulin from polymeric carriers containing (CBAM 1:12) as a function of time at 37 °C.

Conclusions

For colon-selective drug delivery, a polymer is needed that is able to withstand lower pH values, but disintegrates at the slightly alkaline pH values of the ileocecal junction and the large intestine. The use of carriers made of natural

polysaccharides has arisen as a promising alternative for drug delivery. The chemical modification of natural polymers by grafting has received considerable attention in recent years because of the wide variety of monomers available. Novel pH-responsive hydrogels containing chitosan and alginate were synthesized by the graft copolymerization poly methacrylic acid onto chitosan blended alginate matrix (CBAM). In our study all the hydrogels containing chitosan has shown greater bioadhesiveness, in the in-situ-study. By regulating the crosslinking percentage of the grafted polymers, pH-sensitive hydrogels with improved optimal hydrolysis rates were obtained. The hydrolysis of the drug-polymer conjugates were performed at pH 1 and 7.4 at 37 °C. The different systems available with varied release kinetics and bioadhesivity may help in tailoring the system suitable for the oral delivery of insulin for the management of diabetes.

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Composition Of Tree Species In Ehor Forest Reserve, Edo State, Nigeria

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Abstract: The tree composition of Ehor Forest Reserve in Uhunmwode Local government area of Edo State was evaluated by laying out fifteen sample plots of 30m × 30m in three different compartments of 1.6 kilometer square each. Ninety-nine species of trees distributed into 36 families and 87 genera were identified. A total of 2,062 tree stands were encountered in these three compartments (81, 95 and 112) studied with *Celtis zenkeri* as the most abundant having 129 stands. This was followed by *Baphia nitida, Musanga cecropioides, Pentaclethra macrophylla* and *Uvariopsis dioica* with 75, 68, 67 and 64 stands respectively. Conversely, eighteen other species including *Afzelia africana, Albizia zygia, Bombax brevicuspe, Milicia excelsa, Cordia millenii* and *Irvingia gabonensis* had only one stand in the three compartments combined with an area of 4.8 Km.sq. This signified that these plants are under threat of extinction from the reserve. Eighty-three percent of the tree species encountered were wildlings having a diameter at breast height of ≤ 10 cm. Less than one percent (0.63%) of the trees were of merchantable size. This situation is quite alarming and calls for a more resourceful and sustainable management techniques. Among others, it is suggested that the reserve be protected from further timber and fuel wood exploitation in order to allow it regenerate itself fully. [Nature and Science. 2009;7(8):8-18]. (ISSN 1545-0740).

Keywords: Compartments, diameter at breast height, density, stands.

1. Introduction

A reserve is a forest kept aside /protected or saved for future use or a special purpose. Reserves are established to conserve habitats in their natural state, conserve areas for scientific research and education and to protect vulnerable or endangered species or landscapes. A protected or reserved area is "an area of land especially dedicated to the protection and maintenance of biological diversity; and of natural and associated cultural resources, and managed through legal or other effective means" (IUCN, 1994). Since there are few or no natural forest in the world, the Union described a natural forest as "a forest where human impact has not surpassed the impact of other indigenous species and has not affected the ecosystem structure".

There are 445 gazetted forest reserves located in different parts of Nigeria. Only about 137 of these reserves are located in the forest region harboring the bulk of the natural forest wealth of the country (UNEP, 1992). Of the 560 species of trees present in these reserves in Nigeria, only 60 species are currently considered commercially important with attention restricted to about 35 of them (Nwoboshi, 1982). This has resulted in the overexploitation of the few commercially available species. The current global attention on the conservation and sustainability of biodiversity particularly in the tropical forests is a consequence of the threat posed by overexploitation. This might lead to depletion of such trees if allowed to go on unchecked resulting also in the elimination of other flora or fauna which depend on such trees for survival. The purpose of this work therefore is to evaluate the effects of such uncontrolled exploitation on the tree population of Ehor Forest Reserve.

2.0 Materials and Methods

2.1 Study Location

Ehor Forest Reserve occupies an area of 76.8 square kilometers in Uhunmwode Local Government area of Edo State, Nigeria. It is located between latitudes 6^0 34'N and 6^0 38'N and longitudes 5^0 54'E and 5^0 58'E fifty-six kilometers north of the state capital, Benin-City. It is divided into forty-eight compartments of 1.6 square kilometers each. The Orhionmwon River runs through the reserve. It is surrounded by nine villages viz: Ohe, Eguaholor, Egbisi, Ugieghudu, Uhi, Iriwe, Erhue, Evbowe and Ekudo. There are no settlements within the reserve.

It was constituted into a forest reserve by the native authority notice number 73 of 1950 contained in the Forestry Ordinance Chapter 73 of the Federal Republic of Nigeria. It was originally subdivided into the west and east areas of 16/1 and 16/2 respectively but the later has been de-reserved. This study was carried out in area 16/1. Farming is commonly practiced within the reserve which is situated in the lowland rainforest zone. It had a sizeable number of timber species which made it attractive to logging companies. Apart from logging, cassava production which is the second main cause of forest destruction and soil degradation (WWFN, 1992) is the most commonly encountered crop in the reserve.

The vegetative profile of the reserve is mainly two storey with a few scattered emergents as the third storey. The canopies are mostly opened except in a few places where they are closed. This state of the forest explains the level of exploitation that has taken place over the years.

2.2 Survey method

Three compartments were sampled for this study. They were compartment 81 on the western end of the reserve, 95 which is centrally located and 112 at the eastern end of the reserve (Figure 1). This is to have an adequate representation of the whole forest reserve. Five sample plots of 30m × 30m were laid out in a randomized complete block design in each compartment using improvised wooden pegs. The trees were identified and the density of each species per compartments noted.

Plant identification was done by using Keay *et al*.(1964) and Hopkins (1974). The timber species were confirmed using Anonymous (1973) and Gill and Okoegwale (1991).

The girths of the trees at 1.3 meters from the ground level(diameter at breast height) were measured by means of a measuring tape and recorded. All wildlings above 4 cm circumference were measured while those below were just noted. The number of species and the density of each species per sample plot were also noted.

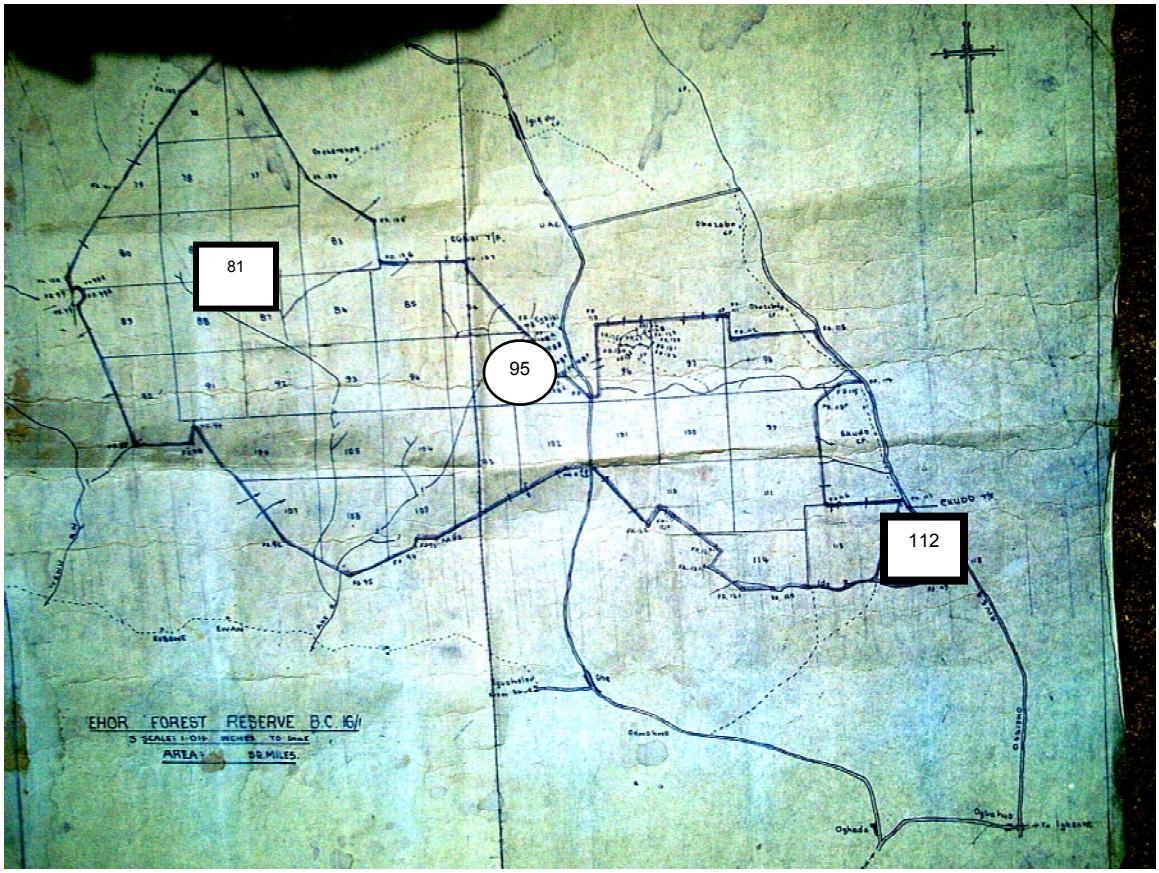

Figure 1: Map of Ehor Forest Reserve showing Compartments of Study

2.3 Analysis of field data

The following parameters were studied

a) Relative diversity which is the number of species in each family represented.
b) Diameter at breast Height using the formula

$$\frac{\text{Girth}}{\pi}$$

where π is a constant of 3.142

3.0 Results

A check list of the trees species, their families, density and habits in Ehor Forest Reserve are presented in Table 1. A total of ninety-nine (99) species of trees distributed into thirty-six (36) families and eighty-seven (87) genera were encountered. Compartment 81 was the richest with sixty-three (63) species while compartment 95 and 112 had fifty-three (53) and fifty-seven (57) species respectively. Based on their habit, these

species were classified into 91% trees and 9% of shrubs.

The family Fabaceae has the highest diversity of eighteen (18) species while fifteen families were represented by only one species each. Meliaceae, Annonaceae, Sterculiaceae and Apocynaceae were represented by seven, six, six and five species respectively in the compartments studied.

The result of the various diameter class sizes are presented in Table 2 and Figure 2. Of the 2,062 stands encountered in the three compartments of study, 1,711 were in the diameter class of ≤ 10 cm making up about 82.98% of the total trees encountered. This was followed by the diameter class of 10-20 cm with 162 stands which is 7.6% of tree population in the reserve. The diameter class of 91-100 cm had no stand while that of 81-90 cm had the least stand of two (2). The most abundant species was *Celtis zenkeri* with a total of 129 stands in the three compartments of study while eighteen species were represented by only one stand. These figures translate to less than one when calculated per hectare.

Results of further breakdown of the proportion of trees making up the 82.98% of tree ≤ 10cm is presented in Figure 3. The highest percentage of 34.42% in this case also belongs to the least diameter class of ≤ 2 cm.

Table 1: Tree species, Habits and families represented at Ehor Forest Reserve

FAMILIES	SPECIES	DENSITY/ HECTARE	HABIT
Anacardiaceae	*Antrocaryon micraster* A. Chev.	0.008	Tree
	Lannea welwitschi (Hiern) Engl.	0.019	Tree
Annonaceae	*Anonidium mannii* (Oliv.) Engl.and Diels	0.027	Tree
	Cleistopholis patens (Benth.) Engl. And Diel	0.050	Tree
	Polyalthia suaveolens Engl. And Diels	0.021	Tree
	Polyceratocarpus parviflorus (Bak. F) Chesq.	0.008	Tree
	Uvariopsis dioica (Diels) Robyn and Chesq.	0.133	Tree
	Xylopia aethiopica (Dunal) A. Rich	0.002	Tree
Apocynaceae	*Alstonia boonei* De Wild.	0.040	Tree
	Funtumia elastica (Preuss) Stapf.	0.056	Tree
	Hunteria umbellata (K. Schum) Hailier	0.067	Shrub
	Rauwolfia vomitoria Afzel.	0.002	Shrub
	Tabernaemontana pachysiphen Stapf.	0.019	Tree
Arecaceae	*Elaeis guineensis* Jacq.	0.006	Tree
Asteraceae	*Albizia ferruginea* (Guill. and Perr.) Benth.	0.045	Tree
	Albizia lebbeck (L.) Benth.	0.004	Tree
	Albizia zygia (DC.) J.F. Machr.	0.002	Tree
Bignoniaceae	*Newbouldia laevis* (P.Beauv.) Seeman ex Bureau	0.046	Tree
	Spathodea companulata P.Beauv	0.046	Tree
Bombacaceae	*Bombax brevicuspe* Sprague	0.002	Tree
	Ceiba pentandra (L.) Garten	0.004	Tree
Boraginaceae	*Cordia millenii* Bak.	0.002	Tree
Burseraceae	*Canarium schweinfurthii* L.	0.023	Tree

	Dacryodes edulis. (G. Don.) H.J. Lam	0.002	Tree
Clusiaceae	*Allanblackia floribunda* Oliv.	0.006	Tree
	Garcinia kola Heckel	0.002	Tree
	Pentadesma butyracea Sabine	0.010	Tree
Combretaceae	*Terminalia ivorensis.* A. Chev.	0.002	Tree
Ebenaceae	*Diospyros alboflavescens* (Gurke) F. White	0.045	Tree
	Diospyros dendo Welw. Ex Hien.	0.006	Tree
	Diospyros mesipiliformis Hochst ex D. AC	0.017	Tree
Euphorbiaceae	*Hevea brasiliensis* (Knuth.) Muell. Arg.	0.002	Tree
	Maesobotrya bateri (Baill.) Hutch.	0.008	Tree
	Ricinodendron heudelotii (Baill.) Pierre	0.104	Tree
	Tetrorchidium didymostemon (Baill.) Pax and K. Hoffm	0.027	Tree
Fabaceae	*Afzelia africana* Sm.	0.002	Tree
	Amphimas pterocarpoides Harms	0.029	Tree
	Angylocalyx zenkeri Harms	0.010	Tree
	Anthonotha macrophylla P. Beauv.	0.069	Shrub
	Baphia nitida Lodd.	0.156	Tree
	Berlinia grandiflora (Vahl.) Hutch. And Dalz.	0.088	Tree
	Brachystegia nigerica Hoyle and A.P.D Jones	0.169	Tree
	Cylicodiscus gabunensis Harms	0.006	Tree
	Daniellia ogea (Harms) Rolfe ex Holl.	0.094	Tree
	Distemonanthus benthamianus Baill.	0.006	Tree
	Gossweilorodendron balsaminiferum (Verm.) Harms	0.004	Tree
	Guibourtia sp. Benn.	0.013	Tree
	Hymenostegia afzelii (Oliv.) Harms	0.048	Tree
	Lonchocarpus griffonianus (Baill.) Dunn.	0.013	Shrub
	Pachyelasma tessmannii (Harms) Harms	0.006	Tree
	Pentaclethra macrophylla Benth.	0.140	Tree
	Piptadeniastrum africanum (Hook F.) Brenan	0.027	Tree
	Pterocarpus osun Craib	0.006	Tree
Irvingiaceae	*Irvingia gabonensis* (Aubry-Lecomte ex O'Rorke)	0.002	Tree
	Irvingia grandifolia (Engl.) Engl.	0.004	Tree
Lecythidaceae	*Combretodendron macrocarpum* (P.Beauv.) Keay	0.046	Tree
Melastomataceae	*Memocylon blakeoides* G. Don.	0.21	Tree
Meliaceae	*Entandrophragma angolense* (Welw.) C.DC	0.013	Tree
	Guarea cedrata (A. Chev.) Pellgr.	0.121	Tree
	Khaya grandifoliola C. DC.	0.002	Tree
	Khaya ivorensis A. Chev.	0.056	Tree
	Lovoa trichilioides Harms	0.006	Tree
	Trichilia lanata A. Chev.	0.036	Tree
	Trichilia prieuriana A. Juss.	0.002	Shrub.
Moraceae	*Antiaris welwitschii* Engl.	0.042	Tree
	Bosqueia angolensis Ficalho	0.054	Tree
	Milicia excelsa (Welw.) C.C. Berg	0.002	Tree
	Musanga cecropioides R. Br	0.142	Tree
	Myrianthus arboreus P. Beauv.	0.013	Tree

Myristicaceae	*Pycnanthus angolensis* (Welw.) Warb.	0.069	Tree
	Staudtia stipitata Warb.	0.015	Tree
Ochnaceae	*Lophira alata* Banks ex Gaertnf.	0.023	Tree
Octoknemataceae	*Okoubaka aubrevillei* Pellgr. And Norman	0.127	Tree
Olacaceae	*Olax subscorpioidea* Oliv.	0.002	Shrub
	Strombosia postulate Oliv.	0.102	Tree
Pandaceae	*Panda oleasa* Pierre	0.002	Tree
Polygalaceae	*Carpolobia lutea* G. Don.	0.017	Shrub
Rhamnaceae	*Maesopsis eminii.* Engl.	0.004	Tree
Rhizophoraceae	*Anopyxis klianeana* (Pierre) Engl.	0.017	Tree
Rubiaceae	*Nauclea diderrichii* (De Wild and Th. Dun.) Merrill	0.002	Tree
	Rothmannia hispida (K. Schum) Fagerlind	0.115	Tree
	Pausinystalia macroceras (K. Schum) Pierre ex Beille	0.023	Tree
Rutaceae	*Fagara macrophylla* Engl.	0.060	Tree
Sapindaceae	*Blighia sapida* Konig.	0.108	Tree
Samydaceae	*Homalium aylmeri* Hutch and Dalz.	0.063	Tree
Sapotaceae	*Chrysophylllum albidum* D. Don.	0.017	Tree
	Chrysophyllum delevoyi De Wild.	0.015	Tree
Simaroubaceae	*Hannoa klaineana* Pierre and Engl.	0.045	Tree
	Pierreodendron africanum (Hook F.) Little	0.004	Tree
Sterculiaceae	*Cola acuminata* (P. Beauv.) Schott and Engl.	0.006	Tree
	Mansonia altissima A. Chev.	0.002	Tree
	Nesogordonia papaverifera (A.Chev.) R. Capuron	0.023	Tree
	Sterculia oblonga Mast.	0.035	Tree
	Sterculia tragacantha Lind.	0.013	Tree
	Triplochiton scleroxylon R. Schum.	0.008	Tree
Tiliaceae	*Desplatsia subericarpa* Bocq.	0.004	Shrub
Ulmaceae	*Celtis mildibraedii* Engl.	0.002	Tree
	Celtis zenkeri Engl.	0.269	Tree

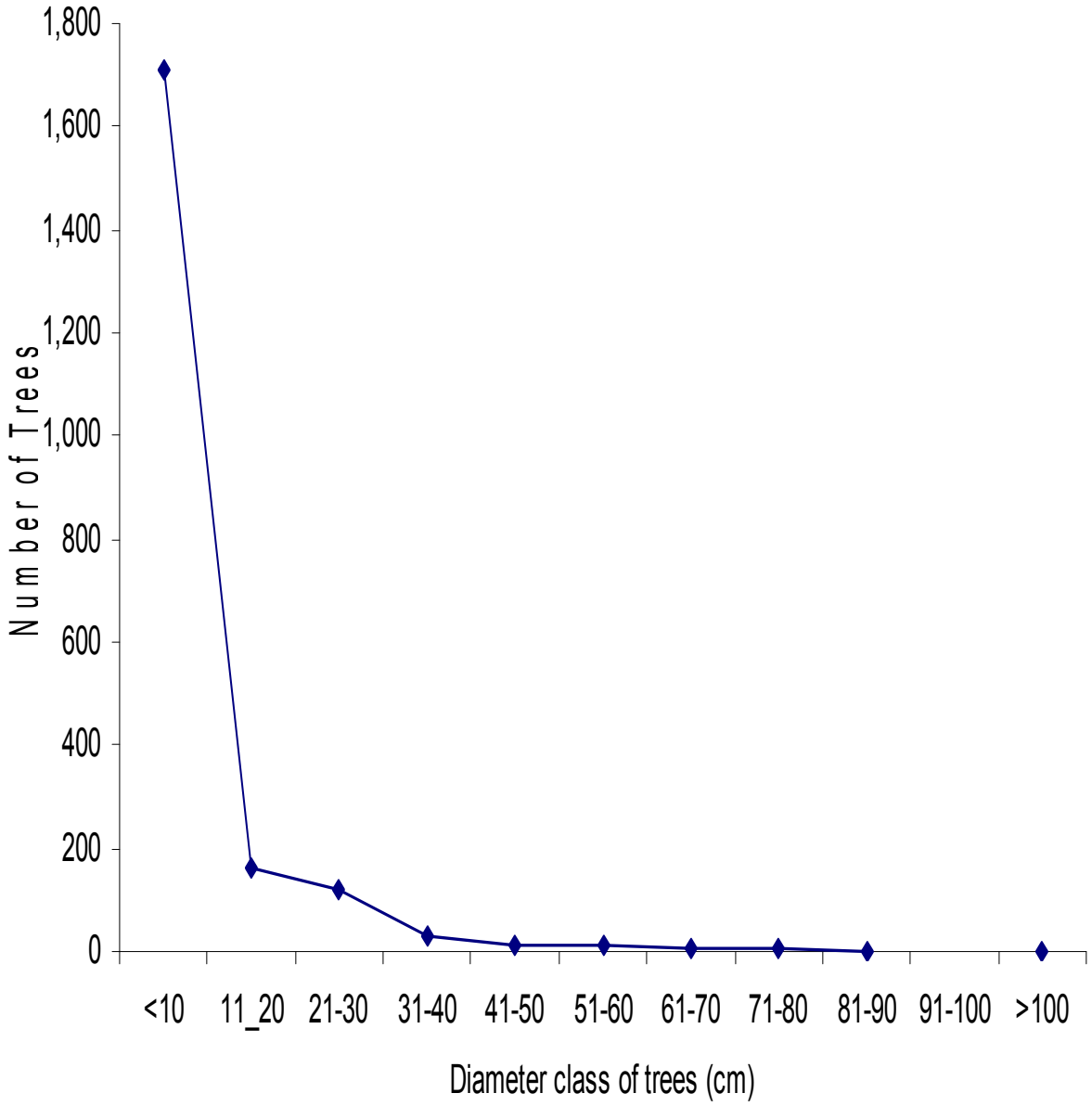

Figure 2: Diameter Class Distribution of trees in Ehor Forest Reserve

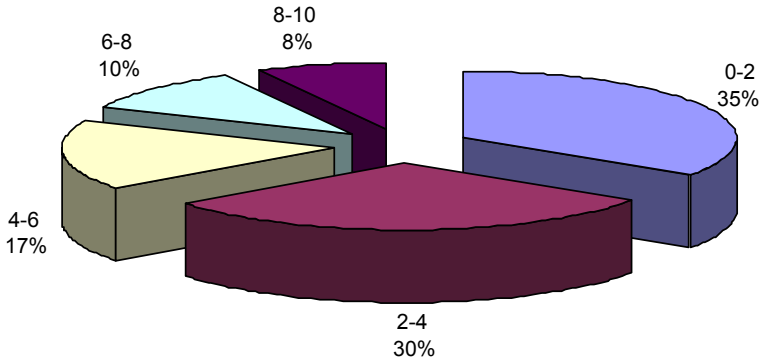

Figure 3: Percentage Distribution of stem diameter classes≤ 10 cm in Ehor Forest Reserve, Edo State

Table 2: Percentage distribution of the various diameter class sizes

Diameter class (cm)	Number of trees	Percentage Proportion
≤ 10	1,711	82.98
11-12	162	7.86
21-30	118	5.72
31-40	32	1.55
41-50	14	0.68
51-60	12	0.58
61-70	4	0.19
71-80	4	0.19
81-90	2	0.10
91-100	-	0.00
> 100	3	0.15
Total	2,062	100.00

4.0 Discussion

4.1 Relative diversity

The family Fabaceae has the highest diversity of eighteen species in this study carried out in Ehor Forest Reserve, Edo State in Southern Nigeria. Omorogbe (2004) reported fourteen species from this same family also with the highest species diversity

in Sakponba Forest Reserve, Edo State. Fabaceae was distantly followed by Meliaceae with seven spp; Annonaceae and Sterculiaceae with six spp. respectively. Moraceae and Apocynaceae had five while Euphorbiaceae had four. These were the dorminant families represented. Apocynaceae, Sterculiaceae, Euphorbiaceae, Ebenaceae, Olacaceae and Rubiaceae were reported by Ojo (2004) as forming 86% of the stand in Abeku sector of Omo Forest Reserve. Osunde (2004) in an unpublished work on Okomu Forest Reserve also reported high species diversity in Fabaceae, Meliaceae and Apocynaceae. The preponderance of occurrence of species in families with high diversity may be due to their method of seed dispersal. Where explosive mechanism and wind disperse the seeds, they are carried far away from the mother tree where they germinate when conditions are suitable but where dispersal is such that seeds are close to the mother trees, such seedlings may die due to competition for nutrients. Ogunleye *et al.* (2004) reported the dominance of Fabaceae and Meliaceae in Olokemeji Forest Reserve because of easy wind dispersal which enhanced their spread in the study location. Soladoye *et al.* (2005) also observed that the dispersal mechanism plays a strong role in addition to climatic condition and soil type in the preponderance of species of Fabaceae, Euphorbiaceae and Rubiaceae on the Olabisi Onabanjo University permanent site.

On the other hand, fifteen families within the Ehor Forest Reserve had poor species diversity. They all had only one species each. Though compartment 81 had the highest spp. of 62 distributed into 27 families, the other two compartments-95 and112- have 54 and 57 species distributed into 28 families each. Diversity is comprised of two components: the variety of species present and the relative abundance of these species (Young and Swiachi, 2006). Hence compartment 95 could be said to be richest in terms of plant population because of its high relative abundance, compared to the other two compartments. The species diversity in the three compartments of study could be attributed to the intensity of logging. This is because only a few trees of merchantable size were left standing resulting in the study sites being populated mainly by wildlings. Brown and Gurevitch (2004) reported that the impact of logging does not only negatively affect the forest diversity but that it exposes the forest to invasive species which is also a major predictor of reduced native species diversity thereby preventing the re-colonization of native species. This could be the case with compartment 95 where we have fewer species but more abundant stands.

4.2 Diameter at breast height (dbh)

Eighty-three percent of the trees encountered were in the diameter class of ≤ 10 cm. This then meant that the majority of the trees were wildlings and so were not merchantable. Oduwaiye *et al.* (2002) reported that all plots studied by them had the largest number of trees in the smallest diameter class of below 10 cm at the Okomu permanent sample plots. They also had the smallest number of trees in the diameter class of 25-30 cm. Conversely, Oduwaiye and Ajibode (2005) reported the highest number of trees for diameter class of 11-30 cm followed by those of between 0-10 cms at Onigambari Forest Reserve, Ibadan. Timber trees are logged at 60 to 90 cm dbh depending on the species (ITTO,2007). Only a few trees amounting to 0.63% (thirteen stands) were in that diameter class. There was no stand in the diameter class of 91-100 cm in the three compartments of study at Ehor Forest Reserve. The three trees above the the diameter class of 100 cm were not accessible to loggers because these species were close to Orhionmwon River. This river is a barrier to moving the logs out of the logging sites hence they are still standing.

These trees were *Piptadeniastrum africana* with a dbh of 136.80 cm in compartment 81, *Alstonia boonei* with a dbh of 115.50 cm in compartment 95 and *Hannoa klaineana* with a dbh of 175.00 cm in compartment 112. These three tree stands were located in sample plot demarcated at the centre of the various compartments.

Felling of both timber and fuel trees in Ehor Forest Reserve have gone on for years hence the reserve has been turned to a forest of wildlings. There is therefore need to reverse this trend.

5.0 Conclusion

The compartments of study in Ehor Forest Reserve were sparsely populated with ninety-nine species of wildlings mostly in the diameter class of ≤ 10 cm. The low density of these stands is an evidence of the degree of devastation the forest has been subjected to by loggers and other exploiters of non-timber forest products. This calls for an urgent solution so as not to drive some of this tree species particularly those already threatened into extinction. It is therefore suggested that Ehor Forest Reserve be protected from further exploitation to give it enough time to regenerate itself.

Acknowledgement.

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Timber Resource Status Of Ehor Forest Reserve In Uhunmwode Local Government Area Of Edo State, Nigeria

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Abstract: Five sample plots of 30m × 30m were laid out in each of three selected compartments (81, 95 and 112) of 1.6 square kilometer within the Ehor Forest Reserve of Edo State, Nigeria. Fifty-four timber species were identified with compartment 81 having the highest number of species of thirty-four (34) and compartment 112 the least with thirty (30) species. Compartment 95 had thirty-three while the three compartments have eighteen species common to them. The following eleven species *Afzelia africana, Albizia zygia, Bombax brevicuspe, Cordia millenii, Dacryodes edulis, Irvingia gabonensis, Khaya grandifoliola, Mansonia altissima, Milicia excelsa, Nauclea diderrichii, Terminalia ivorensis* were represented by only one stand in the three compartments covering an area of 4.8 square kilometers. This translates into 0.002 stand/hectare. It was therefore deduced that these eleven timber species were under threat of extinction from the reserve and therefore need to be conserved for their values. [Nature and Science. 2009;7(8):19-25]. (ISSN 1545-0740).

Keywords: Timber, threatened, extinction, conservation.

Introduction

Timber is wood in a form suitable for construction or carpentry, joinery or for reconversion to manufacturing purpose. Standing trees or felled trees capable of being converted for these purposes. In carpentry, a structural member (Anonymous 1973). According to Cunningham *et al.* (2005) timber accounts for about half of worldwide wood consumption. This exceeds the use of steel and plastic combined. It occurs in low density in most tropical forests hence large areas tend to be exploited diffusedly to extract a few prized logs. Yield of the most valuable timber species often decline owing to initial overcutting and failure to leave sufficient seed trees (Kellman and Tackaberry, 1993). There were 58 commercial timber trees in Nigeria (Anonymous 1973). Gill and Okoegwale (1991) added sixteen (16) more species.

In order to reduce pressure on the popular timber species, newer species are being added to the popular ones. For instance, *Celtis zenkeri* which was not regarded as economic is now being heavily logged and currently has become one of the popular timber species (Isichei, 1995). Ola-Adams and Iyamabo (1977), cited by Omoregbe (2004) reported that only seventeen species of timber were thought to be of economic importance in 1950 but by 1975 the number had increased to forty-seven (47). This increase is as a result of technological advancement in forestry leading to improvement and multipurpose utilization of the non-timber resources. As at now, the number of commercial species is much more than that of ten years ago.

This study was undertaken to investigate and document the number of timber species present in Ehor Forest Reserve in order to provide a baseline

information on the current timber resource richness of the reserve.

2.0 Materials and Methods

2.1 Study Location: Ehor Forest Reserve occupied an area of 76.8 square kilometers in Uhunmwode Local Government area of Edo State, Nigeria. It is located between latitudes 6^0 34'N and 6^0 38'N and longitudes 5^0 54'E and 5^0 58'E fifty-six kilometers north of the state capital, Benin-City. It is divided into forty-eight compartments of 1.6 square kilometers each. The Orhionmwon River runs through the reserve. It is surrounded by nine villages viz: Ohe, Eguaholor, Egbisi, Ugieghudu, Uhi, Iriwe, Erhue, Evbowe and Ekudo. There are no settlements within the reserve.

2.2 Survey Method: Three compartments based on their state of degradation were sampled. They were compartment 81 on the western end of the reserve which is the least degraded, 95 which is centrally located and the most degraded of the three compartments and 112 at the eastern end of the reserve (Fig 1). This is to have an adequate representation of the whole forest reserve. Five sample plots of 30m $\times$ 30m were laid out in a randomized complete block design in each compartment using improvised wooden pegs. The timber trees in each plot were identified and the density of each species per compartments noted. The density was assessed by physically counting the number of stands for each species.

Plant identification was done by using Keay *et al*.(1964) and Hopkins (1974). The timber species were confirmed using Anonymous (1973) and Gill and Okoegwale (1991).

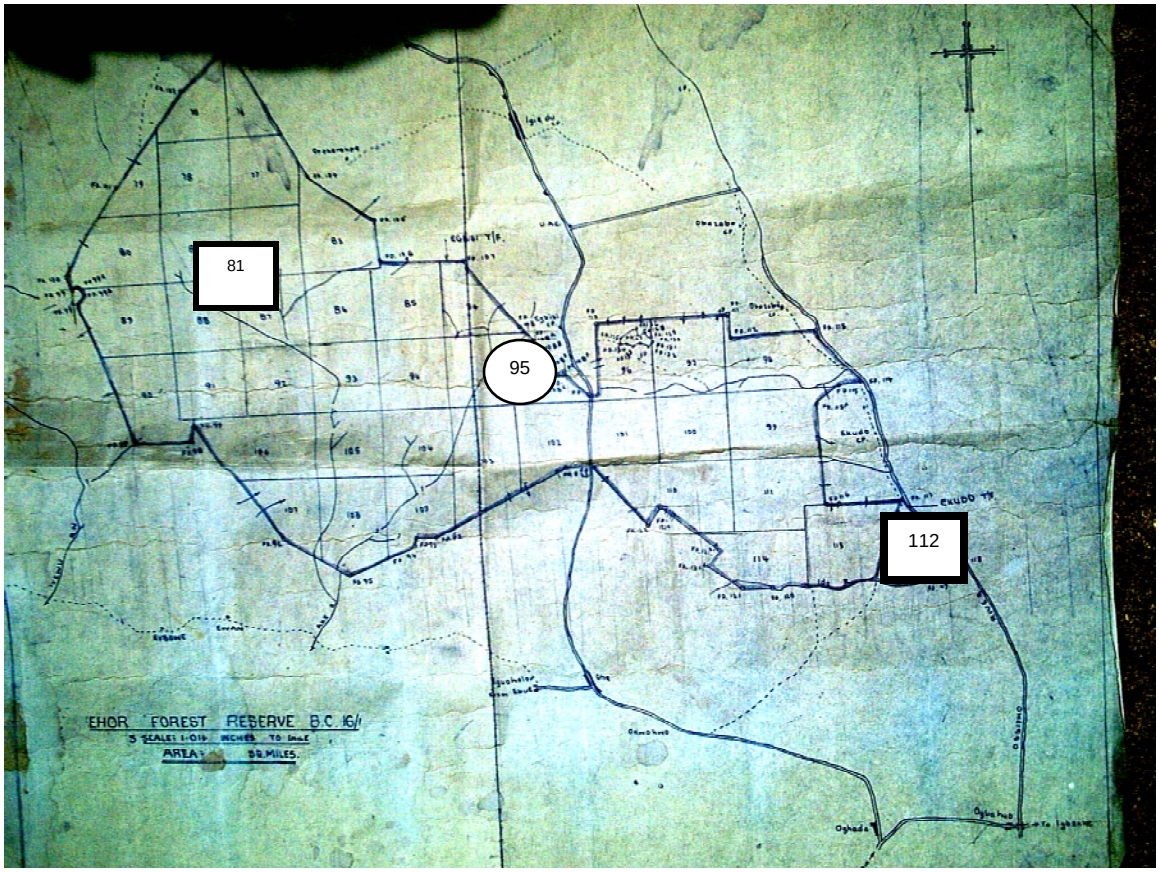

Figure 1: Map of Ehor Forest Reserve showing Compartments of Study

3.0 Results

Fifty-four timber species encountered in the reserve are presented in Table 1. Eighteen of them were common to the three compartments of study while seven (7), eight (8) and ten (10) were peculiar to compartments 81, 95 and 112 respectively (Table 2).

Compartments 81 and 95 have *Alstonia boonei, Antiaris africana, Khaya ivorensis, Piptadeniastrum africanum* and *Triplochiton scleroxylon* in common; 81 and 112 have *Allanblackia floribunda, Ceiba pentandra, Nesogordonia papaverifera* and *Pterocarpus osun* in common while 95 and 112 have *Diospyros mesipiliformis* and *Hannoa Klaineana* in common. Compartment 81 has the highest timber species of thirty-four (34) followed by 95 with 33 and 112 with 30 species. *Celtis zenkeri* had the highest density of 0.269 stand per hectare while ten (10) species had the lowest density of 0.002 stand per hectare.

TABLE 1: Timber species in the compartments of study

Timber species	Density/hectare
Afzelia Africana Sm.	0.002
Albizia ferruginea (Guill & Perr.) Benth	0.045
Albizia lebbeck (L.) Benth	0.004
Albizia zygia (DC.) J.E. Machr.	0.002
Allanblackia floribunda Oliv.	0.006
Alstonia boonei De Wild.	0.040
Antiaris africana Engl.	0.013
Antiaris welwitschii Engl.	0.042
Berlinia grandiflora (Vahl) Hutch. & Dalz.	0.088
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Bombax brevicuspe Sprague	0.002
Bosqueia angolensis Ficalho	0.054
Brachystegia nigerica Holye & A.P.D. Jones	0.169
Canarium schweinfurthii L.	0.023
Ceiba pentandra (L.) Garten	0.004
Celtis zenkeri Engl.	0.269
Chrysophyllum delevoyi De Wild	0.015
Cleistoppolis patens (Benth.) Engl. & Diels,	0.050
Combretodendron macrocarpum (P.Beauv.) Keay	0.046
Cordia millenii Bak.	0.002
Cylicodiscus gabunensis Harms	0.006
Dacryodes edulis (G.Don) H.J. Lam	0.002
Daniellia ogea (Harms) Rolfe ex Holl.	0.094
Diospyros alboflavescens (Gurke) F. White	0.045
Diospyros dendo Welw. Ex Hien.	0.006
Diospyros mesipiliformis Hochst ex A. DC.	0.017
Distemonanthus benthamianus Baill.	0.006
Entandrophragma angolense (Welw.) C.DC	0.013
Fagara macrophylla Engl.	0.060
Funtumia elastica (Preuss) Stapf.	0.056
Gossweilorodendron balsaminiferum (Verm.) Harms	0.004
Guarea cedrata (A. Chev.) Pellgr.	0.121
Hannoa klaineana Pierre & Engl.	0.045
Irvingia gabonensis (Auby-Lecomte ex O. Rorke) Baill.	0.002
Irvingia grandifolia Engl.	0.004
Khaya grandifoliola C.DC	0.002
Khaya ivorensis A.Chev.	0.056
Lophira alata Banks ex Gaetnf.	0.023
Lovoa trichilioides Harms	0.006
Mansonia altissima A. Chev.	0.002
Milicia excelsa (Welw.) C.C. Berg	0.002
Musanga cecropioides R.Br	0.142
Nauclea diderrichii (De Wild & Th. Dun.) Merrill	0.002

Nesogordonia papaverifera (A.Chev.) R. Capuron	0.023
Pachyelasma tessmannii (Harms) Harms	0.006
Pentaclethra macrophylla Benth.	0.140
Piptadeniastrum africanum, (Hook F.) Brenam	0.027
Pterocarpus osun Craib	0.006
Pycnanthus angolensis (Welw.) Warb.	0.069
Ricinodendron heudelotii (Baill.) Pierre ex Pax.	0.104
Sterculia oblonga Mast.	0.035
Strombosia postulate Oliv.	0.102
Terminalia ivorensis A. Chev.	0.002
Triplochiton scleroxylon R. Schum.	0.008

TABLE 2: Timber species according to their distribution in the three compartments

Common to all Compartments	Peculiar to each compartment of study		
	Compartment 81	Compartment 95	Compartment 112
Antiaris welwitschii	*Afzelia africana*	*Albizia ferruginea*	*Albizia lebbeck*
Berlinia grandiflora	*Cordia millenii*	*Canarium schweinfurthii*	*Albizia zygia*
Blighia sapida	*Cylicodiscus gabunensis*	*Chrysophyllum delevoyi*	*Bombax brevicuspe*
Bosqueia angolensis	*Diospyros alboflavescens*	*Combretodendron macrocarpum*	*Diospyros dendo*
Brachystegia nigerica	*Gossweilorodendron balsaminiferum*	*Dacryodes edulis*	*Irvingia gabonensis*
Celtis zenkeri	*Milicia excelsa*	*Lovoa trichilioides*	*Irvingia grandifolia*
Cleistopholis patens	*Pachyelasma tessmanii*	*Sterculia oblonga*	*Khaya Grandifoliola*
Daniellia ogea		*Terminalia ivorensis*	*Lophira alata*
Distemonanthus benthamianus			*Mansonia altissima*
Entandrophragma angolense			*Nauclea diderrichii*
Fagara macrophylla			
Funtumia elastica			
Guarea cedrata			
Musanga cecropioides			
Pentaclethra macrophylla			
Pycnanthus angolensis			
Ricinodendron heudelotii			
Strombosia postulate			

Discussion

Of the seventy-four documented timber species in Nigeria (Anonymous 1973; Gill & Okoegwale 1991) fifty-four were found in the Ehor Forest Reserve so the reserve could be said to be rich in timber species though low in density of such species. Species like *Afzelia africana, Albizia zygia, Bombax brevicuspe, Cordia millenii, Dacryodes edulis, Irvingia gabonensis, Khaya grandifoliola, Mansonia altissima, Milicia excelsa, Nauclea diderrichii* and *Terminalia ivorensis* were represented by only one stand. The results from the present investigations showed that these species were under threat of extinction from the reserve due to over harvesting. *I gabonensis* which also doubles as food crop was reported by Okafor (1980) as having a very low density of less than three stems per hectare in the forest. Almost thirty years later, the situation has degenerated. The density per hectare of these threatened timbers which are mostly the prized species translates to 0.002. Another timber which also doubles as food crop is *D.edulis* which has been domesticated in many communities in Edo State as a means of conserving the plant due to its depletion in the forest because of the frequency of its use.

Timber and its products play very important roles in our daily lives. In the house construction industry alone, it account for about 10-15% of the total cost of the building (Ratra and Panwar, 1980) so efforts should be made to conserve these timber species.

Conclusion

This study revealed that Ehor Forest Reserve is relatively rich in timber species though very poor in density. None of the timber species encountered translates to one stand per hectare. *Celtis zenkeri* was the most abundant with a total of 129 stands in the three compartments of study. This translates to 0.269 stands per hectare. Urgent steps therefore need to be taken to arrest the dwindling density of timber in our forests by restocking the forest with these timber species and domesticating those that double as food crops.

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Profitable and Eco-friendly Bio conversion of "White Button Mushroom" (*Agaricus bisporus*)

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Abstract: The large scale production of mushrooms has opened up new vistas of export earnings to improve the economic status of our country. With the view point of profitable eco-friendly bioconversion of *Agaricus bisporus* (Lange) Imbach, the study conducted upon the morphological features of fruit bodies of different strains, the yield of different strains, growth rate of different strains in compost and casing soil of *A. bisporus* in which, strain P1 and NCS 5 were found superior over the other strains. The yield of strains varied from 11.0 kg to 13.75 kg per quintal compost. The strains NCS 5 (13.75 kg) and P1 (13.10 kg) were statistically at par over the check. [Nature and Science. 2009; 7(8):26-35]. (ISSN 1545-0740).

Key words: Profitable, Eco-friendly, Bioconversion, White Button Mushroom

Introduction

Mushrooms are non-conventional source of human food. These are used as food since the beginning of human civilization. These are delicious, nutritionally rich and have their own importance as medicines. These are excellent low caloric meat substitute. The widespread use of mushrooms in ancient times is also confirmed by the hypothesis of Wason (1971) that the "Soma" of Rigveda was a preparing of mushroom, *Amanita muscaria.* For the profitable eco-friendly bioconversion of lignocellulosic wastes of agro-industry, the production of mushroom is regarded as the second most commercial microbial technology next to the yeast. The large scale production of mushrooms has opened up new vistas of export earnings to improve the economic status of our country.

Mushroom has been defined as a "Macro-fungus with a distinctive fruiting body which can be either epigeous (above ground) or hypogeous (underground) and large enough to be seen with the naked eye and to be picked by hand '' (Chang and Miles, 1993). These are non-chlorophyllic plants, which occur seasonally all over the world with quite different characters like shape, size, colour, appearance and edibility, depending on their habitat.

Taxonomically (Alexopoulas et al, 1996) these are species of phylum Basidiomycota and Ascomycota. Nearly 300 species belonging to genera of the edible mushrooms are reported in India, out of the 2000 species occurring in the world. Out of these total edible mushrooms, 80 species have been grown experimentally, 20 are being cultivated commercially and 8 are being produced on large scale all over the world. Some species of cultivated edible mushrooms whose production has reached on industrial scale are *Agaricus bisporus, Agaricus bitorquis, Flammulina velutipes, Hypsizygus marmoreus, Lentinula edodes, Pleurotus ostreatus, Tremella fuciformis, Volvariella volvacea* (Chang and Miles, 2004).

In India, *A. bisporus* (white button mushroom), *Pleurotus spp* (oyster mushroom) and *Volvariella volvacea* (tropical mushroom) are cultivated commercially. Annual increment of world *Agaricus* production during 1975-1997 was 5.3% (Delcaire, 1978 and Chang, 1999).

The world production of *Agaricus* was about 2 million metric tons (MT) in 1997 with a comparison of world production of all cultivated edible mushrooms, the percentage of world total production of *Agaricus* decreased from 73.1 in 1975 to 31.8% in 1997, even though the actual production increased from 900.0 thousand MT in 1981 to 1955.9 thousand MT in 1997, a 2.2 fold increase, and the annual increase percentage during the period 1975 to 1997 was 5.3%. This is mainly attributable to the increasing production of other cultivated edible mushrooms, such as the drastically increased production in Asia in recent years of *Lentinula, Pleurotus, Flammulina* and *Hypsizygus,* four speciality mushrooms.

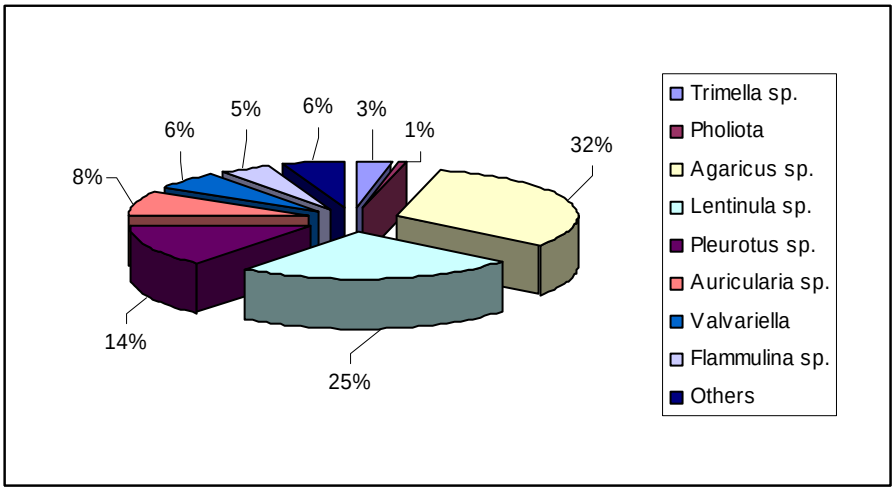

Figure1. Share of Different Mushrooms in Total World Production (6.15 Million Tonnes in 1997)

In 1986, the United States was the largest producer of *Agaricus* (266.7 thousand MT), followed by China (254.6 thousand MT) and France (170.0 thousand MT). However, in 1999-2000 China produced 637.3 thousand MT and jumped to become the largest producer of this important mushroom, followed by United States, Netherlands and France.

In India, Sharma (1997) has reported nearly 85% of the total production (40,000 MT) of edible mushrooms was contributed by *A. bisporus*.

The cultivation of white button mushroom is mostly confined to small, seasonal growing units which are mostly unpasteurised compost and producing 10-14 kg mushroom/qt. compost. However, 18-22 kg mushroom/qt. compost had been harvested under controlled conditions using the pasteurised compost by a limited number of commercial growing units but in the developed countries the production is 25-30 kg mushroom/qt. compost **(**Chaddha and Sharma, 1995).

Mushroom cultivation involves a number of operations. After the completion of spawn run the crop enters the reproductive phase leading to the production of fruit bodies. Even after the colonization of compost, fructification will not take place unless the colonized compost is covered with casing, a process of applying the cover on the surface of compost, is done by a suitable casing mixture. By applying casing layer, which is nutritionally poor, stress conditions for the production of fruit bodies are created. Controlled water supply (spraying of water) for the growth and development of fruit bodies and also to maintain humidity and temperature in the crop room by evaporative cooling is done 2-3 times every day. It also provides a medium of low osmotic value and above all physical support to fruit bodies.

Agaricus is restricted to the saprophytic with a chocolate brown spore print and usually an annulus (ring) around the stalk. The epithet *bisporus* refers to the two spored basidia lining the gills. It is also known as white button mushroom due to its colour and shape. When the mushroom is bruised it becomes brown due to oxidation.

The yield and quality of the mushroom produced is determined by three factors –

1) The genetic makeup of the mushroom strain
2) The environmental conditions in which the mushroom is grown.
3) The physiological and nutritional requirement of different strains.

There are many parameters which can influence the quality of mushroom. In our country, introduction of exotic strains and then evaluation for selection of

better strain is still a continuing process. Some isolated attempts to obtain improved strains through multispores, monospores and tissue culture has started to get better yield. Introduction of better quality strains from other countries cannot solve the problem of better quality strains in our country due to altogether different conditions of growing mushrooms than those 2 of Europe and America. Therefore; we need to develop better performing strain in terms of yield, quality and wider adaptability under diverse growing conditions.

Mushrooms are capable of producing the highest quantity of protein per unit area and time from the worthless agro-wastes. Thus, these are recognised as the alternate source of good quality protein.

Mushrooms contain 20-35% protein on dry weight basis which is higher as well as of higher quality than those of the vegetables and fruits. Many vitamins such as Thiamine (B1), Riboflavin (B2), Nicotinic acid, Pantothenic acid, Vitamin C and Vitamin K are also present in the mushrooms (Manning, 1985). These are good sources of minerals. *Agaricus bisporus* is reported to contain a considerable amount of potassium, phosphorus, copper and iron (Manning, 1985). Mushrooms have long been considered to have medicinal value.

Bioactive polysaccharides having antitumor and anticancer effects are isolated from mushroom species are *Coriolus versicolor, Lentinula edodes, Schizophyllum commune, Ganoderma lucidum, Aagaricus baize, Grifola frondoca* (Chang and Miles, 2004).

Many bioactive substances with immune-modulating effects have been isolated from mushrooms include polysaccharides, glyco-proteins, triterpenoids and fungal immune-modulating proteins.

In view of the above background, present studies were undertaken with the following objectives:

(1) To study the morphological features of fruit bodies of different strains.
(2) To study the growth rate of different strains in compost and casing.
(3) To study the yield performance of the strains.

Edible fungi belong to widely different taxonomic subdivisions among the Ascomycotina and Basidiomycotina. *Agaricus bisporus* is a secondary homothallic basidomycetes belonging to the order agaricales and family agaricaceae (Webster, 1980 and Alexopoulas et al., 1996).

This mushroom is one of the most acceptable edible fungi contributing 31.8% of total world mushroom production (Chang and Miles, 2004). Although cultivation of this mushroom is being practised since17thcentury.A brief account of the pertinent literature available on various aspects of present studies has been reviewed.

Morphological:

Atkinson (1961) described different species of *Agaricus* on the basis of morphological characteristics. However, a detailed outline to study the fungal fruiting bodies was documented by Smith and Smith (1973). Mehta and Dhar (1991) studied vegetative and sporophore characters of 15 different multispore cultures namely, MS 1 to 15 of *Agaricus bisporus* (Lange) Sing and they found an average of pileus diameter 3.43 cm, pileus thickness 0.98 cm, stipe length 3.3 cm, stipe diameter 1.49 cm and gill cavity 3.55 cm. Thakur and Dhar (1993) studied morphology of Agaricus strains and they reported the average pileus diameter 3.78 cm, pileus thickness 1.08 cm, stipe length 2.79 cm, stipe diameter 1.64 cm and gill cavity 4.05 cm.

Growth rate in compost:

Straatsma et al. (1991) studied growth of *A. bisporus* mycelium on sterile compost. The mycelium on sterile compost extended at a linear rate of 4mm/day. Mehta and Dhar (1991) recorded mycelial growth rate of multispore cultures after 7 days of incubation. Singh (1994) studied the growth rate of different strains of *A. bisporus* (P1, P2, MS 39, NCS 5, NCS 15, NCS 12 AND S 11) on compost up to 17 days from inoculation and found maximum and minimum growth rate per day as 4.62 and 2.88mm, respectively for the strains NCS 12 and P2. Singh (1998) working with *A. bisporus* strains X 13, X25, NCS 12, NCS 5, NCS 11, S 11, AbP 7, U 3, MS 39, P1 and one strain of *A. bitorquis*, NCB 13 reported

that strains MS 39 and U 3 showed maximum growth in compost after 17 days of inoculation and NCS 12 followed by NCB 13, X 25, S11, P 1 and AbP 7 were also grown. The average downward mycelial growth rate of multispore cultures afer 7 days of incubation. The average downward mycelia growth varied between 2.0- 4.3 cm.

Growth rate in casing soil:

Before initiation of fruiting, the growth of fungus forms mycelia pad or stroma in casing soil. The growth of *Agaricus bisporus* in casing soil was recorded to be 5 mm/day by Rainey et al. (1986).

Singh (1994) studied the growth rate of different strains of *A. bisporus* (P1, P 2, MS 39, NCS 5, NCS 15, NCS 12 and S11) in the casing soil upto 17 days from inoculation and reported that the maximum and minimum growth rate per day occurred in the strains P1 (4.03mm) and S 11 (1.76mm), respectively. Singh (1998) reported that in casing soil maximum growth rate per day was found in the strain 512-H whereas, NCS 5 showed minimum growth rate per day.

Yield of strains:

Jin (1990) studied the yield performance of fluffy and appressed type mycelium of the same strain of *A. bisporus*, he reported that fluffy type tended to give high yield whereas, the appressed type showed good quality. Mehta and Dhar (1991) evaluated 9 strains of *A. bisporus* for yield performance. The strains NCS 14, NCS 5, NCS 11, NCS 6 and NCS 15 were at par in terms of yield and yielded significantly higher than S 1, MS 39, P2 and NCS 12. Singh (1990, 1991) conducted the yield evaluation trials of different strains of *A. bisporus.* He reported that strains S 11 was introduced in sixties while strains RRL 89, S 22 and S 649 were introduced during 1975- 1983. The strain S 11 and S 310 were good yielders, while TM 7 and L 20 were identified as moderate yielders.

Methodology:

The experiment was conducted at "Mushroom Research and Training Centre", Centre of Advanced Studies in Plant Pathology, G. B. Pant University of Agriculture and Technology, Pant Nagar.

A. Selection of the strains:

Four strains of *A. bisporus* namely P1, NCS5, NCS12 and S11 (check) selected based on their yield performance under All India Coordinated Mushroom Improvement Project at G. B. Pant University of Agriculture and Technology, Pantnagar (UK).

B. Morphology:

The morphology of various structures of the fruiting body produced by the different strains were studied selecting 100 fruiting bodies per strain randomly per day from flush of a crop grown on pasteurized compost. The pileus, stipe and gill cavity of each fruiting body was measured with the help of Vanier Callipers, to know the morphological difference between the strains.

C. Growth rate of strains in compost:

Glass tubes (180 mm long) plugged at one end with cotton wool. 4 g. of spawn were placed at the bottom of the test tube and then covered to a depth of 100mm compost. All the treatments were replicated three times. The first observations were made after 5days of inoculation and the next four observations were made at interval of three days each. The pH and humidity of the compost used for the experimentation was 7.20 and 73.0% respectively.

Growth rate of strains in casing:

The experiment was laid down using glass tubes as earlier experiment on growth rate of strains in compost. Instead of compost, a casing mixture of FYM+ soil (3:1) treated with Formaldehyde (4%) was used. Each treatment was replicated three times and kept in a B.O.D incubator at 20 ± 2° C for growth. The first observation was made five days after inoculation followed by four observations at an interval of three days each.

Yield of strains:

The yield of strains was assessed by growing them on pasteurized synthetic compost. The synthetic compost of 2.2% nitrogen level was prepared as per following formulation:

Wheat straw	: 1000 kg
Chicken manure	: 600 kg
Urea	: 14.5 kg
Wheat bran	: 100 kg
Gypsum	: 50 kg

For compost making, short composting method was employed giving 7 days out and 6 days indoor composting period. Casing mixture (FYM+ soil) 3:1 sterilized with 4 % Formaldehyde was used. 10kg compost was filled in polythene bags of 70 X 45 cm in size and spawning was done using 0.75% grain spawn of compost weight. Each treatment was replicated 3 times and bags were kept in crop room at prevailing temperature of 20 ± 2°C. The 3.5 cm thick casing was done on 17th day from the date of spawning. The yield of strains obtained from 30 days harvesting period were compared with each other.

Statistical analysis:

Statistical analysis of the data was done as per the requirement of the experiment. Critical differences (CD) were calculated at 5% level of significance for comparison of differences between the treatment means.

Results:

The structures of fruit body like diameter, thickness gill cavity of pileus length and diameter of stipe were measured and the data recorded are summarized in Table 1.

Table1. Structural Measurements of Different Strains of *A. bisporus*

S. No.	Strains	Pileus (cm)			Stipe (cm)	
		Diameter	Thickness	Gill cavity	Length	Diameter
1.	P1	1.90	1.10	0.80	2.30	1.50
2.	NCS5	3.00	0.90	1.60	1.60	1.45
3.	NCS12	3.60	1.00	0.70	0.70	2.55
4.	S11 (Check)	2.30	0.80	2.00	2.00	1.60
	C D at 5%	0.314	0.236	0.518	0.570	0.399

A perusal of the data in Table 1 shows that highest pileus diameter (3.60 cm) recorded from strain NCS 12. There was significant difference among the treatments. The lowest pileus diameter was recorded in P1 (1.90cm). The thickness and of pileus did not vary significantly. However, the strain P1 (1.10 cm) and S11 (0.80 cm) were scaled maximum and minimum, respectively for thickness.

However, the strain S 11 (check, 1.75 cm) and NCS 12 (0.70cm) proved to be maximum and minimum size for gill cavity of the pileus. The maximum and minimum length of stipe was measured in the strains P1 (2.30cm) and NCS 12 (0.70 cm), respectively whereas, the length of stipe measured in strain S11 (check, 2.00 cm) and NCS (1.45 cm), respectively. The strain S 11 (check, 1.60cm) and P1 (1.50cm) were at par in diameter of stipe.

Growth rate of strains in compost:

The growth rate of strains in compost plays an important role in mushroom production. The fast growing strains reduce the period required for casing and ultimately it results early fruiting in contrary to slow growing strains. Therefore, this experiment was aimed to assess the growth rate of strains. The compost filled in test tubes were inoculated with grain spawn of different strains and incubated for 17 days at 20 ± 2 C˚. The growth rate recorded at 5^{th,} 8th, 11th, 14th and 17th days of incubation is summarized in Table 2.

The growth rate of strains varied significantly from each other at different intervals (Table 1). On 5th day of their growth, the strain NCS 5 (11.42mm), NCS 12 (11.40 mm) and S 11 (9.20mm, check) gave

significantly higher growth rate in comparison to P1 strain. However, the strains NCS 12 (31.85), NCS 5 (29.25 mm) and S 11 (24.25mm, check) gave significantly higher growth rates than those of other strain which included P 1 (13.22mm) on 8th day of incubation. Thus NCS 12 has started superseding. The strain NCS 12 (49.65) superseded all the strains on 11th day of its growth. While on 14th day the growth rate of NCS 12 (60.45) and NCS 5 (59.45mm) were statistically at par and high than other strains. The similar growth trends of these two strains were observed on 17thday of incubation but the strains NCS 5 had superseded the strain NCS 12.

It may be concluded from the above results that the strains NCS 5 and NCS 12 were statistically higher in growth rates at different intervals and on the basis of per day as well.

Growth rate of strains in casing:

The quick growth of strains in casing soil results early formation of stroma from which the fruit bodies come out. Therefore, the present study was undertaken to determinate the growth of strains in casing as described in "Material and Methods". The growth of strains in casing measured at different intervals for a period of 17th days is given in Table 3.

Table2. Growth Rate of Different Strains of *A. bisporus* in Compost

Observation	**Average growth rate (mm) in 17 days from inoculation**			
	P1	**NCS 5**	**NCS 12**	**S 11 (Check)**
I 5th day	4.81	11.42	11.40	9.20
II 8th day	13.22	29.25	31.85	24.25
III 11th day	37.45	35.05	49.65	38.45
IV 14th day	52.85	59.45	60.45	53.25
17th day	67.20	80.45	78.65	70.85
Growth rate/day	3.94	4.73	4.62	4.16

CD at 5% for: Strains (A) : 3.15

Observations (B) : 3.52

Observations X Strains (A) X (B) : 7.05

Table 3. Growth Rate of Different Strains of *A. bisporus* in Casing Soil

Observation	Average growth rate (mm) in 17 days from inoculation			
	P1	**NCS 5**	**NCS 12**	**S 11 (Check)**
I 5th day	6.01	10.05	6.83	3.00
II 8th day	16.21	18.00	12.60	7.05
III 11th day	35.65	31.45	25.45	15.80
IV 14th day	46.22	43.65	34.22	21.05
V 17th day	68.65	62.62	49.05	30.00
Growth rate/day	4.03	3.68	2.88	1.76

CD at 5% for: Strains (A) : 3.45

Observations (B) : 3.86

Observations X Strains (A) X (B) : 7.72

The data presented in Table 2, shows that the strains varied significantly from each other in terms of growth rate at different intervals. On 5th day of incubation the strain NCS 5 (10.05mm) gave significantly higher growth rate than S 11 (3.00 mm, check) while other strains NCS 12 (6.83mm) and P1 (6.01) were at par with each other. On 8th day of incubation, again NCS 5 (18.00mm) gave significantly higher growth rate as compared to strain S 11 (7.05mm) and NCS 12 (12.60mm) though it was at par with strain P1. But all the strains were significantly at higher at higher growth rate on 8th day as compared to 5th day. On 11th day of growth in casing the strain P1 (35.65) and NCS 5 (31.45mm) were statistically at par and significantly higher than those of other strains. The strains NCS 5 and P1 (46.22mm) gave significantly higher growth rate than S11 (21.05mm, check) and NCS 12 (34.22 mm) on 14th day of incubation. On 17th day the growth of strain P1 (68.65mm) gave significantly higher growth rate than S 11 (30.00 mm, check) and NCS 12 (49.05) but was at par with the strain NCS 5 (62.62).

Yield of strains:

The yield performance of different strains was recorded using pasteurized compost. The experimentation was done on prevailing room temperature during January, 2006 onwards. The

humidity in crop room was maintained by sprinkling of water on walls, floor and beds. The yields obtained from different strains are summarized in the table given below.

Table 4. Yields performance of different strains of *A. bisporus* in 30 days cropping period

S. No.	Strains	Average yield in kg/qt. Compost		
		Number	Weight	Weight / Fruit body (gm)
1.	P1	2280	13.10	5.74
2.	NCS 5	2482	13.75	5.53
3.	NCS 12	2230	12.50	5.60
4.	S 11 (check)	1920	11.00	5.72
	CD at 5 %	263.72	1.140	-

It is evident from the data in the above table that yield of strains varied from 11.00 kg to 13.75kg. The strains P1 (13.10 kg), NCS 5 (13. 75 kg) and NCS 12 (12.50 kg) were statistically at par in terms of yield. The yield obtained from these strains was significantly higher than strain S 11 (check, 11.0 kg). The number of fruit bodies obtained from the strains NCS5, P1 and NCS 12 were significantly higher as compared to strain S 11(1920, check). It is interesting to record that the maximum weight per fruit body harvested was from the strain S 11 (check) followed by P1, a poor and a moderate yielders, respectively.

The environment, substrate and strain are equally important factors for the mushroom production. Since, the present studies were evaluated for their yield. The rest of the strains NCS12 were at par with that of check (S 11). Earlier workers Mehta and Dhar (1991); Singh (1990, 1991) found NCS 5 to be one of the best yielders.

Discussions & Conclusion

The number of fruit bodies obtained from the strains NCS 5, P1and NCS 12 were significantly higher as compared to strain S 11 (1920, check). It is interesting to record that the strain S 11(check) followed by P1, a poor and a moderate yielders, respectively.

Serious efforts are being made world-wide to discover economic methods for upgrading the low cost bulk plant wastes such as cereal, straws, and leaves, wood bark etc. into higher value complex of terrestrial plants is the most abundant biological materials on the earth. It consists of three major components cellulose, hemicelluloses and lignin. The major viable biological processes utilizing significant quantities of lignocelluloses wastes include ruminant's feeding and the cultivation of edible mushrooms. Only Button mushroom is being cultivated on wide scale in different parts of the world. The strains, which play an important role in mushroom production, were evaluated for variability in their morphological and agronomical aspects in present investigation are discussed herein light of the results obtained by other workers. Possible explanations have also been cited wherever feasible.

The compost, spawn of different strains and casing soil were purchased from Mushroom Research and Training Centre, Pantnagar to obtain the fruit bodies for morphological studies. The morphological features of fruit bodies of different strains viz., diameter, thickness and gill cavity of pileus, length and diameter of stipe measured in present investigation have at least, in part, in similarly with the works carried out by earlier workers (Mehta and Dhar, 1991; Thakur and Dahr, 1993). Their observations were based on 4 different strains of *A. bisporus* named P1, NCS 5, NCS 12 and S 11 (check) and recorded mean of different structures like pileus diameter, pileus thickness, stipe, length and diameter etc. which varied to limited level from the measurements of these structures by the authoress in present investigation. The variations might be due to harvesting of sporophores at different growth stage and size. Besides other factors, the growth rate of a strain in compost and casing plays an important role on yield of the cultivated mushrooms. Experiments were conducted to study the growth rate of different

strains in compost and casing. The yield performances of the strains were also assessed. In the present study the growth rate of strain were determined using synthetic compost and casing soil in test tubes spawned with the grain spawn. The growth rate of different strains, varied in compost and casing. In compost, NCS 5, NCS 12 and S 11 gave maximum growth rate on 5th day from inoculation. While, with approach of 8th day the strain S11 (check) slowed down trend was maintained till 17th day. Mehta and Dhar (1991) also recorded variations in the mycelia growth of multispore cultures on 7th day of incubation. However, no report is available in the literature so far on periodical growth rate of strain in compost. The casing used for *Agaricus* cultivation forms the most important part of commercial cultivation. In casing ,on 5th day of incubation the strains NCS 5, NCS 12, P1 and S 11 (check) gave equally the same growth rate i.e. 10.05mm, 6.83mm, 6.01mm, 3.00 mm respectively in comparison to other strain while on 11th day the maximum growth was observed in case of strain P1. On the 14th day the strain NCS 5 was found statistically at par with strain P1.

Finally, the strain P1ranked first in terms of growth rate in casing per day. Rainey et al. (1986), reported growth of *A. bisporus* in casing soil 5 mm per day. The difference in growth rate of present investigation may be because of the difference in casing material used by the authoress and above earlier workers. There is no information in literature on growth rate of the strains being evaluated in present studies. The environment, substrate and strain are the equally important factors for the mushroom production. Since, the present studies were evaluated for their yield. The rest of the strains NCS 12 were at par with that of check (S11). Earlier workers Mehta and Dhar; Singh (1990, 1991); found NCS 5 to be one of the best yielders. It may be concluded from the foregoing discussions that the strain NCS 5 and P1 have superiority over the other strains studied in present investigations.

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Effect of cold stratification on the germination of seeds of chirpine (*Pinus roxburghii* Sargent) from Indian Himalayan Region.

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Abstract: The seeds of *Pinus roxburghii* germinate well in nature, but due to increasing demand of large quantities of seeds for afforestation programmes, ways to accelerate the rate of germination are needed. Present study reports the beneficial effect of pre-chilling on the germination of seeds of 30 provenances of chirpine collected from the Uttarakhand and Himachal Pradesh states of India. The results are being discussed in terms of value of the practice for raising nursery seedling production. Cold stratification for 15 days improved the rate and percentage of germination of the non-dormant chirpine seeds, when germinated at 20°C and 25°C, whereas percentage of germination decreased in most of the provenances, germinated at 30°C. Germination of pre-chilled seeds of the Kalimath and Pokhal seed sources was found maximum (96% each), while minimum (62%) germination was observed for Chotti Singri seed source at 20°C. [Nature and Science. 2009;7(8):36-43]. (ISSN 1545-0740).
Key words: Germination percentage, Gibberellic acid, dormancy, temperature, afforestation.

1. Introduction

For enhancing the rate and percentage of germination and for breaking the dormancy, moist chilling or cold stratification has been widely used as a pre-sowing treatment (Schopmeyer, 1974; AOSA, 1992; ISTA, 1999; Wang and Berjak, 2000). This is simple, inexpensive and effective technique for overcoming the seed dormancy. Though the phenomenon is not yet fully known, but the effects of moist chilling in establishing hormonal levels have been proved due to initiation of appropriate enzyme activity (Nikolaeva, 1977).

Gibberellic acid (GA_3) has been shown to promote germination of seeds (Vogt, 1970; Krishnamurty, 1973; Chandra and Chauhan, 1976; Ghildiyal, 2003). The germination percentage increased in the seeds of *Nothofagus obliqua*, when pre-chilled after soaking in GA_3 solutions for 24 hours (Shafiq, 1980). Singh (1973) reported that spruce seeds germinate comparatively more profusely than silver fir, and every year enough seeds become available for raising sufficient planting stock. Parma Nand and wright (1922) have noticed that germination capacity of spruce seeds collected from trees of different diameter classes, have shown maximum 47% germination. Singh et al. (1975) have recorded 56% germination of spruce seeds in B.O.D. incubator and maximum 39.5% germination in pot culture experiments. On the other hand, pre-chilling or cold stratification has been found to be an effective method for germinating seeds of many other species (Shafiq and Omer, 1969; Miller, 1971; Stilinovic and Tucovic, 1971).

Work on seed testing of various provenances of *Pinus roxburghii* from Uttarakhand and Himachal Himalaya has been done by Sharma et al. (2001), Ghildiyal et al. (2007, 2008, 2009) and Ghildiyal and Sharma (2005, 2007). Studies on both physiologically dormant *Picea glauca* and non-dormant *Picea mariana* (black spruce) seeds have shown that moist chilling was beneficial in accelerating the rate of germination (Wang, 1987). Moreover, the phenomenon of cold stratification has long been recognized in overcoming physiological dormancy of seeds of many species. The work of Wang (1973 and 1987) on *Pinus* species and *Picea* species indicates that short periods of moist chilling of such non-dormant seeds may generally be efficacious, particularly if damage has accumulated due to natural deterioration or as a result of an imposed accelerated ageing regime. The hypothesis, therefore, is that the repair mechanism may be activated during moist chilling on non-dormant, cold temperate gymnospermous seeds, which are naturally aged or have been subjected to accelerated ageing. If this is generally applicable, cold stratification could have far-reaching implications for nursery practice, at least for such

gymnosperms, in which aged, stored seed lots are used. In the present study we have tried to explore the effect of cold stratification on seeds of *Pinus roxburghii*, taking into account various physiological processes in relation to post harvest desiccation and chilling requirements.

2. Materials and Methods

The seeds were collected from the natural chirpine forests of two states viz., Uttarakhand (which is further divisible into Garhwal and Kumaon Himalaya or Central Himalaya) and Himachal Pradesh (Himachal Himalaya), situated in the Western-central Himalayan region of India. In Garhwal region the seeds of *Pinus roxburghii* were collected from the natural forests of four districts viz.; Pauri, Tehri, Rudraprayag and Chamoli (between 29° 20' to 31° 5' 30" N latitudes and 78° 15' to 80° 8' E longitudes). Whereas in Kumaon Himalaya from three districts viz., Nainital, Pithoragarh and Almora (the region extends between 28° 43' 24" N and 31° 27' 50" N latitudes and between 77° 34' 27" E and 81° 02' 22" E longitudes). In Himachal Himalaya the seeds were collected from five districts viz., Mandi, Solan, Hamirpur, Kangra and Shimla, situated between 30° 22' N to 33° 13' N latitudes and 75° 23' 24" E to 79° 00' 50" E longitudes. The details of the study areas have been presented in Table 1 and Figure 1.

Table 1. Geographic and climatic details of the selected seed sources of *Pinus roxburghii*.

Provenance	District /State	Latitude (N)	Longitude (E)	Altitude (m)	Temperature (°C)		Mean Annual rainfall (mm)
					Min.	Max.	
Ashtavakra	Pauri (U.K.)	30° 13'	78° 48'	960	5.76	37.70	705.0
Agustmuni	Rudraprayag (U.K.)	30° 23'	79° 02'	875	4.31	36.59	833.0
Badiyargarh	Tehri (U.K.)	30° 17'	78° 50'	1080	7.50	36.30	930.0
Chhoti Singri	Mandi (H. P.)	31° 49'	76° 59'	1220	0.30	32.50	1025.0
Dhulcheena	Almora (U.K.)	29° 42'	79° 49'	1850	-0.14	26.40	1125.0
Gallu	Mandi (H. P.)	31° 42'	77° 01'	1520	-0.20	31.40	1100.0
Ghansali	Tehri (U.K.)	30° 27'	78° 39'	890	5.00	34.60	1230.0
Godnar	Chamoli (U.K.)	30° 30'	79°16'	1680	1.30	24.00	1890.0
Jasholi	Rudraprayag (U.K.)	30° 16'	79° 04'	1520	1.60	34.10	1025.0
Jaiharikhal	Pauri (U.K.)	29° 47'	78° 32'	960	7.54	37.00	1150.0
Kaligad	Almora (U.K.)	29° 38'	79° 25'	1800	0.42	26.86	1060.0
Kalimath	Chamoli (U.K.)	30° 34'	79° 05'	1540	1.60	26.10	1257.5
Khamlekh	Pithoragarh (U.K.)	29° 47'	80° 04'	1450	3.10	31.20	1230.0
Lansdowne	Pauri (U.K.)	29° 50'	78° 41'	1703	-0.90	25.80	1260.0
Matiyal	Nainital (U.K.)	29° 10'	79° 20'	1740	3.80	23.40	2270.0
Matnoh	Hamirpur (H. P.)	31° 45'	76° 43'	980	0.80	33.6	1150.0
Mayali	Tehri (U.K.)	30° 23'	78° 47'	1400	2.60	25.10	1030.0
Nagali	Solan (H. P.)	30° 54'	77° 12'	1545	0.50	34.1	1000.0
Nihari	Hamirpur (H. P.)	31° 29'	76° 28'	800	1.20	35.4	1125.0
Pabo	Pauri (U.K.)	30° 15'	79° 01'	1640	1.8	32.4	875.0
Patwadangar	Nainital (U.K.)	29° 16'	79° 20'	1500	7.40	28.50	2850.0
Pauri	Pauri (U.K.)	30° 09'	78° 48'	1660	-0.48	26.30	1792.0
Pokhal	Tehri (U.K.)	30° 25'	78° 59'	820	5.70	37.63	800.0
Ranital	Kangra (H. P.)	31° 10'	76° 05'	960	0.20	32.5	1350.0
Seshan	Shimla (H. P.)	31° 07'	77° 45'	1540	0.50	30.3	1075.0
Seuri	Mandi (H. P.)	31° 50'	77° 02'	1460	0.15	33.2	925.0
Soni	Almora (U.K.)	29° 12'	79° 24'	1650	2.3	28.00	1040.0
Tangni	Chamoli (U.K.)	30° 29'	79° 28'	1480	4.20	25.50	990.0
Thalisain	Pauri (U.K.)	30° 02'	79° 03'	1640	1.9	31.00	1025.0
Vana	Chamoli (U.K.)	30° 38'	79° 05'	1610	1.30	24.00	1660.0

U.K.= Uttarakhand, H.P.= Himachal Pradesh

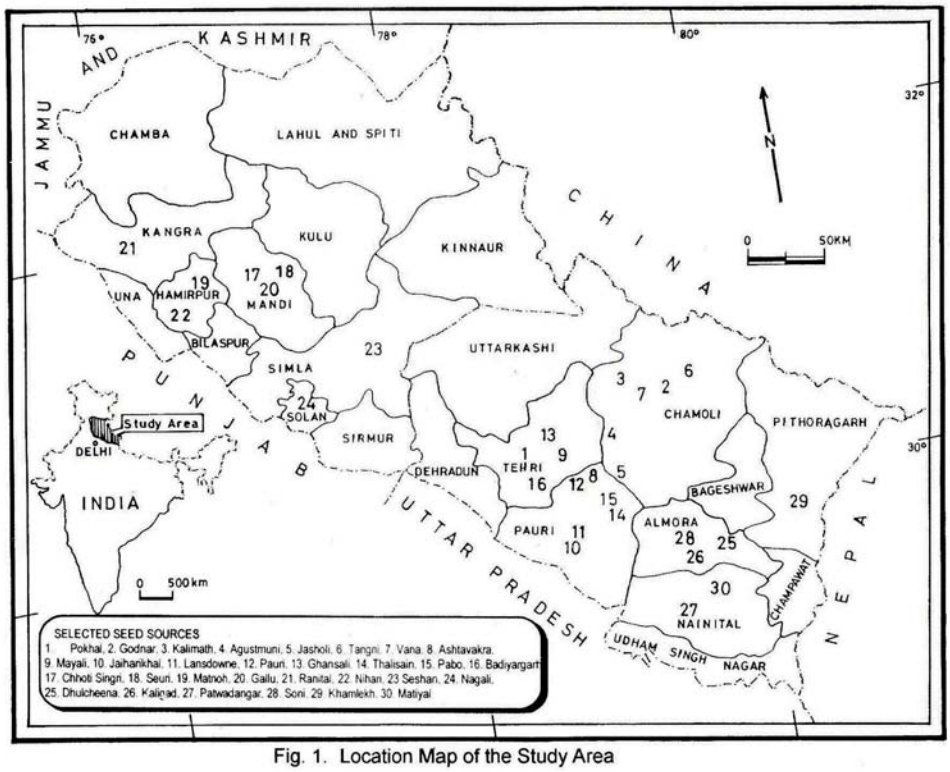

Fig. 1. Location Map of the Study Area

The germination tests on all the 30 seed sources were carried out under laboratory conditions at various temperatures *viz*., 20°C, 25°C and 30°C using regime of 12h light alternating with 12h dark inside a seed germinator (Model No. 8LT-SGL CALTAN). In each temperature regime, the seeds of all the seed sources were tested for germination with the following treatments:

Treatment 1- Soaking of the seeds in distilled water at room temperature (25°C) for 24 hours.

Treatment 2- Seeds treated with Gibberellic acid (GA_3 10 mg l^{-1}) as above at room temperature (25°C) for 24 hour and then chilled for 15 days (at 3°C).

Chilling of non-dormant seeds was carried out in folded-over-polythene bags at 3°C for a period of 15 days (as per method suggested by Tompsett and Pritchard, 1998), after soaking the seeds for 24 hours in 10 mgl^{-1} Gibberellic acid, followed by subsequent drying. The chilled seeds were then subjected to various temperature treatments. Seed germination tests were carried out under similar conditions as described above. The progress of germination was monitored daily, until there was no further germination for a few days.The seeds of different provenances, having same level of ripeness were collected and subjected to viability test by floating method to select only the viable seeds. For germination, the seeds in five replicates of 100 seeds each were placed in Petri dishes (diameter- 10 cm) containing two filter papers, kept in the germinator, and maintained at desired temperature. Observations were recorded daily up to 21 days. Radical emergence was taken as the criteria for germinability.

The collected data were further quantified in terms of percent germination and germination value. Percent germination was the value of seeds germinated at the completion of the germination period, whereas, germination value is an index, combining both speed and completeness of germination; which according to Czabator (1962) can be expressed as: GV= PV X MDG, where, GV is germination value, PV is the peak value of germination, and MDG is the mean daily germination. The statistical analysis of each parameter was carried out on mean values and the analysis of variance (ANOVA) was performed using SPSS package (version 12.0). The critical difference (CD) was calculated as: CD = SEd X $t_{0.01}$, Where, SEd is the standard error of difference calculated as SEd = $\sqrt{2}$Me/r, where Me= mean sum of square and r= number of replicates.

3. Results

Germination of seeds of various provenances after pre-chilling treatment under different temperature regimes, (i.e., 20°C, 25°C and 30°C) has yielded significant differences. The data analysed for its variance, has revealed prominent differences amongst different seed sources, which have been presented in Table 2 & 3. The detailed treatment-temperature interactions are given below:-

Table 2. Mean germination percentage of pre-chilled and un-chilled seeds in various provenances of *Pinus roxburghii* at different temperatures (Mean ± SE).

	Pre-chilled			*F-value*	P-*value*	Un-chilled			*F-value*	*P-value*
Provenance	20°C	25°C	30°C			20°C	25°C	30°C		
Agustmuni	86.2 ±4.94	84.5 ±0.91	79.2 ±4.64	0.25	0.79	85.2 ±2.10	81.2 ±1.10	87.8 ±1.50	1.29	0.34
Ashtavakra	75.0 ±8.95	92.0 ±1.28	56.4 ±1.92	4.14	0.07	70.0 ±8.95	89.6 ±1.17	54.0 ±1.52	8.43*	0.02
Badiyargarh	73.4 ±2.19	67.0 ±1.25	72.4 ±4.23	0.09	0.91	69.6 ±5.62	61.2 ±1.10	81.0 ±1.18	0.23	0.80
Chhoti Singri	62.0 ±4.26	78.4 ±0.86	51.3 ±2.56	1.12	0.39	56.2 ±3.92	73.2 ±1.20	48.1 ±1.65	0.72	0.52
Dhulcheena	94.6 ±2.56	98.8 ±0.92	63.2 ±3.24	1.43	0.31	92.6 ±1.78	93.4 ±1.75	65.5 ±1.31	1.48	0.30
Gallu	78.6 ±8.23	70.4 ±1.48	68.4 ±2.83	0.20	0.82	72.6 ±6.65	66.0 ±1.30	66.9 ±1.41	1.00	0.42
Ghansali	68.5 ±4.36	66.4 ±1.18	62.6 ±3.54	0.44	0.66	63.6 ±4.35	63.2 ±1.10	70.0 ±1.70	0.25	0.79
Godnar	92.4 ±5.62	93.2 ±1.86	82.4 ±2.86	2.25	0.19	90.2 ±1.12	90.2 ±1.10	89.6 ±1.63	0.08	0.93
Jaiharikhal	70.8 ±3.87	68.5 ±0.75	61.8 ±4.27	1.09	0.39	68.4 ±3.63	62.8 ±1.20	59.8 ±1.72	0.96	0.43
Jasholi	72.5 ±7.26	70.8 ±1.19	46.5 ±6.12	16.91**	0.00	69.6 ±4.72	65.6 ±1.72	47.6 ±1.21	2.07	0.21
Kaligad	90.2 ±3.68	94.5 ±1.36	75.6 ±2.48	3.04	0.12	88.2 ±3.25	90.2 ±1.60	73.1 ±1.85	1.81	0.24
Kalimath	96.0 ±8.00	88.0 ±1.52	88.0 ±8.92	0.17	0.85	94.0 ±4.00	82.0 ±1.42	86.6 ±1.81	0.39	0.69
Khamlekh	83.2 ±4.63	66.0 ±1.24	58.2 ±2.52	0.53	0.61	75.4 ±0.85	60.8 ±1.86	56.1 ±1.32	2.20	0.19
Lansdowne	82.0 ±5.59	76.3 ±1.42	74.5 ±2.75	0.48	0.64	75.8 ±6.12	73.6 ±1.17	80.6 ±1.29	0.17	0.85
Matiyal	87.5 ±2.27	86.2 ±1.39	88.6 ±1.82	0.01	1.00	83.2 ±1.93	83.8 ±1.36	97.2 ±0.86	0.03	0.97
Matnoh	90.3 ±5.45	94.0 ±0.96	72.0 ±3.16	1.23	0.36	87.4 ±2.74	90.6 ±0.75	79.4 ±2.02	3.21	0.11
Mayali	77.2 ±6.22	84.2 ±0.96	60.2 ±2.63	65.44**	0.00	72.2 ±5.27	79.6 ±1.17	64.2 ±1.99	1.62	0.27
Nagali	81.6 ±6.23	97.8 ±1.56	81.3 ±1.76	1.36	0.33	79.4 ±5.56	94.6 ±1.47	85.8 ±1.56	0.44	0.66
Nihari	77.5 ±6.46	88.0 ±1.72	52.8 ±2.94	8.47*	0.02	74.8 ±1.21	85.4 ±1.66	49.9 ±1.51	2.63	0.15
Pabo	76.2 ±6.66	69.2 ±1.62	84.2 ±2.68	0.35	0.72	72.4 ±7.28	65.2 ±1.50	82.4 ±1.36	0.01	0.99
Patwadangar	75.0 ±2.38	72.2 ±1.68	78.0 ±4.15	0.55	0.61	70.0 ±2.82	66.0 ±1.82	81.0 ±1.73	0.17	0.85
Pauri	88.6 ±4.73	90.6 ±1.26	44.0 ±5.09	21.68**	0.00	82.6 ±2.16	88.0 ±1.62	58.0 ±1.64	2.63	0.15
Pokhal	96.0 ±7.48	71.4 ±2.14	86.0 ±5.10	8.69*	0.02	94.0 ±2.45	65.2 ±1.40	94.0 ±1.23	6.77*	0.03
Ranital	80.0 ±2.17	75.8 ±1.19	75.6 ±4.28	1.32	0.33	75.0 ±4.32	73.6 ±0.75	73.9 ±1.50	2.22	0.19
Seshan	82.2 ±4.92	96.0 ±1.64	78.6 ±3.48	1.28	0.35	80.2 ±3.84	92.8 ±1.86	82.2 ±1.72	1.38	0.32
Seuri	84.8 ±7.67	86.2 ±1.12	80.7 ±1.84	0.02	0.98	80.0 ±8.39	83.8 ±0.68	86.4 ±0.82	1.78	0.25
Soni	90.8 ±6.59	88.4 ±1.45	80.4 ±2.91	7.22*	0.03	86.6 ±4.46	86.2 ±1.69	85.2 ±1.37	0.94	0.44
Tangni	70.3 ±5.75	67.2 ±1.73	68.3 ±4.36	0.24	0.79	66.0 ±2.42	62.0 ±1.42	66.8 ±1.72	0.71	0.53
Thalisain	88.0 ±5.83	87.6 ±1.21	78.0 ±6.63	0.91	0.45	86.0 ±6.00	83.2 ±1.10	89.4 ±1.36	0.57	0.60
Vana	94.0 ±2.44	92.1 ±0.89	80.0 ±2.84	23.48**	0.01	90.0 ±1.56	89.0 ±1.42	84.0 ±1.58	0.98	0.43
F- value	3.780**	2.505**	3.658**			4.182**	1.848**	3.202**		
P-value	6.75E-06	0.001594	1.51E-05			1.43E-06	0.022626	7E-05		
Mean	76.4	81.2	62.7			83.4	81.4	72.8		
Range	62.0-96.0	66.0-98.8	44.0-88.6			63.6-94.0	43.81-98.4	37.6-97.2		
C.D. at 1%	7.42	7.82	6.76			7.24	7.2	8.6		

***significant at 5% and *significant at 1% level*

Table 3. Germination value of pre-chilled and un-chilled seeds in various provenances of *Pinus roxburghii* at different temperatures.

Provenance	Pre-chilled			*F-value*	*P-value*	Un-chilled			*F- value*	*P-value*
	20°C	25°C	30°C			20°C	25°C	30°C		
Agustmuni	8.38 ±1.12	8.76 ±1.70	10.28±0.92	0.06	0.94	12.16±0.48	14.08±1.41	14.79±0.97	0.78	0.50
Ashtavakra	1.10 ±0.28	12.10 ±2.89	4.18 ±0.62	2.95	0.13	2.22 ±0.56	49.61±2.90	2.60 ±0.67	31.02**	0.00
Badiyargarh	2.27 ±0.68	5.37 ±1.43	6.25 ±0.84	0.61	0.57	2.18 ±0.32	6.38 ±1.13	4.47 ±0.96	1.34	0.33
Chhoti Singri	1.67 ±2.42	5.77 ±1.39	2.14 ±0.36	0.52	0.62	1.94 ±1.54	3.72 ±1.57	0.48 ±0.04	3.09	0.12
Dhulcheena	9.32 ±1.84	9.75 ±1.87	2.06 ±0.54	2.20	0.19	11.39±1.73	11.14±1.41	1.70 ±0.38	5.94*	0.04
Gallu	8.16 ±1.25	3.72 ±0.75	4.64 ±1.37	4.48	0.06	7.25 ±0.89	6.19 ±0.66	2.66 ±0.47	44.51**	0.00
Ghansali	3.88 ±1.61	7.86 ±1.66	5.35 ±0.42	0.81	0.49	3.52 ±0.59	5.40 ±1.24	7.27 ±0.86	1.51	0.29
Godnar	4.03 ±0.88	9.42 ±0.94	9.36 ±1.26	0.16	0.85	5.42 ±1.70	7.49 ±1.41	8.15 ±0.72	0.15	0.86
Jaiharikhal	6.16 ±0.91	6.48 ±1.54	8.74 ±0.86	0.02	0.98	8.62 ±0.81	8.34 ±1.42	10.38±0.98	0.29	0.76
Jasholi	2.71 ±0.96	5.28 ±1.36	3.42 ±0.56	13.72**	0.01	2.76 ±1.32	4.92 ±1.30	1.52 ±0.33	18.78**	0.00
Kaligad	6.44 ±1.12	4.63 ±0.74	2.35 ±1.07	1.96	0.22	6.67 ±0.96	5.98 ±0.98	1.83 ±0.34	33.94**	0.00
Kalimath	2.46 ±0.64	6.26 ±1.15	6.14 ±0.68	2.28	0.18	3.06 ±0.50	5.24 ±1.28	4.60 ±0.57	62.00**	0.00
Khamlekh	4.39 ±0.67	2.47 ±0.66	1.92 ±0.74	1.21	0.36	4.23 ±0.36	1.80 ±0.67	1.67 ±0.32	56.56**	0.00
Lansdowne	4.32 ±1.74	6.72 ±1.33	8.92 ±1.42	0.30	0.75	6.36 ±1.23	5.53 ±1.35	11.98±1.33	1.77	0.25
Matiyal	6.78 ±1.19	6.54 ±1.72	5.68 ±1.28	0.11	0.90	3.07 ±1.55	5.25 ±1.45	9.16 ±0.96	14.58**	0.00
Matnoh	5.63 ±0.69	6.48 ±1.36	2.26 ±0.54	9.76*	0.01	4.19 ±0.52	5.38 ±1.49	1.74 ±0.08	8.65*	0.02
Mayali	3.78 ±0.56	11.36 ±1.66	7.46 ±1.32	1.87	0.23	16.27±0.36	43.81±1.42	4.64 ±0.82	18.20**	0.00
Nagali	7.13 ±0.98	10.62 ±1.46	6.74 ±1.28	1.40	0.32	9.36 ±2.19	10.95±1.29	8.81 ±1.22	0.59	0.58
Nihari	6.86 ±1.54	6.23 ±1.17	1.68 ±0.39	7.28*	0.02	8.71 ±1.46	5.14 ±1.23	0.28 ±0.03	14.15**	0.01
Pabo	5.52 ±1.28	3.25 ±0.88	11.08±1.72	0.71	0.53	5.92 ±1.42	7.21 ±1.54	13.93±1.09	32.86**	0.00
Patwadangar	5.98 ±0.89	4.39 ±1.19	2.60 ±0.92	1.52	0.29	4.24 ±1.64	3.36 ±1.07	1.95 ±0.38	4.92	0.05
Pauri	7.25 ±1.33	10.42 ±1.21	4.56 ±0.78	0.94	0.44	10.71±0.77	17.46±1.17	2.30 ±0.56	4.75	0.06
Pokhal	2.00 ±0.54	4.34 ±0.76	5.21 ±0.82	1.23	0.36	3.43 ± 0.32	5.29 ±1.36	7.18 ±0.73	3.17	0.11
Ranital	4.62 ±1.77	2.08 ±1.26	1.52 ±0.42	1.69	0.26	2.63 ±1.21	4.03 ±1.33	0.25 ±0.02	28.64**	0.00
Seshan	5.92 ±1.27	8.76 ±0.98	4.25 ±0.64	8.86*	0.02	5.54 ±1.70	9.32 ±1.33	6.35 ±0.55	3.29	0.11
Seuri	4.34 ±0.94	6.32 ±0.84	1.94 ±0.33	7.27*	0.02	6.26 ±0.79	5.24 ±0.86	1.85 ±0.07	156.0**	0.00
Soni	8.75 ±1.36	10.08 ±2.03	3.21 ±1.15	5.38*	0.05	12.56±1.48	12.36±2.15	5.05 ±1.85	11.14**	0.01
Tangni	7.42 ±0.85	7.42 ±1.23	6.78 ±2.46	0.38	0.70	4.50 ±1.04	6.32 ±1.15	5.38 ±0.56	0.45	0.66
Thalisain	2.48 ±0.40	6.29 ±1.75	4.37 ±0.69	0.60	0.58	3.79 ±0.60	6.10 ±1.55	5.32 ±0.89	9.91*	0.01
Vana	5.25 ±1.48	7.94 ±0.92	4.53 ±1.21	1.36	0.32	13.64±0.43	15.86±0.95	6.26 ±0.67	66.53**	0.00
F- value	1.734**	1.446*	1.682**			21.325**	17.789**	10.348**		
P-value	0.036362	0.11393	0.045131			4.89E-22	5.33E-20	2.74E-14		
Mean	5.6	6.2	4.7			6.7	5.2	4.6		
Range	1.10-9.32	2.08-12.10	1.52-11.08			1.94-16.27	1.80-43.81	0.25-14.79		
C.D. at 1%	1.4	1.2	0.94			0.76	1.2	1.8		

***significant at 5% and *significant at 1% level*

Pre-chilling effects on germination

The presowing treatment of seeds by cold stratification enhances the rate and percentage of germination of seeds in many tree species. The results on the germination of pre-chilled and un-chilled seeds of 30 provenances of chirpine by subjecting some of the seeds to a chilling temperature of 3°C for 15 days and others without pre-chilling, after soaking with GA_3 and subsequently germinated at different constant temperatures i.e., 20°C, 25°C and 30°C have been presented in Table 2. The data reveals that in the pre-chilled seeds at 20°C, maximum germination (96.0 ±8.00%) was recorded in Kalimath seed source, whereas, minimum germination (62.0 ±4.26%) was

recorded in the Chhoti Singri seed source. However, with a great similarity at 20°C the maximum and minimum germination percentages for un-chilled seeds were also recorded in Kalimath (94.0 ±4.00%) and Chhoti Singri (56.2 ±3.92%) seed sources, respectively. On the other hand, at 20°C the maximum germination value for pre-chilled seeds was recorded in Dhulcheena seeds source (9.32 ±1.84), and minimum in Ashtavakra seed source (1.10 ±0.28). However, at this temperature the maximum and minimum germination values for un-chilled seeds were recorded in Mayali (16.27 ±0.36) and Chhoti Singri (1.94 ±1.54) provenances, respectively (Table 3).

The pre-chilling of seeds followed by a temperature treatment of 25°C have shown highest germination percentage (98.8 ±0.92%) in Dhulcheena seed source, closely followed by several other seed sources, *viz*., Nagali (97.8 ±1.56%), Seshan (96.0 ±1.64%), Kaligad (94.5 ±1.36%) and Matnoh (94.0 ±0.96%). At 25°c the lowest germination percentage (66.0 ±1.24%) was recorded in Khamlekh seed source. Similarly for un-chilled seeds, maximum and minimum germination percentages were again recorded in Dhulcheena (95.4 ±1.75%) and Khamlekh (60.8 ±1.86%) seed sources, respectively. For pre-chilled seeds the highest germination value was recorded in Ashtavakra seed source (12.10 ±2.89), and lowest in Ranital seed source (2.08 ±1.26). On the other hand the highest and the lowest germination values for un-chilled seeds were recorded in Ashtavakra (49.61 ±2.90) and Khamlekh (1.80 ±0.67) provenances respectively.

The results obtained for pre-chilled and subsequent germinated seeds at 30°C (Table 2) ranged from 44.0 ±5.09% (Pauri) to 88.6 ±1.82% (Matiyal). The Kalimath (88.0 ±8.92%), Pokhal (86.0 ±5.10%), Pabo (84.2 ±2.68%) and Godnar (82.4 ±2.86%) seed sources were close to the maximum value. Minimum (44.0 ±5.09%) germination was recorded in Pauri seed source which was comparable to Jasholi (46.5 ±6.12%), Chhoti Singri (51.3 ±2.56%) and Nihari (52.8 ±2.94%) seed sources. A perusal of Table. 3 revealed that differences in germination values were wide-ranging i.e., 1.52 ±0.42 (Ranital) to 11.08 ±1.72 (Pabo) amongst the seed sources. It is clear from the results (Table 2) that a number of seed sources, particularly Agustmuni, Godnar, Lansdowne and Jaiharikhal, excelled over other seed sources. On the other hand, germination percentage and germination value in the un-chilled seeds of various provenances ranged from 47.6 ±1.21% (Jasholi) to 97.2 ±0.86% (Matiyal), and 0.25 ±0.02 (Ranital) to 14.79 ±0.97 (Agustmuni) respectively.

The analysis of variance (ANOVA) calculated between seed source and temperature for the germination of pre-chilled seeds has shown highly significant relationship at 5% level for some seed sources i.e., Jasholi, Mayali, Pauri and Vana, whereas, some other sources (Nihari, Pokhal and Soni) have shown significant relationship at 1% level. On the other hand, for the germination of un-chilled seeds, significant (at 1% level) relationship was obtained for Ashtavakera and Pokhal seed sources. The germination of the pre-chilled and un-chilled seeds of all the provenances has reflected highly significant relationship at 5% level.

4. Discussion

Cold stratification or chilling under moist conditions has long been recognised as a useful method of treating seeds to improve the rate and the percentage of germinability (Outcall, 1991). Heydecker and Coolbear (1977), suggested many other presowing treatments that increased germination percentage and rate. Thapliyal (1986) found that under favourable conditions, the seeds of *Pinus roxburghii* germinate well (60-80%) within 7-21 days. Because most of the seeds of chirpine are non-dormant, therefore it is often assumed that pre conditioning treatments are unnecessary in this species. However, our results have shown that pre-chilling treatment enhances rate of germination significantly at 20°C and 25°C. Our results are in conformation with Barnett (1971) who found that soaking of seeds in aerated water, promotes the germination of non-dormant seeds of South Pine. There are significant differences in germination between 15 days pre-chilled and un-chilled seeds of chirpine at 20°C and 25°C. Similar results were reported by Wang and Berjak (2000) between 14 days pre-chilled and un-chilled seeds of *Picea mariana*, which were subjected to different constant temperatures. Jones and Gosling (1994) and Jinks and Jones (1996) also reported that for the shallowly-dormant seeds of Douglas fir (*Pseudotsuga menziesii*), Lodgepole pine (*Pinus contorta*) and Sitka spruce (*Picea sitchensis*), moist-chilling is a requirement to alleviate dormancy. The beneficial effects of germination speed or rate (as a result of cold stratification), on quality and quantity nursery-grown seedlings were reported by Venator (1973) in Caribbean pine (*Pinus caribaea*), Mexal (1980) and Barnett and McLemore (1984)

in Loblolly pine (*Pinus taeda*), and Logan and Pollard (1979) in Japanese larch (*Larix kaempferi*).

Although, the moist-chilling for 15 days did improve the rate and percentage of germination of the non-dormant chirpine seeds at 20°C and 25°C for over 21 days, but contradictory results were observed for seeds germinated at 30°C temperature. Pre-chilling treatment reduced germination percentage in most of the provenances at 30°C, whereas in remaining provenances there was not much difference in germination percentage between pre-chilled and un-chilled seeds at 30°C.

The effect of moist-chilling on the activation of germination in the chirpine seeds has not been previously reported and, therefore, is a new facet in understanding the benefits of short-term maintenance of seeds in a moistened condition at 3°C. The present results substantiate the beneficial effects of cold stratification on the release of dormancy and enhancing the rate of germination of chirpine seeds, which can be used to raise nursery for large scale afforestation programmes.

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Veterinary Ethno-Medicinal Plants in Uttarakhand Himalayan Region

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Abstract: Drug research has enriched human life in many ways. The health care and resulting social and economic benefits of new drugs to society are most remarkable, are quite well recognized. Drug research has been the driving force for many basic scientific developments, such as that of many new synthetic methods, of the understanding of the physiology and pharmacology of biological systems and has contributed much too molecular recognition. The Uttarakhand Himalayas have a great wealth of medicinal plants and traditional medicinal knowledge. The medicinal plant that has been widely used as veterinary ethno-medicine in Uttarakhand(India) region has been studied. These do not either occur elsewhere or have not so far been exploited commercially. Attempts have been made to explore the new possible species having medicinal importance especially for veterinary and to grow them in suitable areas so as to meet national industrial demands. The present paper deals with the traditional uses of 100 plant species employed in ethno-medicine and ethno-veterinary practice in Uttarakhand(India) Himalayan region.
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Key Words: Ethno-Medicinal Plants, Traditional knowledge, Uttarakhand Himalaya, Veterinary

1.Introduction

The Himalayas have a great wealth of medicinal plants and traditional medicinal knowledge. The Central Himalayan Region covers the new state of India, provides excellent opportunities for studying the Traditional Knowledge Systems. The Indian Himalayan region alone supports about 18,440 species of plants (Angiosperms: 8000 spp., Gymnosperm: 44 spp., Pteridophytes: 600 spp., Bryophytes: 1736 spp., Lichens: 1159 spp. and Fungi: 6900 spp.) of which about 45% are having medicinal properties. According to Samant *et al.*, out of the total species of vascular plants, 1748 spp. species are medicinal. Uttarakhand is a storehouse of a rich variety herbs and medicinal and aromatic plant species. The Government intends to exploit this advantage. Uttarakhand has observed an increase in the area under cultivation of aromatic and medicinal plants. The number of farmers engaged in cultivation of aromatic plants in Uttarakhand has dramatically increased from 301 in 2003-04 to 2714 in 2006-2007 and the area under aromatic plants has increased ten fold.

2. Traditional Knowledge

United Nations University proposal defines Traditional Knowledge System (TKS) as "Traditional Knowledge or 'local knowledge' is a record of human achievement in comprehending the complexities of life and survival in often unfriendly environments. Traditional knowledge may be technical, social, organizational, or cultural was obtained as part of the great human experiment of survival and development." Traditional knowledge provides the basis for problem-solving strategies for local communities, especially the poor. Traditional Himalayan medicine is a good example of TKS, which has affected the lives of poor people around the globe. TKS is of particular relevance to the poor in the following sectors: agriculture, animal husbandry and ethnic veterinary medicine, management of natural resources, primary health care (PHC) and preventive medicine, psycho-social care, saving and lending, community development, poverty alleviation, etc. According to an estimate of the World Health Organization, approximately

80% of the people in developing countries depend on traditional medicine for primary health care needs; a major portion of these involves the use of medicinal plants.

The Traditional Himalayan Medicine System (THMS) is a living example of TKS where small communities fight even incurable diseases through the traditional methods. They also cure their animals through these traditional methods. These traditional methods are totally oral and non-documented. They use generally herbal products like resin, bark, root, leaves, fruits etc., minerals, animal products and *tantric* practices. For millennia human societies have been depending on plants and plant products for various remedies. In certain areas these folk medical prescriptions are endemic and have survived through ages from one generation to the next through the word of mouth. They do not exist as written knowledge. Generally these systems of medicine depend on old people's experiences. Indigenous systems of medicine are specially conditioned by the cultural heritage and myths.

2.1 History of medicine

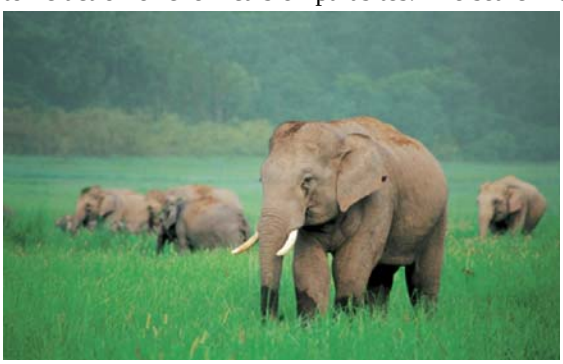
Search for drugs to improve the quality of life and cure diseases has been a part of human life right from its beginning. In many of the well developed ancient civilizations this knowledge was evaluated, codified, recorded and formed an essential part of the texts of their traditional systems of medicine, such as *Ayurveda* in India. Drug research is a well structured and organized endeavor. The starting point of the story of modern era of drug research could actually be the observation by Paul Ehrlich around the turn of last century that dye stuffs stain some cells selectively and destroy them. He exploited this idea and increased the toxicity of a dye towards a pathogen by introducing a toxic element like arsenic leading to the antisyphilis agent 'salvarasan' in 1907, the first designed drug and coined the word 'chemotherapy' for this selective toxic action of chemicals on parasites. The search for Domagk for azo dyes that might be effective antibacterial agents ultimately/resulted in 1935 in the discovery of prontosil which protected mice against lethal streptococcal infections leading to widespread clinical use of a variety of sulphonamides for a wide range of bacterial infections. This was the beginning of the modern era of chemotherapy. The discovery of the powerful antimicrobial activity of a 'penicillium notatum' by Flaming in 1928, followed by isolation by Florey in early 1940's highlighted the microbes as an important source of new drugs and of molecular diversity, and the interest in this resource has continued unabated ever since. The demonstration in early 1950s of the tranquillising and hypotensive activity of Reserpine obtained from 'Rauwolfia Sepentina' a drug commonly used in traditional systems of medicine for insanity, focussed attention on plant especially those used in traditional system of medicines.

2.2 Himalayan Therapies

In Uttarakhand(India), people uses magico-religious therapies as Bhbhuti, Tantra-mantra and Jagar to placate the local gods and supernatural powers but in natural therapies , like Ayurveda they use herbal products. According to the mode of application, the natural therapies have three categories:

1. Herbal products used in systematized system of medicine like Ayurveda, Siddha.
2. Herbal products used in ethno-medicine or indigenous medicine like HMS based on oral tradition.
3. Herbal products used in modern medicine, based on active chemical principles of the herbal products.

Despite significance development of rural health services, village people still use herbal folk medicines to a good extent for treatment of common ailments like cough, cold and fever, headache and body-ache, constipation and dysentery, burns, cuts and scalds, boils, ulcers, skin diseases and respiratory troubles and others.



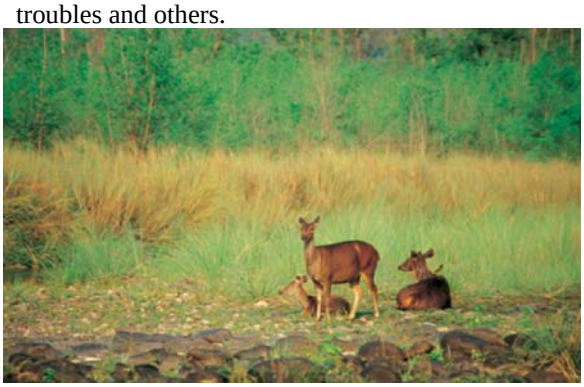


Figure 1. Showing Elephants and Dear in Jim-Corbet park at Uttarakhand(India)

2.3 Challenges

The hill districts of Uttarakhand have tremendous potential. The vast natural resources add to the state's attractiveness as an investment destination, especially for tourism(Jim Corbett park, Uttarakhand is especially rich with elephants and Dear in Fig. 1) and agriculture and forest- based industries. Horses and mules are the backbone of the rural transport system in Uttarakhand(Fig.2). Sheep-rearing for wool can be good opportunity for alternative livelihood, whereas Yak which is used for tourism in few districts of Uttrakhand. Buffaloes are the main milch animals, contributing 62 per cent in milk production.(State Focus Paper 2006-07, NABARD). Attempts should, therefore, be made to explore the new possible species having medicinal importance especially for veterinary and to grow them in suitable areas so as to meet national demands. It is now well established that one major potential area, amongst some others where botanist can make a positive contribution, in the field of molecular medicines and drug research, is that of topological and topographical analyses and system analysis. Development of such analyses leads to a fundamental understanding of the mechanisms of action of biochemically important compounds, including their side effects.

Figure 1. Horses are the backbone of the rural transport system in Uttarakhand(India)

3. Discussion

Table includes the medicinal plants that have been widely used as veterinary ethno-medicine in Uttarakhand region. Such aromatic plants which occur locally in the Uttarakhand himalaya and their medicinal importance for veterinary. These do not either occur elsewhere or have not so far been exploited commercially.

Table: Ethno-Medicinal Plants used as Veterinary Medicine

S. No	Botanical Name	Local Name	Parts Used	Uses	Mode of treatment
1	Capsella *bursa-pastoris*, Moench.	Torighash	Whole plant	For Sikka Rog	Two palmful whole plant decoction in water given two times for vigor
2	Cardamine *impatiens*, Linn.	-	Whole plant	For Tantrka in calf	One palmful whole plant decoction in one liter water given two times for vigor
3	Viola *biflora*, Linn.	Banpansa	Whole plant	In calf for heart & faint problem	Two palmful whole plant two times a day for attack. Three/four parts of two palmful whole plant & a spoon honey given two times for heart & skin problem
4	Viola *patrinii*, DC	-	Root	For liver	Two palmful root decoction in one liter water given two times for vigor

5	V.*serpens*, Wall.	-	Root	For Liver	Two palmful root decoction in one liter water given three times with honey
6	Hypericum *cernum*,	Vaya, Culi	Whole plant	For Hoskins, For wound	Two palmful whole plant decoction in one liter water given two times for vigor
7	Linum *usitatissinum*,Linn.	Alsi	Whole plant	For strength	Two palmful whole plant decoction in 1& 1/4 liter water given two times for strength
8	Melilotus *alba*, Lamk.	Banmethi	Whole plant	For stomach problem and Indigestion	One palmful whole plant given three times in a day for vigor
9	Trifolium *repens*, Linn.	Garila	Whole plant	For Satrika	Four palmful whole plant given two times a day
10	Agrimonia *pilosa*, Ledeb.	Kafliya	Whole plant	For purification of blood	Half palmful whole plant decoction in three/ four liter water given one fourth part with gur in morning
11	Fragaria *vesca*, Linn.	Pudalia Kafal	Leaf	To protect abortion	Two palmful leaves given daily
12	Potentilla *argyrophylla*,	Danti, Brajdanti	Leaf/Root	For stomach problem	One palmful leaves/two matured root decoction in 3/4 liter water given thrice in a day.
13	Rhamnus *virgata*, Roxb.	Chaitula	Fruit	In Leg swelling	Five matured fruit decoction in ¼ liter water given daily
14	Rosa *moschata*, Herrm.	Kunj pani	Fruit	For leucorrhea, bleeding,Pregnancy termination.	Two palmful fruit with one spoon honey given daily
15	Rubus *paniculatus*,Sm.	Kala Hisalu(Kadula)	Leaf	In pregnancy	Two palmful leaves decoction in ½ liter water given its one cup twice a day
16	R. *lasiocarpus*, Sm.	Kala Hisalu	Leaf	In pregnancy	Leaf is useful for cow specially in pregnancy pain
17	Bergenia *ciliata*, Moench.	Silfhora	Root	For Hydrophobia	Two palmful root decoction in ½ liter water given its one cup thrice a day
18	Ribes *grossularia*,Linn.	Caktu	Whole plant	For preventing abortion	One palmful whole plant given daily
19	Punica *granatum*, Linn.	Darim	Skull of fruit	As antimicrobials	One palmful skull of fruit decoction in ½ liter water given its one cup three times a day with gur
20	Woodfordia *floribunda*, Salisb.	Dhow	Flower	As energy syrup	One palmful dry flower decoction in water is useful for animals
21	Centella *asiatica*, (Linn.)Urban	Brahmi	Leaf	For brain fever	Apply Paste of green leaves on forehead during fever
22	Cuminum *cyminum*, L.	Jeera	Seed	For indigestion	One palmful seed in ¼ liter water given daily
23	Foeniculum *vulgare*, Mill.	Saup	Seed	For Hookworm	One palmful seed in 1/8 liter water given before morning meal
24	Pimpinella *diversifolia*, Dc	Dhanjari	Seed	For Lactation	One palmful seed given daily
25	Abina *cordifolia*, Hook. F	Haldu	Bud& leaf	For Wound & fever	Applying paste of new bud on the wound. Decoction of leaves in ½ liter water given thrice a day in fever
26	Valeriana *hardwichii*, wall.	Samyo, Dhup	Root	For titaini	Four matured root decoction in two liter water given ¼ liter twice a day
27	Aesculus *indica*, Colebr.	Pangar	Fruit	In stomach problem	One palmful fruit decoction in ½ liter water given with gur
28	Artemisia	-	Bud/Leaf	For Indigestion	One palmful bud/leaves decoction in one liter water

	maritime,Linn.				given one cup daily
29	A. *nilagirica*, Pampanini.	Patti,Kunj	Whole Plant	For urinary tract infection	One palmful whole body decoction in one liter water given one cup with gur
30	Artemisia *parviflora*, Roxb.	Patti, Dhopani	Leaf/ Bud	For round worm	One palmful leaves/ bud decoction in a liter water given 1/8 liter in one hour interval
31	A. *sacrorum*, Ladeb.	Kapar Patti,Jholp atti	Leaf/Bud	For hair fall	One palmful leaves & bud decoction in two liter water given one cup twice a daily
32	Senecio *chrysanthemoides*, DC.	Ratpatia	Whole plant	For skin disease	Two palmful whole plant decoction in 3/4 liter water given one cup daily
33	S. *rufinervis*, DC.	-	Seed	For wound	Three palmful seed given twice a daily
34	G. *pretense*, Linn.	Chalmori	Whole plant	In fever, urine problem, eye problem	Two palmful whole plant decoction in 3 /4 liter water given one spoon thrice daily
35	Tanacetum *nubigenum*, Wall.	-	Leaf/ Fruit	As energy syrup, anti microbes.	One palmful leaves/ fruit decoction in one liter water given one spoon with honey
36	Lobelia *pyramidalis*, Wall.	Bran tambacoo	Whole Plant	For liver disease	Two palmful whole body decoction in 3 / 4 liter water given one spoon with honey thrice a daily
37	Anagallis *arvensis*, Linn.	Vish Khaparia	Fruit/Leaf	As pain killer	Two palmful fruit/ leaves given daily
38	Primula *denticulate*, Smith.	Vish Khaparia	Fruit	In cough, useful for mammary glands	Two palmful flower given with gur
39	P. *macrophylla*, D. Don.	-	Whole Plant	As painkiller	This plant works as painkiller
40	Holarrhena *antidysenterica*, Wall.	Quiar, Indraw	Seed & bark	In fever, Gastric & dysentery	One palmful powder of bark/ seed decoction in one liter water given one cup with gur
41	Calotropis *procera*, R. Br.	Ank	Root	In indigestion	One palmful powder of root decoction in one liter water given one cup twice a day
42	Gentiana *tenella,(Roltb*) H. Smith.	Kutuki, Katuwi	Fruit	In hysteria, In weakness	25g of bark of fruits decoction in one liter water given one cup with honey per day
43	Swertia *purpurascens*, Wall.	Ciraita	Whole Plant	In fever, In weak appetite.	Two palmful whole plant decoction in one liter water given one cup thrice a day
44	Capsicum *annum*, Linn.	Khusane, Marac	Fruit	As oil massage.	One palmful fruit decoction in three liter water gives one cup twice a day
45	Datura *metal*, Linn.	Dhatura	Seed	As pain killer (for external use only)	25g roasted seed in one liter oil is used for massage
46	Hyoscyamus *niger*, Linn.	Bran juwan	Leaf , Seed	As pain killer	Paste of leaves and seed is used as ointment
47	Digitalis *purpurea*,Linn.	Prawasit Degitelis tilpushpi	Leaf	In burning	One palmful leaves is roast with oil is used as ointment
48	Verbascum *thapsus*, Linn.	Akalvir	Leaf	In bronchitis	One palmful leaves decoction in 3/4 liter water given one cup thrice a day
49	Clerodendrum *infortunatum*, Gaertn.	Aranyo	Bark	In Efra	Powdered bark decoction in 2 liter water given one cup thrice a day
50	Ajuga *parviflora*, Benth.	Ratpatia	Whole plant	In arthritis	One palmful whole plant decoction in 3/4 liter water given one cup daily

51	Mentha *arvensis*, Linn.	Pudina, Eliachi	Whole plant	In post pregnancy problems	Two palmful whole plant decoction in a liter water given ¼ part thrice a day
52	Ocimum *sanctum*, Linn.	Tulsi	Whole plant	In fever	Two palmful whole plant twice a day
53	Origanum *vulgare*, Linn.	Jangali tulsi	Whole plant	Indigestion	Four palmful whole plant with fibrous food twice a day
54	Salvia *lanata*, Roxb.	Sania, Sunip	Whole plant	For vomiting, painkiller	Two palmful whole plant with gur and fibrous food thrice a day
55	Scutellaria *angulosa*, Benth.	Karuijhar	Whole plant	In acidity	One palmful whole plant decoction in ½ liter water given one spoon with honey thrice a day
56	Thymus *serpyllum*, Linn.	Van ajmain	Whole plant	In chest pain	One palmful whole plant decoction in ½ liter water given one cup twice a day
57	Plantago *major*, Linn.	Vrantank	Leaf	In Injury, teeth problem, fever	Paste of leaves in water useful for injury & teeth pain. Two bunch of leaves decoction in one liter water given 1/6 part thrice a day for fever
58	P. *orata*, Forsk.	Esabgol	Seed	In dysentery	One palmful seed in ½ liter water makes a semisolid paste given thrice a day
59	Boerhaavia *diffusa*, Linn.	Parnata	Leaf	In blood dysentery, In dropsy	Juice of leaves thrice a day
60	Achyranthes *aspera*, Linn.	Chirchira	Whole plant	For teeth problem	One palmful whole plant in ½ liter water is useful in teeth problem
61	A. *bidentata*, Blume.	Dansh	Root	As Laxative	One palmful root decoction in one liter water given two times for vigor
62	Chenopodium *album*, Linn.	Bethuwa	Leaf/ seed	For worm	Two palmful seed is given before breakfast
63	Rheum *emodi*, Wall.	Dolu, Archa	Root	For blood purification, for energy	One matured root decoction in one liter water given three times for vigor
64	Rumex *hastatus*, D. Don	Bhilmora	Whole plant	For skin disease, In fever	One palmful whole plant decoction in 3 / 4 liter water given one cup thrice a day
65	Piper *longum*, L.	Pipal	fruit	In Low appetite, As oil massage	Powder of fruit is useful for low appetite. Oil with powder massage is useful
66	Cinnamomum *tamala*, Ness.	Kiriya, karkiriya, Dalchini	Leaf	In stomach problem, in gastric problem	Powder of Leaves and bark with half palmful fiber food is useful
67	Litsaea *polyantha*, Juss.	Cirira	Leaf	In injury	Powder of bark & leaves in cold water as ointment
68	L. *umbrosa*, Ness.	Circira	Leaf	In bone injury	Paste of leaves in water as ointment in bone injury
69	Viscum *album*, Linn.	Bana	Fruit	In pregnancy problem	Six fruits with milk twice a day
70	Emblica *officinalis*,Gaertn.	Aula, Awla	Fruit	In eye disease/ good health	Two palmful fruits powder with fibrous food
71	Euphorbia *prolifera*, Buch. Ham.,ex.Don.	Duwila	Fruit	Used in dog bite	Powder of fruit is useful
72	Mallotus *philippinensis*, Muell. & Arg.	Roli, Kasela	Fruit	To protect from worm	Fruit extract with one palmful fibrous food is given once a day
73	Ricinus *communis*, Linn.	Erind	Leaf	For internal injury	Oil of this plant is useful. Use of leaves in heat therapy

74	Betula *utilis*,Don.	Bhuj, Bhojpatra	Seed	To protect from worm	Two small pinch is useful
75	Quercus *dilatata*, Lindl.	Banj	Bark	In dysentery	Two palmful powder of bark decoction in one liter water given one cup twice a day
76	Q. *semecarpifolia*, Sm.	-	Bark	In dysentery	Two palmful bark powder decoction in one cup water given twice a day
77	Salix *elegans*, Wall.	Garbainsh	Fruit	In rickets	Three palmful fruits decoction in one liter water given one cup thrice a day
78	Ephedra *gerardiana*, Wall.	Gidjing	Stem	In pain	One bunch of stem pieces decoction in 2 liter water given one cup in early morning
79	Juniperus *communis* ,Linn.	Jhora, khichiya	Fruit	In liver disease	Twelve fruits daily
80	Abies *webbiana*, Lindl.	Raisal barmi radha	Bud	In Cough	One palmful bud decoction in 3 liter water given thrice a day
81	Cassoa *absus*, Linn.	Banar, Chakwar	Seed	In urine problem	One palmful seeds decoction in ½ liter water given one cup thrice a day
82	Satyrium nepalense, D. Don.	-	Root	As tonic	Two palmful roots decoction in 3 / 4 liter water given ½ parts twice a day
83	Zingiber *officinals*	Banhaldi	Root	Internal injury, As anti worm	Paste of root
84	Cureuma *angustifolia*, Roxb.	Banhaldi	Root	In gastric problem, anti worm	Paste of root
85	Acorus *calamus*, Linn.	Banj	Root	Fever, pain	Two matured root with fibrous food given daily
86	Allium *stracheyi*,Baker.	Jambu	Whole Plant	For stomach problem	Two palmful whole plant given thrice a day
87	Allium *wallichii*, Kunth.	Jangali Lasun	Root	In infection	Two node given daily
88	Asparagus *racemosus*, willd.	Kairuwa	Bud	In liver problem & To enhance Lactation	One palmful bud given twice a day
89	Aloe *vera*, Linn.	Patquar	Leaf	Stomach problem	Juice of leaves given ½ cup a day
90	Adiantum *venusthum*,G. Don.	Hanshraj	Seed	For Chest problem and hair fall	One palmful seed given with fibrous food
91	Equisetum *arvense*, Linn.	Horsetel	Whole plant	For urinary problem	Half palmful whole plant decoction in one liter water given
92	Althaea *officinalis*, Linn.	Jangalihaul i	Root	For termination of pregnancy	Three/ four matured root decoction in one liter water is given
93	Reinwardtia *trigyna*, Planch.	Pyuli	Root	In wound	One bunch of root decoction in ½ liter water given one cup in a gap of two days
94	Tagetes *arecta*, Linn.	Hazari	Fruit	In vomiting, In healing wound	One palmful fruit is given with fibrous food at the time of vomiting. Its external use is in filling wound
95	Calendula *officinalis*, Linn.	Ganda(Tok ar)	Leaf	In bleeding	Juice of leaves is helping in bleeding
96	Atropa *belladonna*, Linn.	Dhatur Jahar	Leaf	In injury as pain killer	Paste of one palmful leaves burns in oil acts as ointment
97	Datura *stramonium*, Linn.	Dhatura	Leaf	In injury as pain killer	Paste of one palmful leaves acts as ointment
98	Urtica *dioica*, Linn.	Sisauna	Leaf	Skin disease, For	One palmful leaves is given with fibrous food in 1h

				lactation	interval
99	Juglans *regia*, Linn.	Akhore	Leaf/ fruit	In stomach problem, As anti worm	Two palmful leaves or two green fruits decoction in 1 liter water is given one cup with two spoon honey thrice a day
100	Hedychium spicatum,Ham.ex. Smith	Kapur Kachari	Root	For fever & cough	Root is given with gur
101	Canna *indica*, Linn.	Kewara	Root	In disinterest, In afra	Powder of one bunch of root is given with gur
102	Anemona *obtusiloba* Don.	Kakaria	Leaf	In sinus	A cotton bud is made of Paste of leaves with Ghee for cleaning sinus
103	Delphinium *denudatum*, Wall	Nirwishi, Munel	Seed	In tics	One palmful seed decoction in ½ liter water is given
104	Aconitum balfouria, stapf.	Bishjahar	Root	In wound	One matured root burns in one liter oil gives a ointment
105	Paeonia *emodi*, Wall.	Bhoi Pawin	Root	In stomach problem	One matured root decoction in 3 / 4 liter water is given one cup with 100g gur thrice a day
106	Berberis *aristata*, DC	Kilmori	Root & stem	In fever, weakness	One palmful root/ stem decoction in ½ liter water given one cup daily
107	Fumaria *parviflora*, Lamk.	Pitpapara	Whole plant	In skin etching(disease)	One palmful whole plant decoction in one liter is given
108	Brassica *napus*, Linn.	Kali sarso	Seed	In poor appetite	Two palmful seed is given with fibrous food and gur twice a day
109	Geranium *ocellatum*, Camb.	Bhiljari	Whole plant	As insecticide	Four whole plant with fibrous food twice a day. Powder of whole plant is given as insecticide
110	Acacia *catechu*, Wild.	Khair	Stem	In Urine problem, dysentery	One palmful stem decoction in ½ liter water given one cup four times a day
111	Butea *frondosa*, Koen.	Dhank	Flower, Seed	As painkiller	Paste of flower and seed is given

3.1 Special emphasis is on R&D.

An integrated action plan has been drawn up for this purpose in coordination with the Government of India and other concerned agencies in the State and elsewhere in the country. R&D in the area of Medicinal Plants and commercial production of applications and formulations will be developed in conjunction with Research Institutions and reputed companies. A Medicinal and Aromatic Plants Export Zone has been set up covering seven districts of Uttarakhand and Specialized Herbal Parks are in the offing.

The salubrious climate, pollution free environment and the availability of a wide range of flora and fauna in the mountainous terrain, make Uttarakhand an ideal location for developing centres for alternative medicine and health care facilities. A significant portion of Uttarakhand is under forest cover (almost 70 percent). There is, thus, excellent potential for the development of forest resources based Industries in the State. In addition, there is ample scope to develop industries based on forest and agro-wastes such as lantana, pine needles, plant and vegetative fibres such as Rambans, etc.

4. Conclusion

Himalyan people have close relationship with nature. Generally, they believe that diseases are caused by the supernatural powers and they treat them through natural products like plants, herbs, trees, soil etc. Himalayan veterinary medicine system is totally non-systematized. The person, prescribing these medicines has no so-called scientific knowledge about the disease. So, discoveries coming from diverse backgrounds laid down the broad canvas for drug research to follow. Most of the basic concepts and approaches to modern drug discovery research were established. These developments aroused worldwide interest and offered great hope and prospects.

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Influence of Foliar Spray with Paclobutrazol and Ethepon on Growth and Photosynthetic Behavior of *Saussurea costus* (Falc.) Lipsch. - An Endangered Medicinal and Aromatic Herb

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Abstract: Paclobutrazol (PCB) and ethepon were applied as foliar sprays (25, 50, 100 ppm and 1.0 mM and 1.5 mM) to greenhouse-grown plants of *Saussurea costus* (Falc.) Lipsch. seedlings. Under stress conditions the growth of the PCB and ethepon treated seedlings was measured by leaf area, root length and root dry weight. A significant increment in root length was recorded for all treatments and showed a concentration-dependent pattern excluding for 50 ppm PCB treatment where the results were non-significant at 5% P level. Photosynthetic rate (A), transpiration rate (E) and stomatal conductance (g_S) were found decreased in all the treatments as compared to control. A decrease in fluorescence ratio (Fv /Fm) indicated lower photosynthetic efficiency. Based on the results of this study, we presume that the stress caused by PCB and ethepon probably contributes to some extent to the inferior gas exchange but an increase in root length. PCB had a more inhibitory effect than ethepon. [Nature and Science. 2009; 7(8):53-62]. (ISSN: 1545-0740).

Key words: Paclobutrazol, ethepon, root growth, photosynthetic rate (A), transpiration rate (E), stomatal conductance (g_S), endangered medicinal and aromatic plant.

1. Introduction

Saussurea costus (Falc.) Lipsch., locally known as Kuth, belong to family Asteraceae is a perennial aromatic herb. It is 1-2 m tall, with a thick fibrous stem. Leaves radical with long lobately winged stalks. Root stout, carrot like, 60 cm long, possessing a characteristic penetrating odour. Flowers are dark purple or black in colour. (Nayar and Shastry, 1988; Stainton, 1988). Samant et al., 1998 reported that it is distributed between 2500- 3000m asl in the Himalaya, and native to the Himalayan region. Its natural populations are reported from the higher elevations of Jammu and Kashmir and Himachal Pradesh (Aswal and Mehrotra, 1994) and now cultivated in Kashmir, Himachal Pradesh and in some part of Uttarakhand. The medicinal properties of *Saussurea costus* are well documented in different systems of medicine. Its roots have a strong and sweet aromatic odour with bitter taste, and used as antiseptic, in controlling bronchial asthma (Anonymous, 1972). It is also used for curing various diseases like dysentery, rheumatism, cholera, jaundice, cold, fever and stomachache etc. The oil is pale yellow to brownish in color and used in high grade perfumes and in the preparation of hair oil. Because of an endemic species to the Himalaya, the distribution of this species is quite restricted to extremely narrow geographical range (Siddique et al., 2001) which makes it more susceptible to extinction. Being an endangered species (Anonymous, 1972, Nayar and Shastry, 1987, 1988, 1990), enlisted in Appendix I of CITES (Convention of International Trade in Endangered Species of Wild Fauna and Flora), it has been prioritized among the species of high conservation concern (Ved et al., 2003). Its trade is strictly prohibited under Foreign trade Development Act-1992.

Paclobutrazol is a strong growth retardant used in many plants to control their growth and development (Hamid and Williams 1997, Gonzalez et al., 1999). Ethepon, an ethylene releasing compound has also demonstrated a capacity to modify plant growth (Muse and Holcomb 1997, Banon et al., 1998, Cardoso et al., 1998). Both paclobutrazol (Kim et al., 1999) and ethepon (Endres et al., 1999) have been shown to have an additional effect of modifying the color of the leaves of some plants, another aspect which may increase their ornamental value. Recently paclobutrazol has been shown to increase root growth of trees in certain landscape situations (Watson and Himelick 2004).

Photosynthesis is controlled by several intrinsic and extrinsic factors. Of these, plant hormones have received considerable attention in the past in photosynthetic responses of plants. Ethylene is a phytohormone that influences every aspact of plant growth and development (Abeles et al., 1992). It is synthesized by the activity of 1-aminocyclopropane carboxylic acid synthase (ACS). The response of plants to ethylene depends on the sensitivity of plants to the gas. Most of the work related to paclobutrazol and ethepon has been done on tree species. The response of growth retardant on photosynthetic behavior of

herbaceous plant species is still lacking. The present work is focused on the effect of different concentration of Paclobutrazol and ethepon on the photosynthetic parameters as Photosynthetic rate (A), stomatal conductance (g_S) and transpiration rate (E). The chlorophyll fluorescence ratio Fv/Fm is correlated with the efficiency of leaf photosynthesis and a decline in its ratio is a good indicator of photoinhibitory damage caused by incident photosynthetic photon flux density (PPFD) when plants are subjected to a wide range of environmental stresses (Bjorkman and Demming, 1987). Thus Chl a fluorescence parameter (Fv/Fm) was also determined for finding a possible relationship of Paclobutrazol and ethepon-mediated changes in foliar gas exchange and growth parameters of *Saussurea costus.*

2. Materials and methods

Seeds of *Saussurea costus* were sown in Styrofoam seedling trays in soil compositions of soil: sand: litter in (1:1:1, v.v.v) proportion during the month of October 2007 inside polyhouse at High Altitude Plant Physiology Research Centre (600 m asl), Srinagar, Garhwal, Uttarakhand. Onset of germination has taken place after 10 days of seed sown. Emergence of true leaf has taken place 21 days after onset of germination. The most homogeneous seedlings with 20 replicates for each treatment were selected for transplantation in polybags of size of 16 cm diameter and 13 cm height with same composition of soil i.e. soil: sand: litter in (1:1:1,v.v.v) proportion during the month of December 2007. The average air temperature inside polyhouse 12.87±2.23°C, minimum temperature 6.19±1.30°C, maximum temperature 16.26±3.59°C; average relative humidity percentage 53.00±5.50 and average soil temperature 12.32 ±2.56°C was recorded.

After one month of acclimatization inside polyhouse these seedlings were given different concentrations of growth retardants as for paclobutrazol [PCB] (25ppm, 50ppm and 100ppm) and for ethepon (1.0mM and 1.5mM). The foliar spray of these growth retardants were done with a hand sprayer. After 15 days of spraying, the growth and photosynthetic parameters were observed.

2.1 Measurement of growth parameters

Five plants for each treatment were dug out for observing the growth parameters such as leaf area (cm^2), root length (cm) and dry weight (mg) of economically important part i.e. root/rhizome. These plants were taken to laboratory, washed with running water. Further, all samples were dried at 80°C for 24 hours or until constant weight to measure dry weight in milligram per plant. Variation in leaf area, root length and dry weight in different treatments was analyzed using ANOVA.

2.2 Measurement of photosynthetic parameters

Photosynthetic rate (A), stomatal conductance (g_S), transpiration rate (E) and other parameters were measured using infrared gas analyzer (LCPro$^+$, ADC BioScientific Ltd., England.) on fully expanded uppermost leaves at saturation light intensity on five plants from each replicate. The atmospheric condition during experiment between 1100-1200 h were Photosynthetically active radiation (PAR) <400 $\mu molm^2s^{-1,}$ relative humidity 52% and temperature 21°C, atmospheric CO_2 concentration 380 $\mu molmol^{-1}$. Study of chlorophyll fluorescence parameters in different treatments was worked out using a Plant Efficiency Analyzer PEA (Haansatech ltd U.K.). Samples were illuminated homogeneously over an area of 4 mm diameter with an array of 6 LED (650 nm, 600 nm) after 30 minutes dark adaptation. Fluorescence signals were detected using PIN Photocell after passing through a long pass filter (50% transmission at 720 nm). Fluorescence transient were recorded from 10 MS- 1s with data acquisition rate or 10 MS from the first 2 MS and then at 1 MS data obtained from 5 independent measurements for each type of plant.

3. Results and discussion

3.1 Effect of paclobutrazol and ethepon treatment on growth

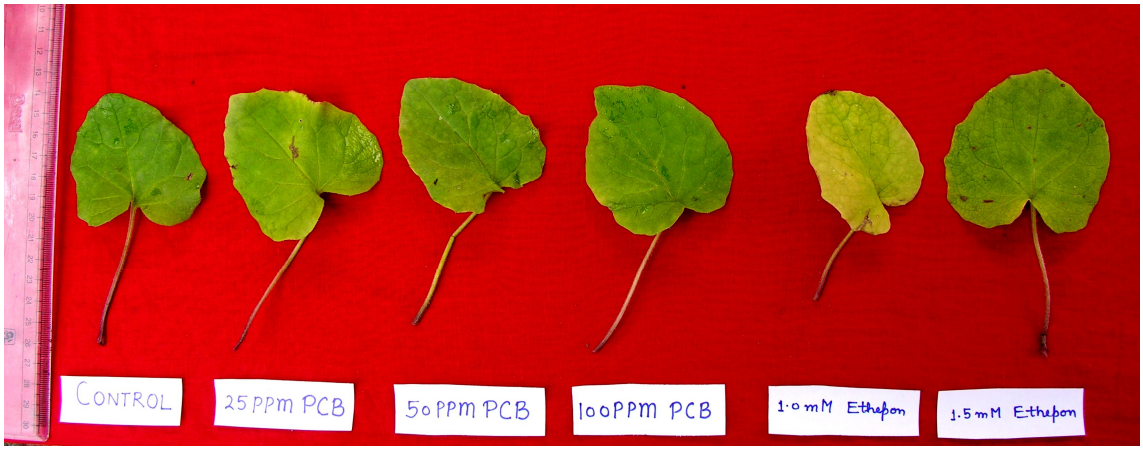
Seedlings of *Saussurea costus* exhibited typical characteristics of triazole treatment as reported in wheat seedlings earlier by other workers (Davis and Curry, 1991, Webb and Fletcher, 1996). There was a significant increase in tap roots of plants treated with 25 ppm PCB and 100 ppm PCB (33.60% and 31.03% respectively) whereas in case of 50 ppm PCB there a decrease of 2.91%. Ethepon treated plants have shown increase of 40.95% and 20.79% in tap root length for 1.0mM ethepon and 1.5 mM ethepon concentration respectively. In case of secondary root length, all treatments have shown significant enhancement in root length. Thus, results for root lengths were found significant for both tap and secondary roots except for tap roots of plants treated with 50ppm PCB (mechanism of which is unknown). (Plate 1-4).

Control 25ppm PCB 50ppm PCB 100 ppm PCB

Plate 1- Effect of paclobutrazol (PCB) treatment on whole plant of *Saussurea costus*

Control 1.0mM ethepon 1.5 mM ethepon

Plate 2- Effect of ethepon treatment on whole plant of *S. costus*



Control 25ppm PCB 50ppm PCB 100 ppm PCB 1.0mM ethepon 1.5 mM ethepon

Plate 3- Effect of PCB and ethepon treatment on leaves of *S. costus*

Control 25ppm PCB 50ppm PCB 100 ppm PCB 1.0mM ethepon 1.5 mM ethepon

Plate 4- Effect of PCB and ethepon treatment on roots of *S. costus*

Effects of foliar spray with paclobutrazol and ethepon on *Saussurea costus* were found non-significant for leaf area as maximum leaf area (352.56±10.50cm^2) was found in control plants, whereas the leaf area decreased in all other treatments as in case of paclobutrazol in 25ppm concentration the leaf area was 338.03±2.37cm^2 and in 50ppm the leaf area was 317.56±1.80 cm^2 but at higher concentration i.e. 100ppm there was a slight increase in leaf area i.e. 329.30±3.44 cm^2, whereas in ethepon treatment the leaf area was minimum (197.20±2.14 cm$^{2)}$ in 1.0mM ethepon. Contrary to this as the concentration of ethepon is increased to 1.5mM ethepon the leaf area increased to 277.96±1.93cm^2.

In overall result the application of PCB and ethepon treatment resulted in decrease in leaf area.

Increase in dry weight of roots have been shown in plants treated with 25ppm PCB concentration and 1.5mM ethepon whereas in other treatments data were found non-significant (Table 1).

Table1. Effect of paclobutrazol and ethepon treatment on growth of *S. costus*

Treatments	Leaf Area in cm^2	Root length (cm) Tap root	Secondary root	Dry weight (mg)
Control	352.56±10.50	4.80±0.26	12.33±0.29	412.33±2.52
25ppm PCB	338.03±2.37 ns	7.23±0.25*	26.76±0.25*	520.33±4.16*
50ppm PCB	317.56±1.80 ns	4.66±0.15 ns	23.50±0.50*	307.33±3.79 ns
100ppm PCB	329.30±3.44 ns	6.96±0.50*	19.66±0.67*	402.33±3.51 ns
1.0mM Ethepon	197.20±2.14 ns	8.13±0.32*	16.73±0.80*	268.66±6.51 ns
1.5mM Ethepon	277.96±1.93 ns	6.06±0.15*	15.30±0.26*	589.00±3.61*
F value LSD (P<0.05)	424.31, 4.95^+	64.72, 0.30^+	334.36, 0.51^+	2539.10, 4.31^+

* Significant, $^+$LSD, ns non-significant

3.2 Changes in photosynthetic parameters of plants treated with PCB and ethepon

In different treatments *viz.,* 25ppm, 50ppm, 100ppm PCB, 1.0mM and 1.5mM Ethepon the photosynthetic rate (A), transpiration rate (E) and stomatal conductance (g_s) were compared with the untreated plants *i.e.* control. In case of PCB treated plants as the concentration of PCB increased, photosynthetic rate decreased as compared to untreated plants. It was found maximum (8.57 $\mu molm^{-2}s^{-1}$) in control plants followed by 25 ppm PCB (7.68 $\mu molm^{-2}s^{-1}$), 50 ppm PCB (5.40 $\mu molm^{-2}s^{-1}$) and 100 ppm PCB (4.20 $\mu molm^{-2}s^{-1}$) (Figure 1). The data for photosynthetic rate were found negatively correlated with percent variation of 97.28. Conflicting results on the effects of ethylene releasing compounds on net photosynthetic rate (A) have been reported. It has been reported to increase A (Pua and Chi, 1993, Khan et al., 2000, 2003) or decrease it (Kays et al., 1980, Rajala et al. 2001). In this study for ethepon treated plants the photosynthetic rate was also found negatively correlated with a percent variation of 83.53 (Figure 2). There was a slight increase in photosynthetic rate at 1.5 mM ethepon concentration. These results were found against the results for crop plants where etehpon treatment resulted in an increase in photosynthetic rate due to ethepon treatment (Subrahmanyam et al., 1992: Pua and Chi 1993 and Khan et al., 2000). Khan et al., 2004 found that increasing concentration of ethepon upto1.5mM increased A, whereas 3mM ethepon concentration was proved inhibitory. But no definite result has been assigned for this. Transpiration rates as well as stomatal conductance have also shown negative correlation with PCB and Ethepon treatments but percent variation for E and gS were higher in ethepon treated plants *i.e.* 92.70 and 92.02 respectively as compared to PCB treated plants with 36.67 and 82.49 percent variation for E and g_S respectively (Figure 3-6).

It was established that paclobutrazol protect stress injury and a similar increase in plant growth parameters was observed earlier by Pinhero and Fletcher (1994). Fluorescence parameters (Fv/Fm) indicated that photosynthetic efficiency of PS II was decreased due to PCB and ethepon treatment as Fv/Fm ratio was found negatively correlated for PCB and ethepon treatments with percent variation of 97.21 and 93.94 respectively (Figure 7-8).

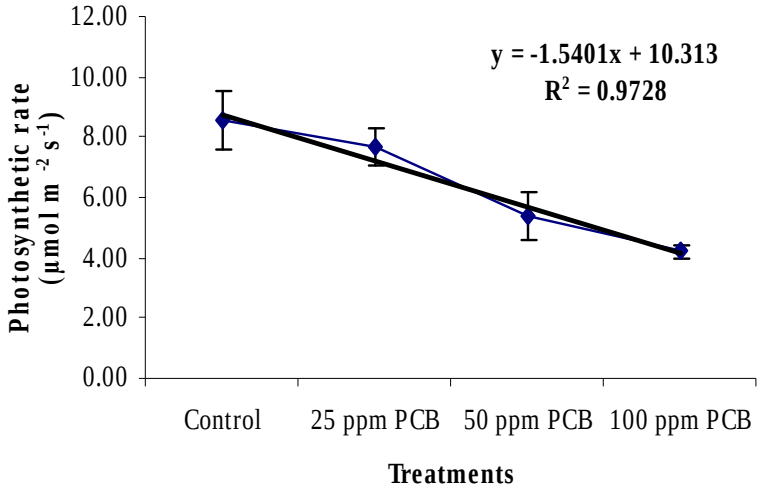


Figure 1- Effect of PCB treatment on Photosynthetic rate of *Saussurea costus*

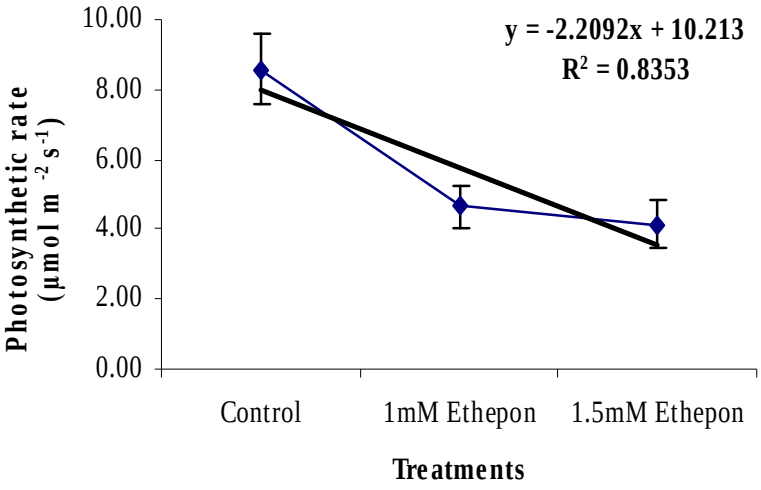


Figure 2- Effect of ethepon treatment on Photosynthetic rate of *S. costus*

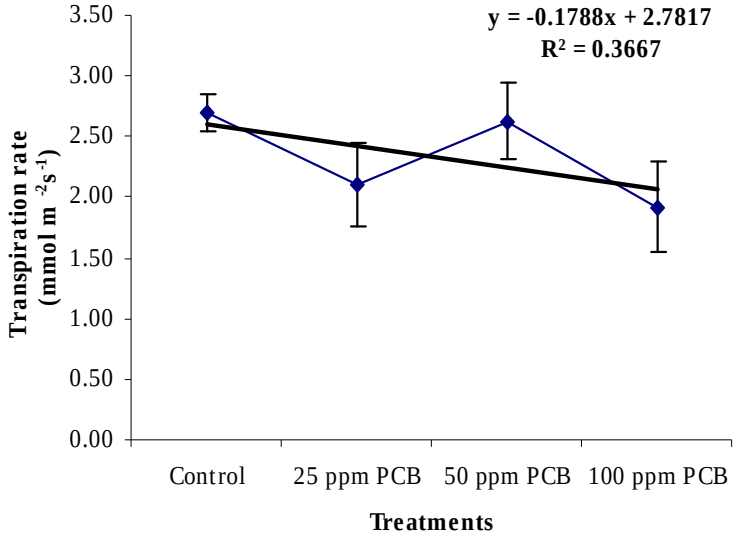


Figure 3- Effect of PCB treatment on Transpiartion rate of *S. costus*

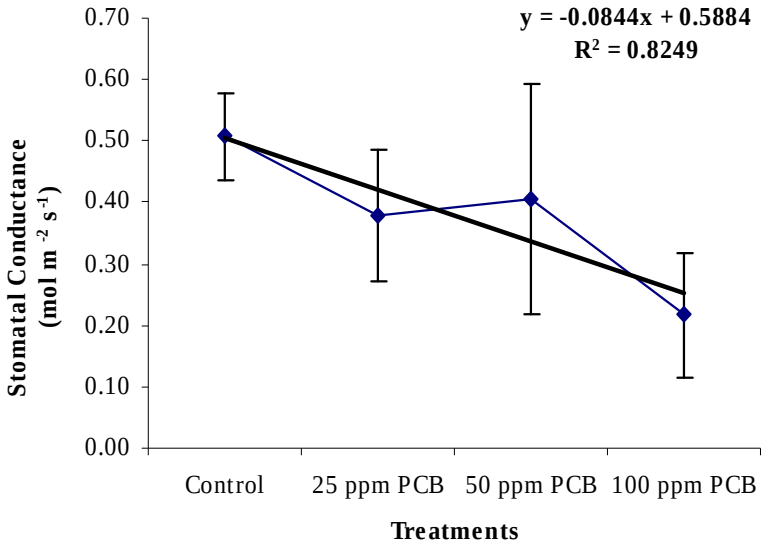


Figure 4- Effect of PCB treatment on Stomatal conductance of *S. costus*

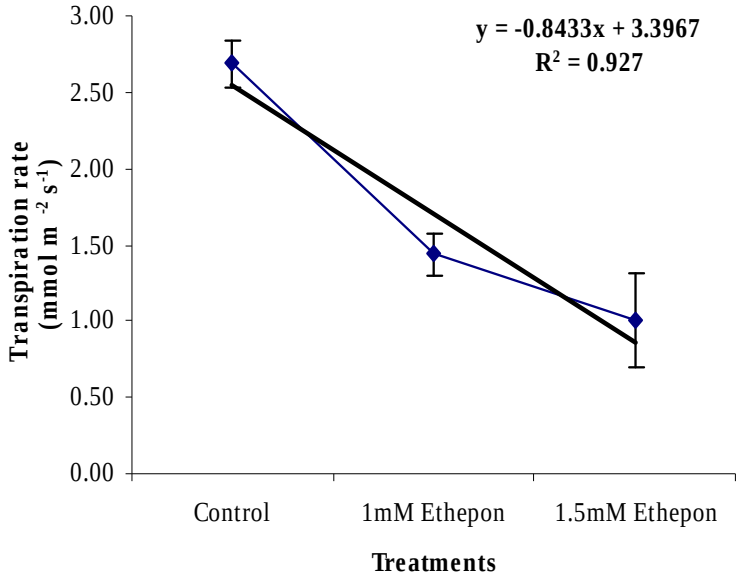


Figure 5- Effect of ethepon treatment on Transpiration rate of *S. costus*

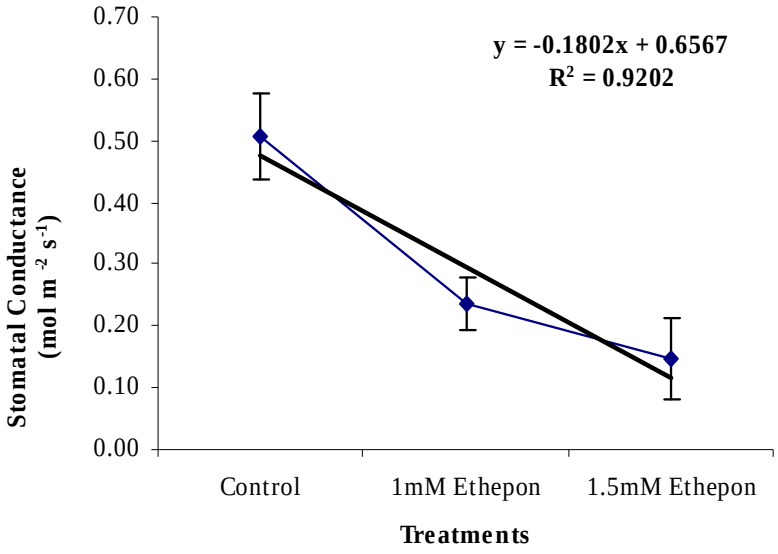


Figure 6- Effect of ethepon treatment on stomatal conductance of *S. costus*

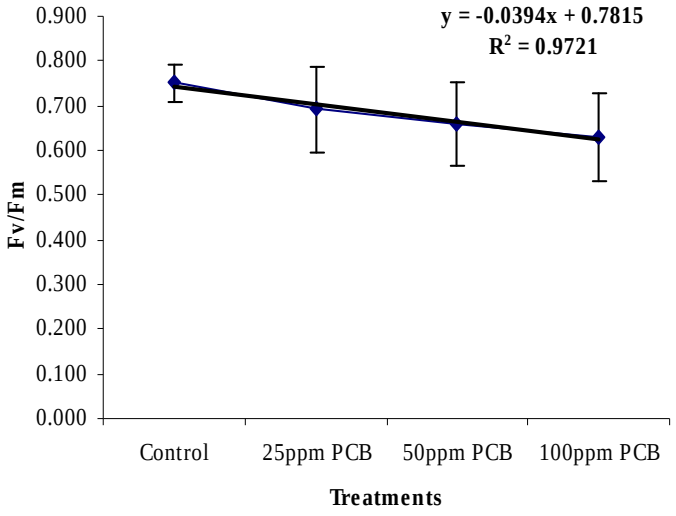


Figure 7- Effect of PCB treatment on fluorescence parameter (Fv/Fm) of *S. costus*

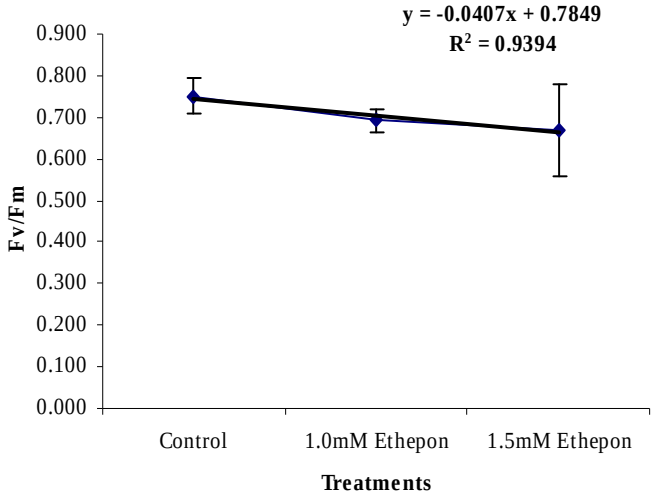


Figure 8- Effect of ethepon treatment on fluorescence parameter (Fv/Fm) of *S. costus*

The overall study shows that both PCB and ethepon affected the gaseous exchange in *Saussurea costus* which resulted in increase in root length. Photosynthetic rate (A), transpiration rate (E) and stomatal conductance (g_S) decreased in all the treatments as compared to control and a decrease in fluorescence ratio (Fv /Fm) indicated lower photosynthetic efficiency. Based on the results of these studies, we presume that the stress caused by PCB and ethepon probably contributes to inferior gas exchange.

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Effect of Enhanced Lead and Cadmium in soil on Physiological and Biochemical attributes of *Phaseolus vulgaris* L

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Abstract: *Phaseolus vulgaris* L. plants were grown in soil supplemented with different Pb and Cd concentrations (2, 4, 6, 8 gKg^{-1} for Lead and 1.5, 2.0, 2.5, 3.0 gKg^{-1} for Cadmium). Germination % was remained unaffected at low concentration of both metals as compared to control plants but at higher concentration of Cd i.e. 3g/Kg soil, germination was completely inhibited. Growth was also decreased as concentration of metals was increased as compared to control plants. Photosynthetic pigments, total soluble sugar, starch content as well as soluble protein content decreased as concentration of metals was increased in comparison of control plants. However, total free amino acid content and lipid peroxidation were increased with increasing concentration of heavy metals. Electrophoretic studies revealed that Acid phosphatase, Peoxidase and Esterase isoenzyme activities were increased with increasing concentration of heavy metals. Electrical conductivity of tissue leachates of leaves of *P. vulgaris* L. was recorded minimum in control plants while maximum was recorded in 8g Pb and in 2.5g/kg Cd treatments. Activity of antioxidative enzymes as ascorbate peroxidase (APX; EC 1.11.11), guaiacol peroxidase (GPX; EC 1.11.1.7) and glutathione reductase (GR; EC 1.6.4.2) was increased while Catalase (CAT; EC 1.11.1.6) activity decreased with increasing concentration of heavy metals. [Nature and Science. 2009; 7(8): 63-75]. (ISSN: 1545-0740).

Key words: Pb toxicity, Antioxidant enzymes, Physiological changes, biochemicals.

1. Introduction

Environmental deterioration has generated an increase of stress in all forms of life. Of these, stress on agricultural crops is of prime importance since agriculture is lifetime of global society. Abiotic stresses like water stress, salinity stress, and high temperature stress are known to adversely affect growth and grain yield of paddy (Pareek et al., 1999). Along these stresses, toxic heavy metal stress is an emerging and more dangerous stress for major crops. Metals like Pb, Hg, Cd, Ar, and Cr have no biological function and are toxic to life even at very low concentration (Salt et al., 1995). Pollution of soil and water due to toxic heavy metals is mostly of anthropogenic origin and there are many records that agricultural land adjacent to industrial areas are polluted to varied extent by many toxic heavy metals (Rao, 1979). Different plants absorb toxic and non-toxic metals from soil and water to varied extent and accumulate in different body parts (Chamber and Sidle, 1991).

Bioaccumulation of toxic heavy metals by various crop plants has been reported by number of workers and is a matter of serious health hazard (Mishra and Singh, 2000). Lead has long residence time in soil due to low solubility and strong binding capacity with soil colloids. In soil it accumulates and enters into food chains. Lead stress causes multiple direct & indirect effects on plant growth and metabolism (Balsberg, 1989).

The Reactive Oxygen Species (ROS) are produced in the young senecing leaf cells excessively under stressful conditions and are removed by complex non-enzymic (ascorbate, glutathione,α-tocopherol) and enzymic (CAT, APX, GPX, SOD, GR etc) antioxidant systems (Prochazkova et al., 2001). Plants have evolved antioxidant pathways that are usually sufficient to protect them from oxidative damage during periods of normal growth and moderate stress (Hauptmann and Cadenas, 1997).When severely stressed, however, the production of reactive oxygen species (ROS) can exceed the capacity of the antioxidant system to neutralize them and oxidative damage can occur. Heavy metals are known to induce free radical formation (Aust et al., 1985) and a consequent oxidative damage in senescing leaf cells under light (Panda and Patra, 2000). The aim of present study is to assess the effect of different concentrations of Lead and Cadmium in soil on physiological and biochemical attributes of *Phaseolus vulgaris* L. (French bean).

2. Materials and Methods

For the present study local cultivar of *Phaseolus vulgaris* L. (French bean) was selected to study the effect of selected heavy metals Lead and Cadmium. For

treatments, nitrate salts of lead & Cadmium Viz. Cadmium nitrate [Cd $(NO_3)_2$ $4H_2O$] and Lead nitrate [Pb $(NO_3)_2$] were used. Soil was collected from the field situated in the backyard of High Altitude Plant Physiology Research Centre. Soil, FYM (Farm Yard Manure) and sand were dried & thoroughly mixed at the ratio of 1:1:1 followed by thorough mixing of calculated amount metal salt. The amended soil was filled in polythene bags of size of 16 cm diameter and 13 cm height. Each bag was filled with 1 Kg of amended soil. The control set is comprised of un-amended soil.

2.1 Toxic metals Treatments

The different concentration of heavy metals chosen were 2, 4, 6, 8 gKg^{-1} for Lead and 1.5, 2.0, 2.5, 3.0 gKg^{-1} for Cadmium. For each treatment five replicates were maintained. After allowing bags filled with amended and unamended soil to stand for 7 days, seeds of *Phaseolus vulgaris* were sown. Watering was done at regular interval. The whole experiment was conducted in the glass house condition. Morphological and biochemical changes were studied at 15 days interval upto one and half months after initiation of heavy metal stress. Enzyme activity and changes in the polypeptides and isoenzymes were studied at final harvest.

2.2 Morphological observations

Morphological observations like root length, shoot length, leaf area, fresh and dry weight were recorded after each harvest. Leaf area was calculated using a graph paper.

2.3 Biochemical estimations

Fresh leaves were used for the estimation of chlorophyll and carotenoid contents (Holm, 1954), soluble sugars and starch (McCready et al., 1950), soluble proteins (Bradford, 1976) and total free amino acids (Moore and Stein, 1954).

2.4 Lipid Peroxidation

Lipid peroxidation in leaves was measured in terms of Malanodialdehyde (MDA), a product of lipid peroxidation content determined by the thiobaribituric acid (TBA), according to the method of Heath and Packer (1968) as modified by Dhindsa et al., (1981).

2.5 Electrical conductivity

For the estimation of leakage of cellular electrolytes, 100 mg of leaf tissue were incubated in 10 ml of deionised water for 6 h. The electrolyte in the tissue leachates were determined by measuring the electrical conductivity (EC) in a conductivity meter (Systronics) and the EC of tissue leachate was expressed in terms of $mS^{-1}g^{-1}$ dry mass (d.m.).

2.6 Electrophoretic analysis of polypeptides and Isoenzymes

Polypeptide pattern was analyzed on 10% SDS polyacrylamide gels using a BIO- RAD MiniPROTEAN II system. Isoenzymes esterase, peroxidase and acid phosphatase were analyzed on 10 % Polyacrylamide slab gels using a BIO- RAD MiniPROTEAN II system.

(i) Esterase: Esterase isozymes were detected on gels by the method described by Bhadula and Sawhney, 1987.

(ii) Peroxidase: Peroxidase isoenzymes were detected on gels by the method as described by Welter (1982).

(iii) Acid Phosphatase: Detection of acid Phosphatase isozymes on gels was done by method of Bhadula and Sawhney, 1987.

2.7 Spectrophotometric estimation of catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.11), guaiacol peroxidase (GPX; EC 1.11.1.7) and glutathione reductase (GR; EC 1.6.4.2) activities

2.7.1 Preparation of enzyme extract

Fresh leaves of 0.1 g fresh weight was homogenized at 4^o C in 5 ml extraction buffer [50mM potassium phosphate buffer (pH 7.0), 1% Triton X-100 and 7 mM 2-mercaptoethanol] with mortar and pestle. The homogenate was centrifuged at 25000 rpm for 20 min. and the supernatant was used as the crude extract for the APX, GPX and GR assay. However, for the estimation of CAT activity the extraction buffer was 0.05 M tris- HCl buffer (pH 7.5) 3 mM $MgCl_2$ and 1 mM EDTA. Protein content was determined using bovine serum albumin as a standard, according to the method of Bradford (1976).

2.7.2 Enzyme essay

CAT activity was assayed by measuring the rate of disappearance of H_2O_2 using method of Maehly and Chance (1959). **APX** activity was determined according to the method Chen and Asada (1989). **GPX** activity was determined according to Upadhyaya et al., (1985). **GR** activity was assayed by measuring the decrease in absorbance at 334 nm due to the oxidation of NADPH (Klapheck et al., 1990). All the enzyme activities were calculated and expressed as enzyme units per milligram of protein per minute.

3. Results and discussion

3.1Growth and Morphological changes

The germination-percentage was affected with different concentrations of Pb & Cd as compared with controls one. The results in relation to the effect of different concentrations of Pb & Cd on germination performance measured in terms of per cent germination were shown in Table 1. Germination declined with

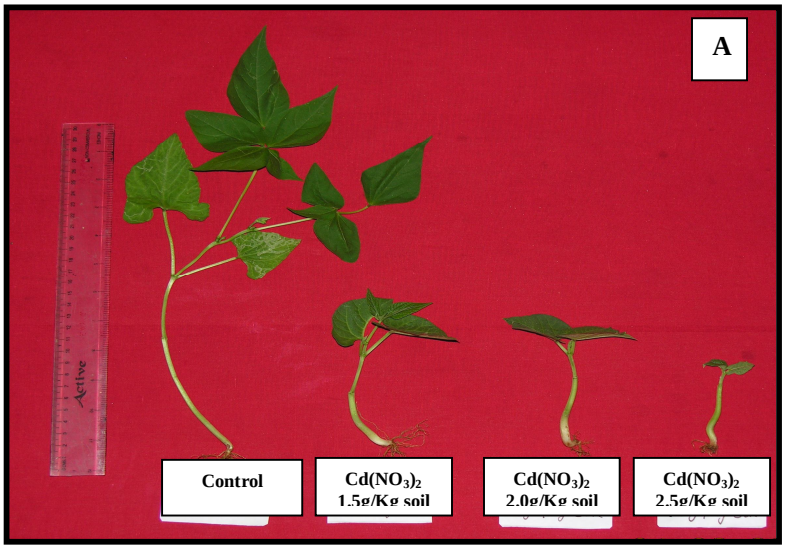
increasing concentration of both heavy metals in a concentration dependent manner. But at the highest concentration of Cd i.e. 3g/kg soil, germination was completely inhibited. For control plants percent germination was 67.71% (Table 1 and Figure 1).

During seed germination a number of hydrolyzing enzymes become active. These include amylases, proteases and lipases that hydrolyse polysaccharides, proteins & lipids respectively into their monomers. The stored materials in the cotyledons & the endosperm are hydrolysed and transferred to the growing embryo. This involves the activation & synthesis of several hydrolyzing enzymes (Bose et al., 1982). Inhibition of seed germination, hydrolyzing enzymes (amylase and proteases) and seedling growth treatment of a number of toxic salts, viz. Pb, Hg, Cu, Ni, Co etc. have been reported in many plants (Sharma et al., 1995; Jain et al., 1998).

Excess supply of heavy metals such as cobalt sulphate, nickel sulphate, sodium molybdate and sodium dichromate with nutrient solution caused depressed germination, length of radicles, lowered the mobilization of reserved materials from the cotyledons to the developing embryo axis and adversely affected a no. of respiratory enzymes in the case of green-gram (Kumar and Brisht, 1986).

Growth is the best indices for evaluating plant response to environmental stress Morphological studies in the present study had shown that minimum growth occurred in the plants in which Pb concentration was 8g/kg soil and for Cd it was 2.5g/kg soil when compared with control plants. Growth was not observed at the highest concentration of Cd i.e. 3g/kg soil. With increase in Pb and Cd concentrations, there was a gradual decrease in plant height. At different concentrations of lead, i.e. 2,4,6,8 g Pb / kg of soil, shoot length decreased by 37.98%, 55.81%, 61.24%, and 70.93% respectively. Root length was decreased 28.43%, 42.74%, 65.24%, and 87.73% respectively as compared to control. For different concentrations of cadmium i.e. 1.5, 2.0, 2.5 g Cd / kg of soil, shoot length decreased by 67.05%, 70.54%, 78.68% and root length by 46.83%, 57.06%, and 69.33% respectively. The more severe decrease in the length of roots may be due to their direct contact with lead and cadmium polluted soil. Similar observations have been observed on *Triticum sativum* and *Lens esculanta* by Mesmar and Jaber (1991). Seedling biomass (Fresh wt. and Dry wt.) also declined proportionately with increasing concentrations of both the heavy metals. Fresh weight decreased by 28.43%, 42.74%, 65.24%, 87.73% and dry weight by 26.67%, 46.67%, 53.33%, 87.78% respectively for different concentrations of Pb.

Xiong (1997) also reported progressive decline in plant dry weight with increasing concentrations of Pb in soil. Fresh weight of seedling at different concentrations of Cd decreases 54.09%, 62.07%, 82.76% and dry weight 75.56%, 78.89%, 81.11% respectively. Leaf area showed significant decline with increase in concentrations Pb and Cd. For different concentrations of Pb it was 16.87%, 56.27%, 68.62%, 79.78% and for Cd it is 22.96%, 63.47%, and 84.91% respectively. (Table 1)



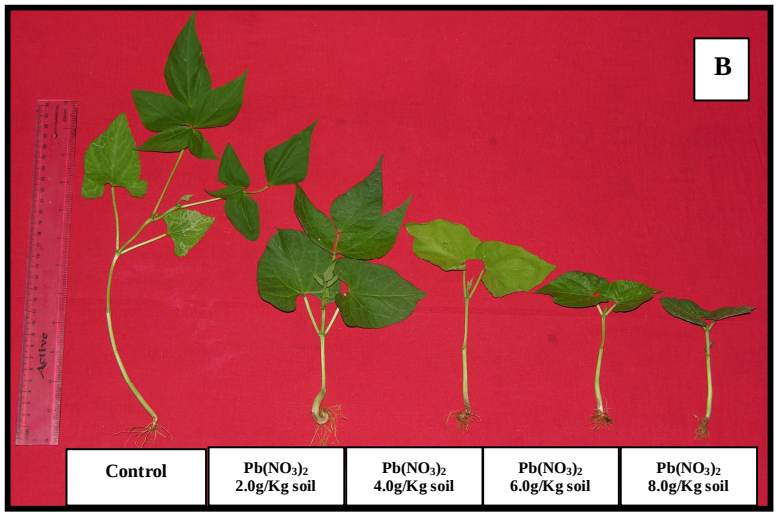


Figure 1.Comparative morphology of *P. vulgaris* seedlings grown under different metals (A, Cd; B, Pb) (after 20 days growth)

Table 1. Morphological changes in *P. vulgaris* grown under different metal concentrations

Treatments	Percent germination (%)	Shoot length (cm)	Root length (cm)	Fresh weight (gm)	Dry weight (gm)	Leaf area (cm^2)
Control	67.71	25.8	4.89	4.64	0.9	52.83
2g $Pb(NO_3)_2$	66.66	16	3.5	3.24	0.66	43.92
4g $Pb(NO_3)_2$	64.66	11.4	2.8	1.56	0.48	23.1
6g $Pb(NO_3)_2$	53.33	10	1.7	1.04	0.42	16.58
8g $Pb(NO_3)_2$	52.32	7.5	0.6	0.98	0.11	10.68
1.5g$Cd(NO_3)_2$	63.33	8.5	2.6	2.13	0.22	40.7
2.0g$Cd(NO_3)_2$	59.99	7.6	2.1	1.76	0.19	19.3
2.5g$Cd(NO_3)_2$	53.33	5.5	1.5	0.8	0.17	7.97
3.0g $Cd(NO_3)_2$	0	0	0	0	0	0

3.2 Biochemical changes

3.2.1Photosynthetic pigments

Along with the growth of plants under increasing concentration of heavy metals, the entire metabolic activity of the plants under metal stress was affected resulting in reduced metabolic activities. Under the metal stress, the levels of photosynthetic pigments, namely Chl. 'a' and Chl 'b' and Carotenoids decreases as the concentrations of Pb and Cd in soil increases. Chl. 'a' was more affected in comparison to carotenoids. At 2, 4, 6, 8 g Pb/kg in soil, Chl 'a' showed a decrease of 25.73%, 43.08%, 54.45%, and 68.23%, Chl 'b'; 21.62%, 41.98%, 49.64%, 64.76% and carotenoids 8.52%,

65.90%, 70.16%, 72.13% respectively. Same decreasing pattern showed with different concentrations of Cd. At 1.5, 2.0, 2.5 g Cd/kg soil, Chl 'a' decreases 21.25%, 40.71%, and 67.26%; Chl 'b' decreases 16.27%, 39.14%, and 63.12%; carotenoids 35.41%,52.46%,70.49% respectively (Figure 2). The decline in the levels of these pigments clearly shown the metal interference with pigment metabolism. Similar observations were made by Mukherji and Maitra (1976) in rice where Pb toxicity resulted in lowering Chl a/b ratio. Lead was found to inhibit δ amino levulinic acid dehydratase activity in mung bean resulting in a decrease in Chl. Content (Prasad and Prasad, 1987). Pb also distorts the membrane structure of chloroplasts, which ultimately leads to decrease in Chl. Content.

3.2.2 Carbohydrates

Soluble carbohydrate contents in plants decreased with increasing concentration of heavy metals. At the highest concentration of Pb i.e. 8g and Cd 2.5 g /kg soil, the carbohydrate content was minimum and maximum carbohydrate content was observed in control.

Soluble sugar: Effect of both heavy metals, Pb and Cd on the sugar content of leaves showed that there was a decrease of 1.85%, 14.68%, 17.43% and 26.45% in sugar content at different concentrations of Pb whereas 2.50%, 13.82% and 18.29% decrease at different concentration of Cd respectively (Figure 3).

Starch: Starch content of leaves decreases 7.09%, 27.90%, 34.98% and 50.22% at different concentration of Pb and 41.93%, 46.48%, and 50.85% at different concentrations of Cd (Figure 4).

3.2.3 Soluble protein content

Similar results as in the carbohydrate content were found for soluble protein content as minimum protein content was recorded in plants at the highest concentration of both heavy metals. Toxicity of Pb & Cd altered the protein content of leaves. There was a decrease of 3.56%, 26.92%, 33.69% and 57.69% at Pb concentrations and 14.48%, 18.62% and 23.17% at Cd concentrations (Figure 5). Kastori et al., (1992) reported in *Helianthus annus* that content of soluble proteins decreased with high concentration of heavy metals. Protein content under heavy metal influence may be affected due to: (i) Enhanced protein hydrolysis resulting in decreased concentration of soluble proteins (Melnichuk et al., 1982), (ii) Catalytic activity of lead (Bhattacharya and Choudhuri, 1997); (iii) Protein synthesis becoming reduced under all stress condition.

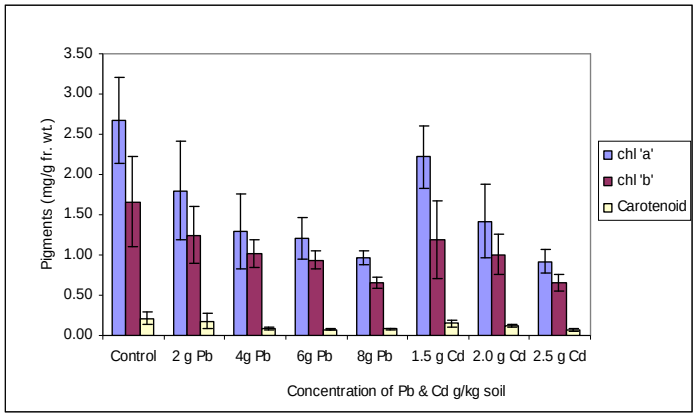


Figure 2 Changes in the leaf pigment contents of *P. vulgaris* grown under different concentrations of Pb & Cd

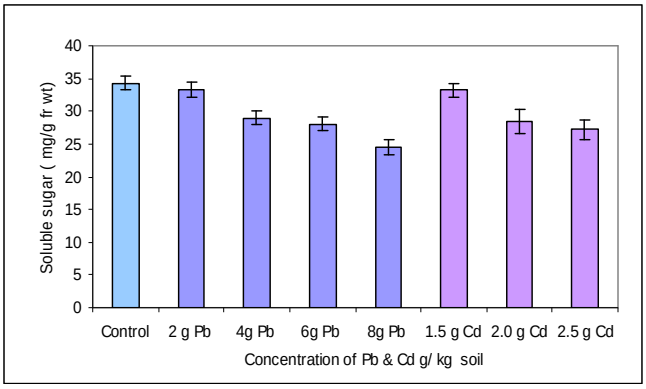


Figure 3. Changes in the soluble sugar content of *P. vulgaris* grown under different concentrations of Pb & Cd

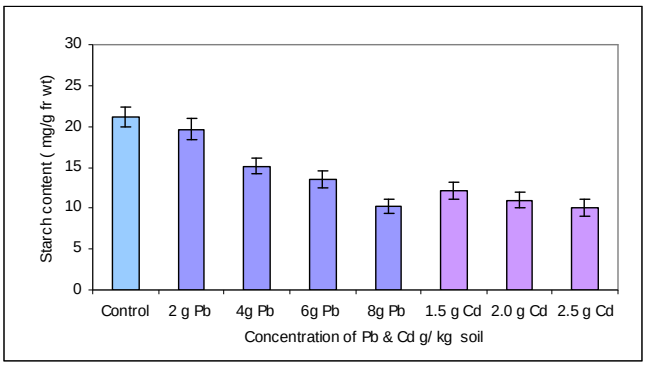


Figure 4. Changes in the starch content of *P. vulgaris* grown under different concentrations of Pb & Cd

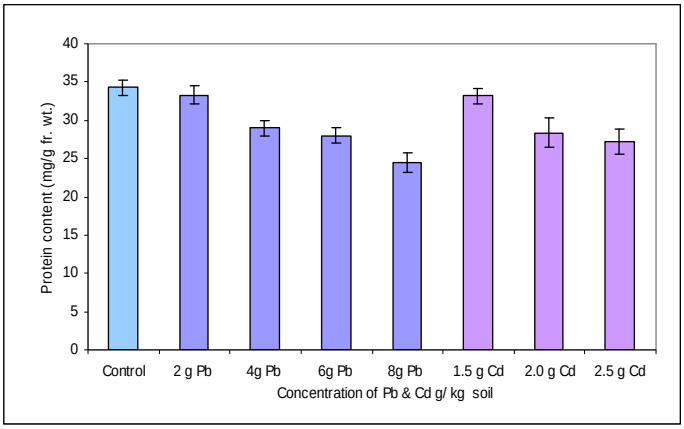


Figure 5 Changes in the soluble protein content of *P. vulgaris* grown under different concentrations of Pb & Cd

3.2.4 Total free amino acids and electrical conductivity

Total free amino acid and electrical conductivity at the increasing concentration of heavy metals increased in the plants. Total free amino acid increased in stressed plant as the metal stress applied. At different concentrations of Pb i.e. 2, 4, 6 and 8 g/kg soil amino acid content increases 40.61%, 42.58%, 46.59% and 65.81% respectively. For different concentrations of Cd i.e. 1.5,2 and 2.5 g/kg soil amino acid content increases 46.77%, 52.30%, 54.08 respectively (Figure 6). Electrical conductivity of tissue leachates of leaves at different concentrations of Pb and Cd was shown in Figure 7. In general, conductivity increases from control to higher concentration of metals. At different concentrations of Pb, there was an increase of 10.26%, 18.60%, 20.45% and 23.61% while for Cd it was 21.35%, 24.73% and 28.57% respectively (Figure 7).

Several studies with heavy metals support the view of changes in membrane architecture and permeability (Jensen and Adal-Steinsson, 1989). Various workers (Vangronsveld and Clijsters, 1994) suggested oxidation of cross linking of protein thiols, inhibition of plasma membrane ATPase as the mechanism of heavy metal induced membrane damage. Green et al., (1980) have suggested interaction of heavy metals with membrane phospholipids or the displacement of membrane-bound Calcium as a cause of heavy metal-induced changes in membrane structure.

3.2.5 Lipid peroxidation

Changes in lipid per oxidation in leaves of *P. vulgaris* at different treatments were shown in fig 10. As the concentration of metal increases, lipid per oxidation increases. For different concentrations of Pb it was 11.61%, 15.2%, 14.81% and 24.19% increase and for Cd it was 17.74%, 19.58% and 21.18% increase respectively (Figure 8).

Excess of Pb and Cd promoted lipid peroxidation with excessive production of MDA content over untreated control in concentration – regulated manner in plants of *P. vulgaris*, Lead & Cadmium – induced membrane damage seems to be largely due to enhanced membrane lipid-peroxidation, corroborating well with the data of Gallego et al., (1999). Membrane lipid peroxidation is also mechanically important from the perspective of production of oxyfree radicals like OH and HO_2 that further causes enhanced oxidative injury (Cakmak and Horst, 1991).

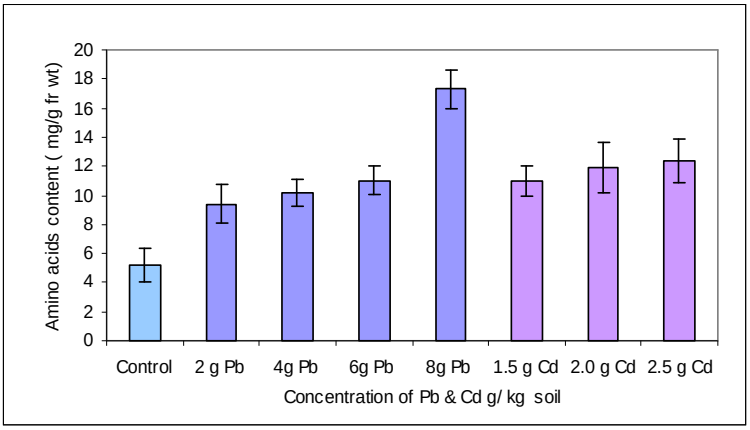


Figure 6 Changes in the leaf free amino acid content of *P. vulgaris* grown under different concentrations of Pb & Cd

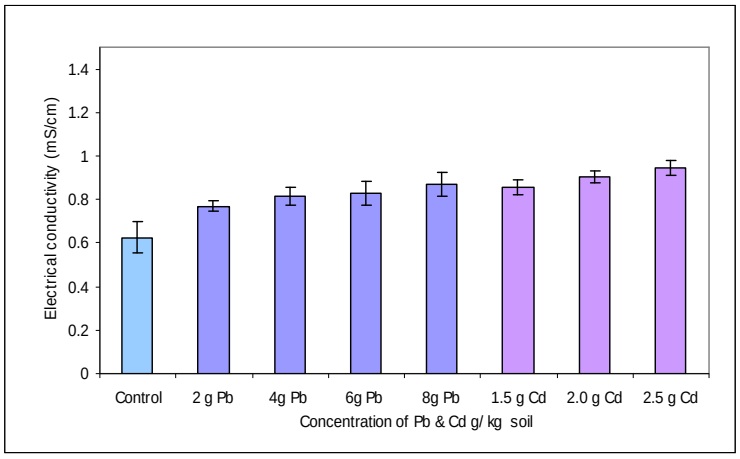


Figure 7 Changes in the electrical conductivity of leaf tissue leachate of *P. vulgaris* grown under different concentrations of Pb & Cd

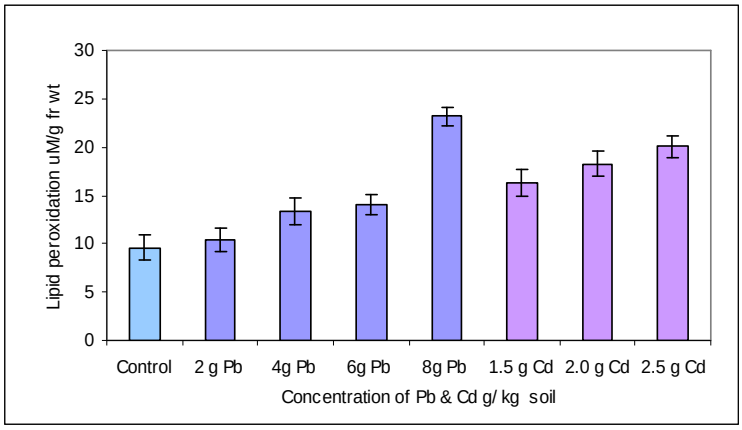


Figure 8 Changes in the leaf lipid peroxidation content of *P. vulgaris* grown under different concentrations of Pb & Cd

3.2.6 Antioxidant enzymes activities (APX, GPX and GR)

Development of oxidative stress in plants exposed to heavy metals (Weckz and Clijesters, 1996) is largely ascribed to heavy metal induced disbalance between the generations of toxic oxygen radicals and their scavenging through the anti-oxidative defense mechanism. The latter provides an efficient system for detoxification and scavenging of the toxic oxygen species through an adaptive mechanism involving upregulation of anti-oxidative enzymes such as SOD, CAT, POD, APX and GR (Foyer et al., 1994) and enhance accumulation of cellular antioxidants such as ascorbate glutathione cycle these reactions also down regulate the conversion of the super oxide ions to the highly reactive and genotoxic hydroxyl (OH) ions.

With increase in the concentrations and time of treatment of heavy metals, there was decrease in activities of antioxidative enzymes but in the present study with the increase of heavy metals increase the activity of anti-oxidative enzymes APX, GPX and GR in the leaves of P. vulgaris while there was a corresponding decrease in the CAT activity as compared to control plants. In the present study, increase in the concentrations of Pb & Cd increase in the activity of APX, GPX and GR in leaves of *P. vulgaris* as compared to the control plants shown in Figure 11-14 while activity of CAT decreases as the concentration of Pb and Cd increases (Figure 9-12). From the result it has been clear that the concentration of heavy metals which was given to the plants do not impose any kind of oxidative stress because the activity of antioxidant enzymes was higher. But from all other parameters which were studies, it is clear that increasing concentrations of

heavy metals significantly affect on the physiology and biochemistry of plants. As we have compared between both the metals, Cd was more toxic than the Pb because the lower concentration of Cd have almost same effect as the higher concentration of Pb.

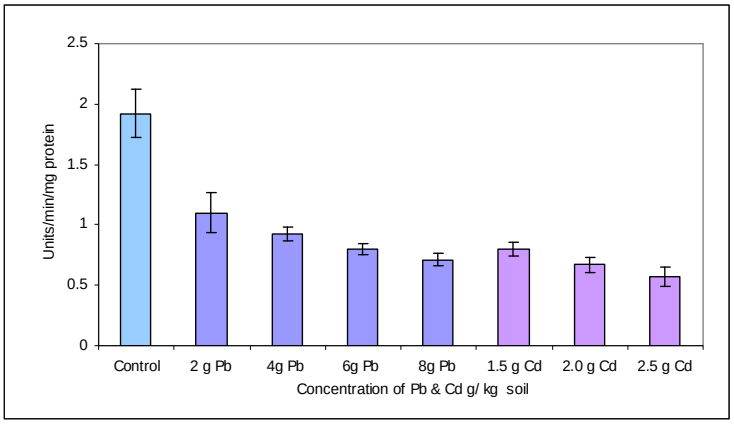


Figure 9. Changes in the leaf catalase activity of *P. vulgaris* grown under different concentrations of Pb & Cd

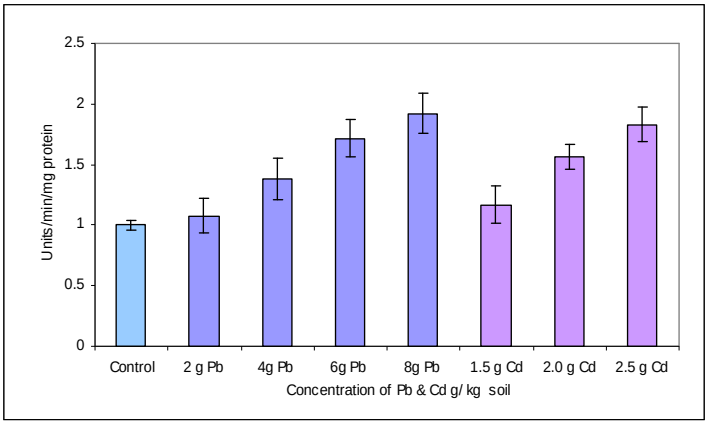


Figure 10. Changes in the leaf APX activity of *P. vulgaris* grown under different concentrations of Pb & Cd

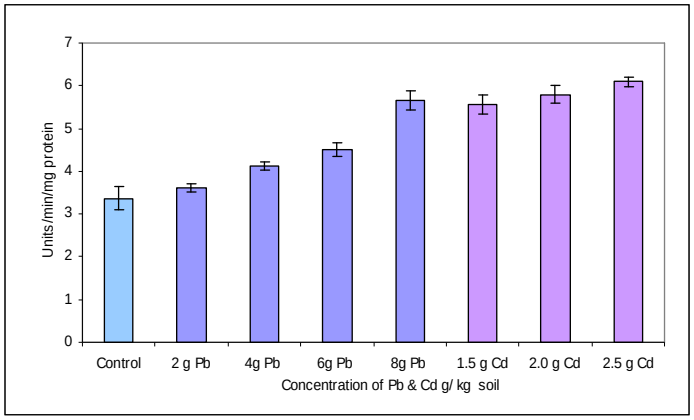


Figure 11. Changes in the leaf GPX activity of *P. vulgaris* grown under different concentrations of Pb & Cd

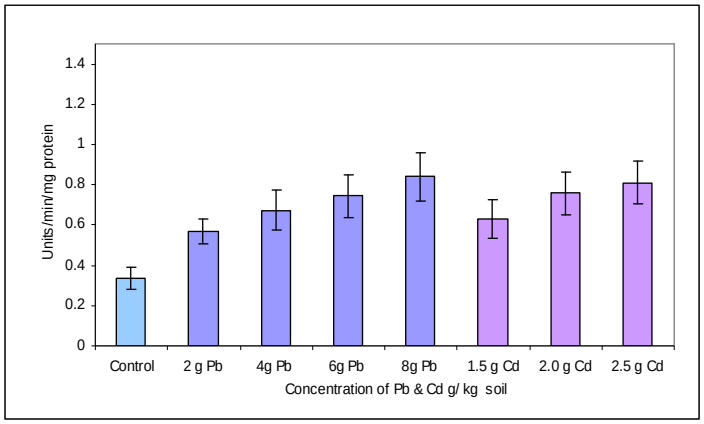


Figure 12. Changes in the leaf GR activity of *P. vulgaris* grown under different concentrations of Pb & Cd

3.2.7 Polypeptides and isoenzyme patterns:

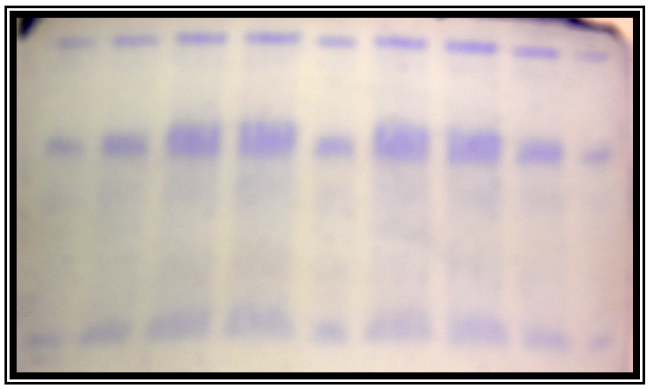
The SDS-PAGE pattern of polypeptides extracted from leaves of *P. vulgaris* at different concentrations of Pb and Cd were analyzed using 10% SDS gel shows that the polypeptides decrease as the concentration of both the metals increase compared with control (Figure 13).

Acid phosphate (APase, EC 3.1.3.2) is widely distributed in plants it has long been recognized that Apase activity in plants typically increased when plants become phosphorus (Pi) deficient the increase Apase activity correlates with a low level of Pi in numerous species and plant parts (Ueki and Sato, 1997). Salt, water and osmotic stresses have also been reported to increase APase activity (Szabo-Nagy et al., 1992).

Isoenzyme activity of Esterase, Peroxidase & Acid phosphatase was studied through electrophoresis. Isoenzyme variation in leaves of *P. vulgaris* at different concentrations of Pb and Cd was shown in Figure 14, 15 and 16 respectively.

In the present study, it was observed that the APase activity in plants was maximum at the highest concentration of Pb and Cd in comparison with control plants (Figure 16). Esterase activity was also increased slightly as the concentration of heavy metal increased (Figure 14). Peroxidase activity in leaves of plants increased with increasing concentration of heavy metal (Figure 15).

An increase in peroxidase activity probably represents an induced protective reaction delaying senescence (Birecka et al., 1977). Since, as we know the importance of peroxidase isoenzyme to catalyze the reaction that protects the plants, against damage by free radicals. Populations showing low peroxidase activity indicated that it may not adapt them at wider range because plants may lose the permeability of membrane and proceed toward the end of life due to the harmful action of free radicals. Lipid of membranes where a peroxidation of unsaturated fatty acids takes place is main cellular components susceptible to damage by free radicals (Monk et al., 1989).

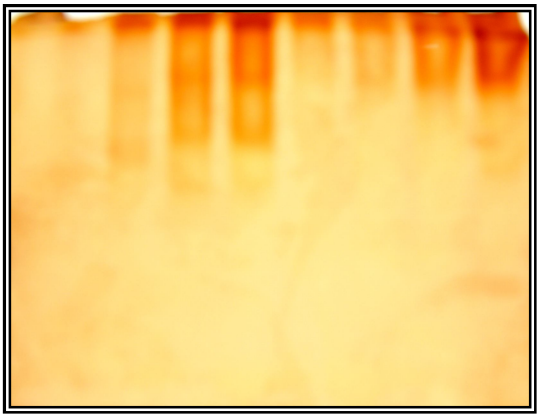


6 Pb 4Pb 2 Pb C 8 Pb C 1.5 Cd 2 Cd 2.5Cd

Figure 13 Changes in leaf polypeptide banding pattern of *P. vulgaris* grown under different metal concentrations

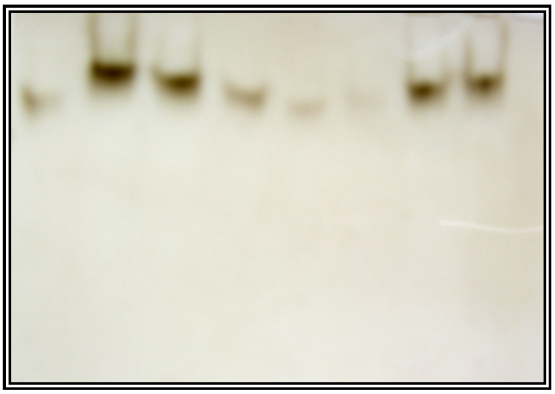
1.5 Cd 2 Cd 2.5Cd C 2 Pb 4Pb 6 Pb 8 Pb

Figure 14. Changes in Esterase activity of *P. vulgaris* grown under different metal concentration



1.5 Cd 2 Cd 2.5Cd C 2 Pb 4Pb 6 Pb 8 Pb

Figure 15. Changes in Peroxidase activity of *P. vulgaris* grown under different metal concentration



8 Pb 6Pb 4 Pb 2 Pb C 1.5 Cd 2 Cd 2.5Cd

Figure 16. Changes in Acid phosphatase activity of *P. vulgaris* grown under different metal concentration

(C=control, 2Pb=2 g Pb/ kg soil, 4Pb=4 g Pb/ kg soil, 6Pb=6 g Pb/ kg soil, 8Pb=8 g Pb/ kg soil, 1.5 Cd=1.5 g Cd/kg soil, 2 Cd=1.5 g Cd/kg soil, 2.5 Cd=2.5 g Cd/kg soil)

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Vegetative propagation of *Angelica glauca* Edgew. and *Angelica archangelica* Linn.: two high value medicinal and aromatic herbs of the Himalaya

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Abstract: *Angelica glauca* Edgew. and *A. archangelica* Linn. (Apiaceae) are high value medicinal and aromatic plant species of the Himalaya. Their *ex-situ* cultivation is recommended for conservation and regular supply of raw material for pharmaceuticals and ethno-medicinal uses. Vegetative propagation of these species was carried out at Pothivasa (2200 m asl): a part of Western Himalaya, Uttarakhand, India. Three treatments *viz.*, IBA, IAA and GA_3 with different concentrations (100, 200 & 500 ppm, each) were tried to stimulate sprouting and rooting. IBA 100 ppm showed better results in both the species. These treatments may be used for mass multiplication of these species. [Nature and Science. 2009;7(8):76-82]. (ISSN 1545-0740).

Key words: *Angelica* spp., conservation, medicinal herb, treatments, vegetative propagation

1. Introduction

Angelica glauca Edgew. and *A. archangelica* Linn., belong to family Apiaceae, are high value medicinal and aromatic plant species of the Himalaya. *A. glauca*, locally called as Choru or Gandhrayan, is native and endemic plant species, distributed along 2600 to 3,700 m asl in Uttarakhand, Jammu & Kashmir and Himachal Pradesh (Butola and Badola, 2004). The species is known for its multiple uses in traditional as well as in modern medicine. The rhizomes of the species are considered as cardio active, cordial and useful in constipation (Anonymous, 1985). Whole herb is reported to be useful to cure stomach troubles, bilious complaints, menorrhagia, infantile atrophy and as a stimulant (Chopra et al., 1956 and Anonymous, 1985). *A. archangelica* locally called as Rickhchoru in Garhwal region and commonly known as European angelica or wild parsnip, is an aromatic, stout, perennial herb with 60-200 cm in height. It is native to Austria, Belgium, Denmark, Germany, Greenland, Hungary, Ice-land, Poland and Central Russia. In India, it is found in Western Himalaya mainly in Kashmir (1000-3900 m), Garhwal and Kumaon regions at altitudes of (2600 m–3900 m); also reported from Sikkim at (3000-3300 m). The herb, including the fruits and roots, is used for flavoring, and is reported to possess carminative properties. The root is aromatic and is reported to posses' diaphoretic and diuretic properties, and is used in flatulent colic. It is sometimes applied externally as a counter-irritant. Internally it is used in digestive complaints, flatulence or as a tonic for cold and respiratory system (Anonymous, 1985).

Market demand of these species for pharmaceuticals and ethno-medicinal utility, are met through harvesting from wild populations. Due to unsustainable harvesting, habitat loss and grazing pressure these species have been assigned as endangered for the Himalayan region (Ved et al., 2003). Vashistha et al. (2006) reported the status of both *Angelica* spp. as endangered on the basis of population survey from Garhwal Himalaya. Both the species are propagated by the seed and vegetative parts. However, the existing report on seed germination is not reliable in view of their low germinability (Butola and Badola, 2004; Vashistha, 2006; Vashistha et al., 2009) and slow growth (Butola and Badola, 2006).

Vegetative propagation is one of the potential and useful methods that need to be tried for those species, which are economically important, difficult to raise through seeds and other means. Plant propagation through vegetative means multiplies these plants and preserves their essential genetic characters. This is an easy and effective technique for multiplication and conservation of plant species. Sexual reproduction is considered less important than vegetative propagation for arctic and alpine species (Bliss, 1971). Plant growth regulators and other chemicals are widely used in vegetative propagation to improve rooting and subsequent growth of cuttings (Nadeem et al., 2000; Butola and Badola, 2007). Present study was carried out to develop vegetative propagation protocol for the selected species using

rhizome segments.

Materials and methods: In the month of October, rhizomes of selected *Angelica* species were collected from natural habitat Tungnath (TN): an alpine zone (3600 m asl) located between 30°14' N Lat. and 79°13' E Long. in Rudraprayag district of Garhwal, Uttarakhand Himalaya, India. The rhizomes were washed thoroughly with running tap water. Each rhizome was cut into small pieces (approx 6 cm long) as there were apical buds present on the rhizome. The root part was not considered for propagation. These were treated with different hormonal concentrations by dipping them in particular hormonal solution for 24 hrs. One lot was dipped in distilled water to treat as control. Hormonal solutions of 100, 200 and 500 ppm concentrations of GA_3, IBA and IAA were used as treatments. For individual treatment three replicates with twenty cuttings each were used. Subsequently, treated segments were planted in soil beds in October 2004 at Pothivasa (PV) situated in temperate zone (2200 m asl) between 30°28' N Lat. and 79°16' E Long. in Rudraprayag district of Garhwal, Uttarakhand Himalaya, India. Soil beds with pH 4.67-5.01, soil organic carbon 1.0-1.23% and nitrogen content 0.04-0.23% while potassium and phosphorus content was very low in the soil of the experimental site (Vashistha et al., 2007). After the onset of next growing season in May 2005 number of sprouted segments and rooting were recorded in each treatment.

Data analysis: The data was analyzed statistically using MS-Excel 2003. Data presented here are mean values of treatments with standard deviation. ANOVA was used to interpret the variation and to identify the best treatment.

Results and Discussion: Results of vegetative propagation of *A. glauca* and *A. archangelica* are shown in table 1 & 2, respectively. Results indicate that maximum sprouting and rooting was observed in 100 ppm of IBA (88.33% and 85.00%, respectively). Rooting of the rhizome cuttings was fairly high in *A. glauca* (68.33%) and *A. archangelica* (78.33%) even without using any intervention. However, further increase in rooting percentage was possible by applying different concentrations of IBA, IAA and GA_3 (100, 200 and 500 ppm). Butola and Badola (2007) have recommended IAA and IBA as promising treatments to improve rooting, growth and biomass in *A. glauca* and *Heracleum candicans*. Variation was found significant among different treatments on the basis of ANOVA ($P<0.05$). When, sprouting as well as rooting was compared with control by using LSD, variation was found significant for IBA 100 ppm and IAA 100 ppm and rest of all treatments were found non-significant. In case of *A. archangelica*, maximum sprouting and rooting was observed in IBA 100 ppm (91.67%, 88.33%, respectively). In this species, variation was significant ($P<0.05$) only for IBA 100 ppm and rest of all treatments were found non-significant (Table 2).

Table 1. Effect of different growth hormones on vegetative propagation of *A. glauca* using rhizome segments.

Treatments	Sprouting Percentage	Rooting Percentage
Control	70.00±5.00	68.33±2.89
IBA 100 ppm	88.33±2.89*	85.00±5.00*
IBA 200 ppm	75.00±5.00	73.33±2.89
IBA 500 ppm	68.33±2.89	65.00±5.00
IAA 100 ppm	85.00±5.00*	83.33±7.64*
IAA 200 ppm	68.33±5.77	66.67±7.64
IAA 500 ppm	63.33±2.89	61.67±2.89
GA_3 100 ppm	75.00±5.00	71.67±2.89
GA_3 200 ppm	61.67±2.22	60.00±5.00
GA_3 500 ppm	51.67±2.89	48.33±2.89
F value & LSD (P<0.05)	20.23* 5.80$^+$	14.85* 6.55$^+$

* Significant

Table 2. Effect of different growth hormones on vegetative propagation of *A. archangelica* using rhizome segments.

Treatments	Sprouting Percentage	Rooting Percentage
Control	83.33±7.64	78.33±2.89
IBA 100 ppm	91.67±2.89*	88.33±5.77*
IBA 200 ppm	80.00±5.00	76.67±2.89
IBA 500 ppm	71.67±2.89	68.33±2.89
IAA 100 ppm	83.33±5.77	80.00±5.00
IAA 200 ppm	71.67±7.64	68.33±2.89
IAA 500 ppm	65.00±5.00	61.67±2.89
GA_3 100 ppm	76.67±5.77	73.33±7.64
GA_3 200 ppm	65.00±3.33	63.33±7.64
GA_3 500 ppm	55.00±5.00	51.67±2.89
F value & LSD (P<0.05)	11.80* 7.59^{+}	14.91* 6.68^{+}

*Significant

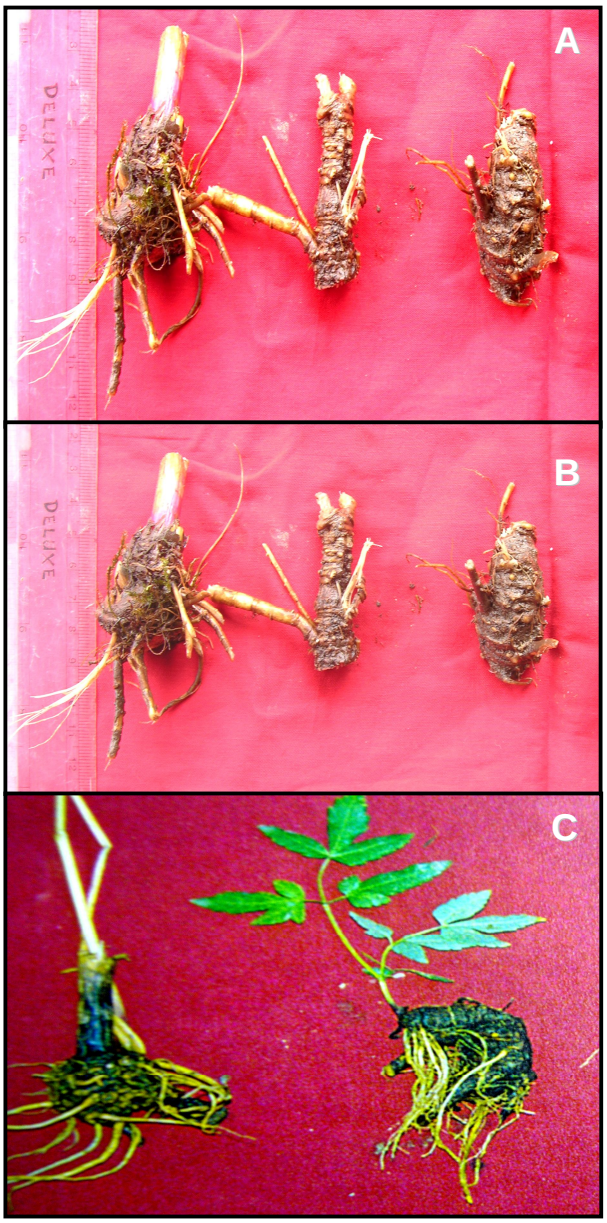

Plate 1. Vegetative propagation in *A. glauca*
A- Rhizome segments; B- Root initiation in rhizome segments;
C- Vegetatively propagated plants

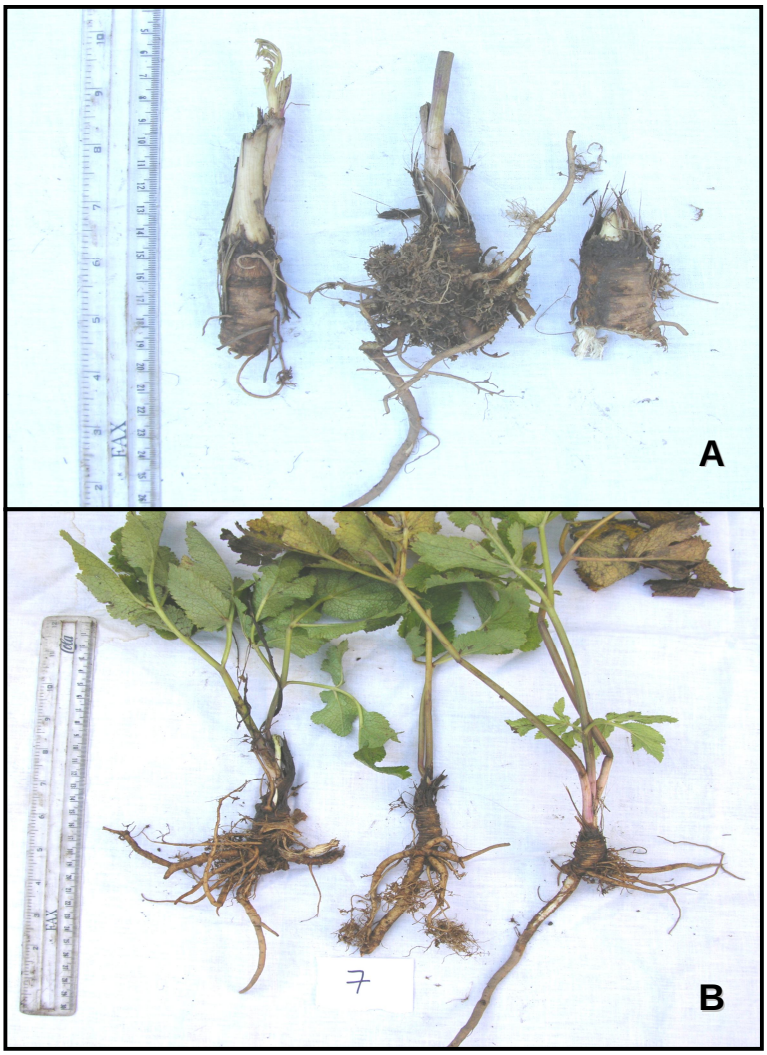

Plate 2. Vegetative propagation in *A. archangelica*
A- Rhizome segments; B- Vegetatively propagated plants

Rooting response under control set, however, indicates availability of natural auxins within the cuttings in adequate quantities to initiate the rooting. Moreover, better responses under IBA were in conformity with the reports of its effectiveness as compared to several naturally occurring auxins in promotion of adventitious roots (Hartmann and Kester, 1983). Higher concentrations of GA_3 (200 and 500 ppm) were found less effective in both the species. In the present study, transverse rhizome segments were used which responded well for differentiation of root/shoot system (Plate 1 and 2). Rawat et al. (1992) reported that the transversely segmented tuber have the potential to regenerate in new plantlets with well-differentiated root and shoot. Kuniyal et al. (2003) attempted to propagate *Aconitum atrox* through tuber segments at lower elevation in the Garhwal Himalaya. Apical segments produced single shoot while sub-apical, middle and basal were also able to regenerate several sprouts.

Vegetative propagation can be used as an efficient tool for mass scale propagation of tuberous roots of medicinally important species as in case of *Aconitum atrox*, the species fails to establish through seeds under natural conditions in an alpine environment (Kuniyal, 1999). In *Picrorhiza kurrooa*, vegetative propagation using stolon segments was found successful for cultivation up to 1800 m altitude with high moisture regime and proper aeration (Nautiyal et al., 2001). Maturity stage of planting material is suitable for multiplication as maximum numbers of buds are found in this stage (Manjkhola

and Dhar, 2002). Therefore, in present study rhizome segments were used after complete maturity stage of the selected species.

Conclusion: In both the *Angelica* species, only rhizome segments can be used for vegetative propagation. The collectors of raw material may be suggested to use the terminal part of rhizome for cultivation and utilize the remaining root part for medicinal purposes. Considering the economic potential of these species, this technology has immense potential of easy adoption by the farmers, and has significant value for both conservation and sustainable utilization of these *Angelica* species.

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6/2/2009

Bisphenol A Toxicity in milk: A Review

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Abstract: Objective: Bisphenol A is constantly discharged at trace levels in food packed in metal cans with PVC lining. This represents a cause for concern because of potential effects of bisphenol A to human health. We compiled data on the analysis of bisphenol A in milk samples, published in the last 10 years. Pubmed and Medline were used to search for articles published in peer-reviewed journals written in the English language since 1999. Information on Bisphenol A concentrations in milk, the source of contamination, year of publication and method of analysis was extracted. [Nature and Science. 2009;7(8):83-85]. (ISSN 1545-0740).

Key words: Milk samples, Bisphenol A, Analysis

Introduction:

Bisphenol A is used as plastic additives, lacquers, resins, or surfactants and can be found in milk due to contact with plastic materials during food processing and storage. [Casajuana N et al 2004].

Human exposure to BPA may arise through BPA leaching from these materials into foods [Health Canada]. Bisphenol A has been known to leach from the plastic lining of canned foods and, to a lesser degree, polycarbonate plastics that are cleaned with harsh detergents or used to contain acidic or high-temperature liquids. A recent Health Canada study found that the majority of canned soft drinks it tested had low, but measurable levels of bisphenol A [Y. Kawamura et al, 1999].

Kang JH et al 2003. Conducted a study to develop a selective and sensitive method for the determination of bisphenol A (BPA) levels in milk and dairy products. A method based on solvent extraction with acetonitrile and solid-phase extraction (SPE) was developed for the analysis of BPA in milk, yogurt, cream, butter, pudding, condensed milk, and flavored milk was developed. The detection limits were (1 microg/liter for milk, yogurt, pudding, condensed milk, flavored milk, and skim milk and 3 microg/liter for cream and butter). These methods are simple, sensitive, and suitable for the analysis of BPA in milk and dairy products.

A highly sensitive and selective method was developed, based on alkaline digestion for the simultaneous determination of bisphenol A (BPA) and 4-nonylphenol (NP). The procedure will be reliable for the trace analysis of BPA and NP in human milk, since alkaline digestion can diminish their documented association with protein. The limits of detection of BPA and NP were 0.09 nag/g and 0.50 nag/g, respectively. [Otaka H et al 2003]. A highly sensitive HPLC method was developed for the determination of xenoestrogenic compound, bisphenol A (BPA) in human breast milk samples. Twenty-three breast milk samples of healthy lactating women were analyzed for the BPA concentration. The mean value was 0.61 +/- 0.20 ng mL(-1), with no correlation to the lipid content of milk samples. [Sun Y et al, 2004]. Authors developed a highly sensitive method of analyzing breast milk for triclocarban (3,4,4'-trichlorocarbanilide) and eight phenolic compounds: bisphenol A (BPA), 4-tert-octylphenol (4-tOP), ortho-phenylphenol (OPP), 2,4-dichlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, and 2-hydroxy-4-metoxybenzophenone (BP-3). The method was validated using pooled breast milk samples. Detection limits for most analytes are below 1 ng/mL in 100 microL of breast milk.

Maragou NC et al, [2006] developed a method for the determination of bisphenol A (BPA) in milk method is simple and reliable based on solid phase extraction (SPE) and liquid chromatography coupled with

electrospray ionization mass spectrometry was. The concentration of BPA found in commercial canned milk samples ranged from <1.7 to 15.2 ng/g.

A method has been developed for the simultaneous determination of nonylphenol (NP), octylphenol (OP) and bisphenol A (BPA) in eggs and milk, based on matrix solid phase dispersion (MSPD) using C18 as dispersant, and a subsequent cleanup step with amino-propyl solid phase extraction cartridges and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS Recovery studies were performed at different fortification levels. The limits of detection (LODs) in eggs were 0.10, 0.10 and 0.25 microg/kg for BPA, NP and OP, respectively. Investigation of the levels in commercial samples indicated that NP was ubiquitous in milk and eggs at levels ranging from 4.24 to 17.60 microg/kg, and the milk samples were more heavily contaminated by NP than were the egg samples. [Shao B,et al 2007]

Ye X et al [2008] developed a sensitive method, to measure in human milk the concentrations of five parabens (methyl-, ethyl-, propyl-, butyl-, and benzyl parabens), triclosan, and six other environmental phenols: bisphenol A (BPA); ortho-phenylphenol (OPP); 2,4-dichlorophenol; 2,5-dichlorophenol; 2,4,5-trichlorophenol; and 2-hydroxy-4-methoxybenzophenone (BP-3), using a unique on-line solid-phase extraction-high performance liquid chromatography-tandem mass spectrometry system with peak focusing feature. The method was validated by use of breast milk pooled samples, showed good reproducibility and accuracy. The detection limits for most of the analytes are below 1 ng mL(-1) in 100 microL of milk.

This study reported a new method for determination of dimethyl phthalate, diethyl phthalate, di-n-butyl phthalate, butylbenzyl phthalate, bis(2-ethylhexyl) phthalate, nonylphenol, bisphenol A, and bisphenol A diglycidyl ether in commercial whole milk. They are all suspected endocrine disrupters or mutagens. Limits of detection were from 0.06 to 0.36 microg/kg.[Casajuana N et al,2004]

Liu X et al [2008] employed a method to determine bisphenol A (BPA) in milk samples. Solid-phase microextraction coupled to high-performance liquid chromatography (SPME-HPLC) with fluorescence detection was used. The proposed method was successfully applied to real samples, BPA being detected within the range 1.6-2.6 ng ml(-1) in four brands of commercial milk but not in soybean milk.

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6/6/2009

Isolation of flavonols from Euphorbia *wallichii* by preparative High Performance Liquid Chromatography.

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Abstract: Flavonoids are a diverse group of natural products found in all plants. In present study three flavonols namely quercetin, kaemferol and myricetin were identified and isolated in Euphorbia *wallichii* in different solvents. Study was carried out on acid hydrolyzed methanolic extracts which was further fractionated into diethyl ether, n-butanol, ethyl acetate and water extracts. Quercetin was found to be the most abundant flavonol present in Euphorbia *wallichii*. [Nature and Science. 2009;7(8):86-88]. (ISSN 1545-0740).

Key words: Antioxidants, Flavonoids, Euphorbia *wallichii*, prep- HPLC

1. Introduction:

Interest in the role of antioxidants in human health has prompted research in the fields of food science and horticulture to assess fruit and vegetable antioxidants (Kalt et al., 1999). The majority of the antioxidant capacity of a fruit or vegetable may be from compounds such as flavonoids, isoflavones, flavones, anthocyanins, catechins and isocatechins rather than from vitamins C, E or B-carotene (Wang et al., 1996; K.hk.nen et al., 1999). Many of these phytochemicals may help to protect cells against the oxidative damage caused by free radicals (Wada and Ou, 2002).

Many of them play important roles as flower and fruit pigments, UV protectants, signaling molecules between plants and microbes, and regulators of auxin transport (Doone, 1991, Dixon, 1995). The flavonoids are also thought to have antioxidant, anti-allergenic, and anti-inflammatory effects, thus contributing to human health (Scalbert, 2005, Ross, 2002).

HPLC is gaining increasing importance for the analysis of plant extracts. The qualitative analysis which produces a "fingerprint" chromatogram obtained under standard conditions can be very useful for quality control of phytochemicals. Although TLC is a powerful and simple technique used for this purpose, there are situations in which it can produce doubtful results. HPLC can also be a useful tool in chemosystematics helping, for example, to characterize species on the basis of their secondary metabolite contents.

Reversed-phase HPLC has been used in a number of occasions for the analysis of flavonoids in plants. In one study it was used to distinguish species based on the quantitative variation of flavonoids among them.

2. Materials and method:

All reagents were of analytical grade. Quercetin , myricetin
and kaemferol were purchased from Sigma Aldrich.

2.1 Extraction Method:

The Euphorbia *wallichii* was collected from Murree hills Pakistan in June. 2008 and a voucher

specimen was deposited at LCWU Herbarium. The plant material (1.00 kg) were dried away from the sunlight, powdered and exhaustively extracted with methanol using Soxhlet extraction method to give solvent free crude methanolic extract (7.056%). The methanolic extracts were then acid hydrolyzed and tested for flavonoid contents using standard myricetin, kampharol, and quercetin by HPLC. The methanol extracts was then fractionated using diethyl ether, n-butanol, ethyl acetate and water to evaluate the most suitable solvent for separation.

2.2 Acid Hydrolysis:

Controlled acid hydrolysis was carried out with 10% acetic acid under reflux for 3.5 hours. These fractionated samples were then analyzed by HPLC without any further separation [Filippo Imperato]

2.3 HPLC Conditions:

The prep HPLC system (Waters) consisted of a UV detector (2487). Column was a C18, (250 x 4.6 mm, 5 mm particle sizes). Acetonitrile was purchased from Merck. Water was HPLC grade and acidified with 1 % acetic acid. Qualitative analysis was made with samples, in isocratic mode, with acetonotrile/water 1:1 at a flow-rate of 1 mL min-1. The injection volume was 10 uL (analytical mode) and 10 mL(prep mode) and elute was monitored at 254 nm. The filtered methanol extracts (0.5 microns) of Euphorbia wallichii and its fractions was injected under these conditions and compared with authentic standards of myricetin, quercetin and kaemferol, injected under similar conditions.

2.4 Qualitative HPLC analysis:

The method developed for HPLC fingerprinting provided a quick analysis of the methanolic extract and fractions obtained after fractionation. The conditions used led to a good separation of the peaks which could be identified by comparing the chromatogram with the chromatogram of the reference compounds obtained under the same conditions. This way a qualitative analysis was made in analytical mode of HPLC.

2.5 Quantitative HPLC analysis:

Quantitative isolation of flavonols was made by HPLC in prep mode. Flavonols were easily isolated using fraction collector according to retention time of different peaks.

2.6 Vacuum evaporation:

Fractions collected containing single compounds were subjected to vacuum evaporation and after complete solvent removal, weights were noted. These compounds were then confirmed by comparison with standards in analytical mode of HPLC.

3. Results and discussion:

Myricetin and kaemferol were not detected in all the fractionated extracts of Euphorbia wallichii while quercetin was the most abundant flavonoid aglycone (20.626%) present in methanol extract, most of it went into n-butanol extract (14.714 %) and the rest in diethyl ether extract (5.712%) when fractionated [table-1]. Quercetin has been reported to have interesting biological activities including the inhibition of the anticancer drug target, heat shock protein-9 (Hsp90) [Nagai et al. 1995; Hansen et al. 1997; Kudo et al. 1999; Wu & Yu 2000]. Euphorbia *wallvichii* extract in methanol presented a better source of Quercetin having antihypertensive properties.

Acknowledgment:

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Table 1. Percentage of Flavanols isolated different extracts of *Euphorbia wallichi*

Sr.No	Extract	Myrcetin	Kampherol	Quercetin
1	Euphorbia Methanol	1.640%	nd	20.626%
2	Euw - Diethyl ether	1.250%	nd	5.712%
3	Euw -n-butanol	nd	nd	14.714%
4	Euw -ethyl acetate	nd	nd	nd
5	Euw -Water	nd	nd	nd

Nd= Not Detected

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Anti-Inflammatory, Anti-Pyretic and Anti-Diarrhoeal Properties of an Anti-Haemorrhoid Tri-Herbal Pill.

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Abstract

A Nigerian tri-herbal decoction branded "Jedi-Jedi Pill" made from *Croton penduliflorus, Cassia podocarpa and Manihot esculenta,* is widely regarded as effective for treating haemorrhoids and associated diseases. The anti-inflammatory, anti-pyretic, anti-diarrhoeal activities and the overall drug safety of the preparation was investigated using carrageenan induced oedema, 2, 4-Dinitrophenol-induced pyrexia, Castor oil-induced diarrhoea models in rats. Doses of the decoction (300 – 1500 mg^{-1}kg body weight (b. wt.)., were orally administered to Albino rats (150 – 200 g) and Mice (15-30 g) of either sex to evaluate side effects and mortality within 72 h and to assess drug safety. Paw inflammation was induced in rats hind paw by the subplanter injection of Carrageenan while Oedema was assessed immediately after, at intervals of 0 – 6 h using the cotton thread method. Pyrexia was induced in the rats by the administration of 10 mg^{-1}kg b. wt., of 2, 4-Dinitrophenol intraperitoneally while measurement was by inserting a clinical thermometer into their anal cavities for about 2 min. Castor oil method was used to induce diarrhoea while adsorbent paper examination was done to determine the presence of wet stool every hour for 4 h. Acute toxicity studies produced no mortality but weakness and bloody eyes especially at the highest dose of 1,500 mg^{-1}kg b. wt was observed. Anti-inflammatory activity studies showed a dose dependent trend in percentage inhibition which peaked at 900 mg^{-1}kg b. wt. This activity was comparable with the reference drug Indomethacin. Activity against hyperthermia was insignificant. The preparation significantly reduced ($p<0.05$) diarrhoeal frequency in rats at 900 mg^{-1}kg b. wt., but this activity was significantly lower than the reference drug. This investigation reports that Jedi-jedi pill possesses anti-inflammatory and anti-diarrhoeal capabilities but lacks anti-pyretic action. Though non toxic, it has some side effects. [Nature and Science 2009;7(8):89-94] (ISSN 1545-0740).

Keywords: *Croton penduliflorus, Cassia podocarpa*, *Manihot esculenta*, Anti-inflammatory, Anti-pyretic, Tri-herbal and Anti-diarrheal.

1. Introduction

Medicinal plants have been of great importance to the healthcare needs of individuals and their communities. The use of herbal preparations made from medicinal plants is widespread in developing countries. In these local communities where medicare is not so easily accessible due in part to lack of healthcare facilities and the high cost of orthodox treatment (Zhu et al., 2002; Okochi et al., 2003), recourse to traditional medicine offers the only hope of staying healthy and alive. The most important bioactive constituents of medicinal plants are alkaloids, tannins, flavonoids and phenolic compounds (Edeoga et al 2005). Crude drugs obtained from medicinal plants have been used to treat all manner of aliments in most traditional societies.

In African traditional medicine, a mixture of herbs and plants are cooked, macerated or made into tincture to treat different diseases. The tri-herbal preparation popularly known as "Ogun Jedi Jedi" likewise is prepared from *Manihot esculenta, Cassia podocarpa and Croton penduliflorus,* mucilage and potash, is believed to be effective for the treatment of haemorrhoids. The plants from which this preparation was formulated have been used in ethno medicine to treat different ailments. Cassava (*Manihot esculenta* Crantz) serves as food and an important tuber in the provision of energy for the teeming millions of people from tropical African countries and has been ranked as an important human calorie source, behind rice, sugar cane and maize (FAO, 1995; Siqueira et al., 2007). Adeyemi et al., (2008) reported that cassava tuber is effective against diarrhoea, fever, headache, aches and pains among other medicinal uses. *Cassia podocarpa* and *Croton penduliflorus* have been reported to have laxative properties (Asuzu et al., 1988, Elujoba et al., 1989). Mucilage from cassava was found to be a very good binding agent (Uhumwangho, 2006), and has antioxidant properties (Fu et al, 2004).

The present study was undertaken to evaluate the pharmacological properties of ogun Jedi-Jedi widely sold in motor parks, markets and other public places to a cross section of our untutored population by medicine hawkers. The tri-herbal preparation was made into a crude pill –like form for aesthetic purposes. The efficacy and safety of such herbal preparation is a source of concern to us as scientists working in this field.

2. Materials and Methods

2.1 Plant materials and Sample preparations:

The tri-herbal formulation locally known as "Jedi-Jedi Pill" was purchased from medicine hawkers at a motor park in Lagos metropolis. They are presented in brownish pill form and weighs approximately 500 mg per pill. The formulation consists of 20 % *Croton penduliflorus*, 25 % *Cassia podocarpa*, 15 % *Manihot esculenta*, 20 % Potash and 10 % starch mucilage. The pills were dissolved in water and administered to the animals according to their body weights (b. wt.).

2.2. Animals

Albino rats (150 – 200 g) and Mice (15-30 g) of either sex obtained from Biochemistry Department of Nigerian Institute of Medical Research (NIMR) Yaba, Lagos were used for the study. Approval was obtained from the University of Lagos Ethical Committee on the use of animals for research purposes. The rats and mice were fed standard laboratory diet and water *ad libitum*. They were maintained under standard environmental conditions as described by Bishayee and Chanterjee, (1994).

2.3. Acute toxicity (LD_{50}) study

Acute toxicity study was carried out as reported by Aniagu et al., (2005). This was done orally (p.o) using mice (n=25) and rats (n=25). The mice and rats were randomly divided into five groups of five animals respectively. The dose levels used ranged from 300 to 1500 mg^{-1} kg b. wt. The mice and rats were observed for signs of adverse side-effects and death within 72 h after treating them with the preparation. The acute toxicity LD_{50} was calculated as the geometric mean of the dose that resulted in 50 % mortality.

2.4. Anti-inflammatory activity

Paw inflammation was induced in rats hind paw by the subplanter injection of phlogistic agent (Carrageenan) as described by Oloyede et al., (2008) with minor modifications.

The albino rats used for this study were fasted for 12 h but allowed access to water. The aqueous preparation dose of 300 to 900 mg^{-1}kg b. wt., was administered orally to the test group of rats, while indomethacin 25 mg^{-1}kg b.wt., dose was administered orally to the reference group (positive control). Distilled water 1 mL^{-1}kg b. wt., was orally given to the control group (negative group).

To induce paw oedema, 0.1 mL carrageenan diluted in distilled water was injected into the sub-planter region of the right hind paw 1 hr after the treatment. Oedema was assessed immediately after carrageenan injection at intervals of 0, 1, 2, 3, 4, 5 and 6 h using the cotton thread method described by Bamgbose and Neomesi, (1981). The increase in paw swelling was measured and percentage inhibition was calculated.

2.5. Anti-pyretic activity

Anti-pyretic activity of the preparation was carried out using the methods of Berken et al (1991). Rats were weighed and randomized into five groups of five rats per group. The baseline body temperatures of the rats were taken by inserting a clinical thermometer into their anal cavities for 2 min. The steady temperature readings obtained were recorded as the pre-treatment temperatures. Pyrexia was induced in the rats by the administration of 10 mg^{-1}kg b. wt., of 2, 4-Dinitrophenol (DNP) intraperitoneally. Hyperthermia developed 30 min later after DNP administration. Different doses of the preparation (ranging between 300 - 900 mg^{-1}kg) were given orally, aspirin (100 mg^{-1}kg i.p) and distilled water (10 mL^{-1}kg b. wt.,) were administered orally to the treatment and control groups of animals. Rectal temperatures were obtained at 1 hr interval for 5 hr.

2.6. Anti-diarrhoeal activity

Anti-diarrhoeal activity of the preparation was evaluated using the castor oil-induced diarrhoeal model in rats (Awouters et al., 1978). Five groups of five rats per group were used for the study. The rats were fasted for 24 h prior to the experiment. Distilled water 10 mL^{-1}kg b. wt., was given to group I (control group) orally. Group II received 100 mg aspirin/kg orally while the other three groups were treated with 300, 600 and 900 mg^{-1}kg b. wt., respectively. One hour after the treatment, rats in all the groups were given 1 mL castor oil/100 g^{-1} body weight orally. The rats were separated into individual cages having adsorbent paper beneath and examined for the presence and frequency of wet stool every hour for 4 h. Absence or delay in production of watery stool was regarded as protective or positive.

2.7. Statistical analysis

Results were expressed as the mean ± standard error of mean (S.E.M). Statistical analysis of data was carried out using Student's *t*-test. Differences in

mean were considered to be significant when $p \leq 0.05$.

3. Results

3.1. Acute toxicity studies

General weakness, sluggishness and bloody eyes were the major behavioural changes observed in the rats and mice at 1200 and 1500 mg^{-1}kg b. wt., doses. No death was recorded at any of the doses administered. Oral LD_{50} was therefore not determined because mortality was not observed.

3.2. Effect on carrageenan induced inflammation

The effect of the tri-herbal preparation on carrageenan induced rat paw oedema is shown in Table 1. The control animals progressively exhibited increasing paw volume in response to carrageenan injection during the study. The anti inflammatory activity become noticeable after the third hour at a dose of 300 and mg^{-1}kg b. wt., whereas the same activity became evident at the second hour in that of 900 mg^{-1}kg b. wt., The oral administration of 300 mg dose did not produce any significant effect but doses of 600 and 900 mg of the preparation produced a significant ($p< 0.05$) inhibition of the rat paw oedema. At the sixth hour the sample exhibited a significant anti inflammatory activity at a dose of 900 mg when compared to control. The maximum paw oedema percentage inhibition of 73.3 % and 93.3 % respectively (Table 2) was observed at doses of 600 and 900 mg^{-1}kg b. wt., when compared to the control group, but lower than that of Indomethacin.

Table 1a: Effect of "Jedi-Jedi Pill" on Carrageenan-induced Rat Paw Oedema

Treatment	Dose	0hr	1hr	2hr	3hr
Control	10mL/10kg	2.4 ± 0.02	2.54 ± 0.04	2.62 ± 0.04	2.78 ± 0.02
Crude	300mg	2.50 ± 0.03	2.66 ± 0.06	2.74 ± 0.05	2.76 ± 0.04
	600mg	2.54 ± 0.02**	2.72 ± 0.04***	2.82 ± 0.04**	2.86 ± 0.02
	900mg	2.56 ± 0.02 * *	2.74 ± 0.05	2.70 ± 0.04 *	2.68 ± 0.04
Indomethacin	25mg	2.40 ± 0.03	2.52 ± 0.04	2.56 ± 0.02	2.70 ± 0.03

Table 1b: Effect of "Jedi-Jedi Pill" on Carrageenan-induced Rat Paw Oedema

Treatment	Dose	4hr	5hr	6hr
Control	10mL/10kg	2.66 ± 0.02	2.68 ± 0.04	2.70 ± 0.03
Crude	300mg	2.70 ± 0.05	2.66 ± 0.04	2.64 ± 0.05
	600mg	2.76 ± 0.04	2.68 ± 0.04	2.62 ± 0.02
	900mg	2.66 ± 0.04	2.60 ± 0.04	2.58 ± 0.04*
Indomethacin	25mg	2.54 ± 0.02**	2.48 ± 0.02***	2.44 ± 0.04***

Values: Mean ± SEM, *P<0.05, **P<0.01, ***P<0.001 significantly different from control (student's t-test)

Table 2: Percentage Inhibition of "Jedi-Jedi Pill" on Carrageenan-induced Rat Paw Oedema

		Percentage inhibition					
Treatment	Dose (mg)	1hr	2hr	3hr	4hr	5hr	6hr
Crude	300	0	0	31.6	23.1	42.9	53.3
600		0	0	15.8	15.4	50.0	73.3
900		0	36.4	68.4	61.5	85.7	93.3
Indomethacin	25	14.3	27.3	21.1	46.2	71.4	86.7

3.3. Anti-pyretic studies

The tri-herbal preparation did not exhibit any anti-pyretic activity at 300-900 mg^{-1} kg dose. The basal rectal temperature of the control was 37.88 ± 0.45 ºC (n=5).Thirty minutes after DNP administration, the mean rectal temperature of the rats was 38 ± 0.21º C. The rectal temperature remained hyperthermic in all the groups throughout the 4 hr period. Aspirin (100 mg/kg) produced significant ($p<0.05$) reduction of hyperthermia throughout the study (Table 3).

Table 3: Effect of "Jedi-Jedi Pill" on D-amphetamine-induced Pyrexia in Rats

Treatment	Dose (mg/kg)	Baseline Temp	Post-DNP Temp	1hr	2hr	3hr	4hr
Control	10mL/kg	37.9±0.5	38.8±0.2	37.6±0.4	27.8±0.3	37.8±0.3	37.7±0.3
Extract 1	300mg	38.1±0.3	38.4±0.2	38.2±0.3	38.3±0.2	38.3±0.1	38.5±0.1
Extract 2	600	37.3±0.4	38.4±0.2	38.5±0.3	37.3±0.4	38.3±0.2	37.9±0.2
Extract 3	900	38.1±0.2	38.7±0.3	37.7±0.4	37.6±0.3	37.5±0.3	37.4±0.3
Aspirin	100	38.2±0.4	38.3±0.3	39.4±0.2**	39.3±0.2**	39.2±0.2**	38.8±0.2*

3.4. Anti-diarrhoeal activity

The 900 mg^{-1}kg b. wt., dose of the decoction significantly ($p < 0.05$) protected the rats against castor oil-induced diarrhoea at 1 hr and 2 hr. Table 4 shows that the percentage inhibition of the Jedi-Jedi pill on castor oil-induced diarrhoea at doses 300-900 mg^{-1}kg b. wt., increased dose-dependently throughout the period of study. Aspirin at 100 mg^{-1}kg b. wt., was also observed to significantly protect the rats against castor oil-induced diarrhoea in 1 and 2hr of the study. It also had a pronounced percentage inhibition especially at 1 and 3hr respectively (Table 5).

Table 4: Effect of "Jedi-Jedi Pill" on Castor oil-induced Diarrhoea in Rats

Treatment	Dose (mg/kg)	1hr	2hr	3hr	4hr
Control	10mL/kg	4.40 ± 1.03	5.40 ± 1.80	3.40 ± 1.5	2.00 ± 0.84
	300mg	1.80 ± 0.86	2.40 ± 1.36	3.80 ± 1.72	2.40 ± 1.12
	600mg	2.80 ± 1.24	0.40 ± 0.24	1.00 ± 0.89	1.00 ± 0.32
	900mg	0.20 ± 0.20**	0.40 ± 0.24*	1.00 ± 0.89	1.00 ± 0.32
Aspirin	100mg	0.40 ± 0.24**	0.60 ± 0.04*	0.06 ± 0.40	1.40 ± 0.87

Values are mean ±SEM *P<0.05 significantly different from control (Student's t-test).

Table 5: Percentage inhibition of "Jedi-Jedi" on Castor oil-induced diarrhoea in rats

		Percentage inhibition in parenthesis			
Treatment	Dose	1hr	2hr	3hr	4hr
Control	10mL/kg	16	16	17	6
	300mg	9 {43.0}	13{18.7}	4 {76.5}	16 {-166}
	600mg	9 {77.7}	10 {37.5}	10 {41.2}	1 {83.3}
	900mg	4 {75.0}	4 {75.0}	3 {82.4}	1 {83.3}
Aspirin	100mg	0 {100}	7 {56.3}	5 {70.5}	3 {50.0}

Values are expressed as percentage inhibition, N = 5.

4. Discussion

Jedi-Jedi Pill is a Nigerian local tri-herbal formula prepared with three plants *Croton penduliflorus*, *Cassia podocarpa*, *Manihot esculenta*, mucilage and potash. It is one of several herbal preparations sold openly in motor parks and market places in Lagos metropolis Nigeria, by medicine hawkers and it is widely used and acclaimed to be effective for the treatment of haemorrhoids. Although acute toxicity studies did not indicate any mortality, adverse side effects like general weakness, sluggishness and bloody eyes were observed.

Carrageenan-induced rat paw oedema is a suitable test for evaluating anti-inflammatory drugs. Its oedema formation in rat paw is a biphasic event which involves various inflammatory mediators (Ahamed et al 2005). Chemical mediators such as histamine and serotonin are released in the first phase (the first 2hrs after carrageenan administration) while in the second phase (3-5 hrs after), Kinins, prostaglandins and other slow reacting substances become active (Hernandez-perez and Gallazo 2002). This pill exhibited inhibitory effect on carrageenan induced rat paw oedema at all doses used in this study. The 600 mg^{-1}kg b. wt., dose inhibited oedema

significantly ($p < 0.05$) throughout the first phase, whereas the 900 $mg^{-1}kg$ b. wt., dose oedema inhibition occurred in both phases. The reference drug (Indomethacin) exhibited inhibition throughout the second phase and the level of its inhibition pattern is comparable with the tri-herbal preparation. The highest percentage inhibitions observed in the carrageenan induced oedema at 600 and 900 $mg^{-1}kg$ b. wt., showed a dose-dependent trend. It is most probable that the inhibition of different types of chemical mediators of inflammation may be involved in the biphasic inhibition pattern observed. Therefore the ability of "Jedi-Jedi Pill" to inhibit the biphasic events of carrageenan induced rat paw oedema by suppressing inflammation confirms its anti-inflammatory properties.

Antipyretics are known to prevent rise in body temperature generally in response to endogenous pyrogens as excessive rise in body temperature may cause irreversible tissue damage and possibly death (Tijani *et al,* 2008). Cyclooxygenase (COX) which is the enzyme that converts arachidonic acid to prostaglandin (PG) is activated by pyrogens. The pill which did not produce any anti-pyretic effect at 300-900 $mg^{-1}kg$ b. wt., shows that there is continued synthesis of prostaglandins and this supports the biphasic pattern of carrageenan induced inflammation. It is therefore not surprising that COX activity which was activated by exogenous DNP administration was not affected by the preparation since synthesis of prostaglandins continued. Therefore, it is suggested that the pill does not compete with arachidonic acid at the active site of COX; hence it does not have antipyretic activity. Antipyretics have been reported to compete with arachidonic acid at the active site of cyclooxygenase (Insel, 1996). Most of the currently available antipyretics inhibit both cyclooxygenase I and cyclooxygenase 2 (COX-1 and COX-2, respectively), inhibiting the synthesis of prostaglandin and thromboxane (Insel, 1996). Inhibition of COX-2 is thought to mediate, at least in part, the anti-pyretic action of aspirin and related antipyretic drugs while inhibition of COX-1 results in the unwanted side effects associated with this drug.

The crude pill inhibited castor oil-induced diarrhoea in rats in a dose-dependent manner producing maximal inhibition at 600 and 900 $mg^{-1}kg$ b.wt., respectively. Inhibition of experimental diarrhoea and reduction in faecal output by a substance are the basis of the pharmacological evaluation of a potential anti-diarrhoeal agent (Akah *et al*., 1999). It was able to protect against castor oil-induced diarrhoea, and the reduction in faecal output in this study. The mode of action may be through the inhibitory action on the transmembrane fluxes of Ca^{2+} as suggested by Seung *et al* (2004) , therefore it may be suppressing diarrhoea by direct inhibition of myolysis via calcium blockade, and possibly by its antimicrobial potential against *Escherichia coli* and other micro-organisms causing diarrhoea.

The mucilage and potash added in the tri-herbal preparation may have re-enforced the crude pill's efficacy. Mucilage from cassava was found to be a very good binding agent (Uhumwangho, 2006), and has antioxidant properties (Fu et al, 2004). *Sijima and Ohzeki (2008) had shown from clinical studies that replacement therapy with NaCl and KCl in Congenital chloride diarrheal patient normalized serum electrolytes and only KCl was administered in adolescence.* A combined effect of the *Manihot esculentus*, starch mucilage and potash may probably have suppressed the laxative and purgative effect of *croton penduliflorus* seed (Asuzu *et al.,* 1988) and *cassia podocarpa* (Elujoba *et al.,* 1989) to produce the observed anti- diarrhoeal effect in the rats.

In summary, this study reports that a crude preparation, "Jedi-Jedi" pill possesses anti-inflammatory and anti-diarrhoeal capabilities but lacks anti-pyretic action. It therefore, partially supports the claim by traditional medicine practitioners and hawkers that this tri-herbal preparation is effective for managing haemorrhoids. The study also reports its non toxicity but with side effects. Further research is therefore recommended to investigate the full implications of observed side effects.

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Nature and Science

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Reference Examples:

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Book: Berkowitz BA, Katzung BG. Basic and clinical evaluation of new drugs. In: Katzung BG, ed. Basic and clinical pharmacology. Appleton & Lance Publisher. Norwalk, Connecticut, USA. 1995:60-9.

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