Pharmacognostic and Preliminary Phytochemical Investigations on *Holoptelea integrifolia*

Padmaja V., Srisailam K.*, Reddy V.M.

Vaagdevi College of Pharmacy, Ramnagar, Hanamkonda, Warangal 506001, AP, India

Abstract

Various parts of *Holoptelea integrifolia*, a roadside plant, are indicated by Charaka Samhitha, Sushrutha Samhitha and other traditional systems for the treatment of inflammations, acid gastritis, dyspepsia, flatulence, colic, intestinal worms, vomiting, wounds, vitiligo, leprosy, filariasis, diabetes, haemorrhoids, dysmenorrhoea and rheumatism. The present study was aimed at pharmacognostic and preliminary phytochemical investigations of *H. integrifolia* leaves and bark. The pharmacognostic investigations were carried out in terms of organoleptic, microscopic and physical parameters. The dried leaves and bark were subjected to successive Soxhlet extraction using petroleum ether, chloroform, ethyl acetate and methanol. These solvent extracts were subjected to a preliminary phytochemical screening to detect the different chemical principles present viz., carbohydrates, proteins, amino acids, steroids, glycosides, alkaloids, tannins and phenolic compounds. The phytochemical analyses indicate that the plant contains carbohydrates, proteins, amino acids, steroids, glycosides, alkaloids, tannins and phenolics.

Keywords: *Holoptelea integrifolia*, Pharmacognosy, Phytochemistry

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***Author for correspondence:** Email: ksrisailam@yahoo.com

INTRODUCTION

Natural products are a source for bioactive compounds and have potential for developing some novel therapeutic agents. Over the last decade there has been a growing interest in drugs of plant origin and such drugs formed an important class for disease control. Among the known plant species, only a small percentage has been investigated for phytochemicals and pharmacological activities. *Holoptelea integrifolia* Planch (Syn. *Ulmus integrifolia*) is a roadside tree of the family Ulmaceae and commonly known as the Indian elm. The plant was found to be distributed in India, Sri Lanka, Myanmar, China, and Malaysia (1). The tree offers several classes of chemical constituents in which triterpenoids and sterols constitute a major portion reported so far. Various parts of the tree were found to be useful in the treatment of bronchitis and obesity (2, 3). The tree was found to possess oviposition deterrent activity and protease inhibitory activity (4, 5). Padmaja et al (6) have reviewed the phytochemical and pharmacological reports related to *H. integrifolia*.

The present investigation dealt with the pharmacognostic parameters of the leaves and bark of *H. integrifolia*, and also with preliminary phytochemical evaluation of different solvent extracts of leaves and bark. The leaves and bark were studied to know their organoleptic, microscopic, and physical parameters. The successive petroleum ether, chloroform, ethyl acetate, and methanol extracts of these two parts of the plant were examined for their phytochemical principles.

MATERIALS AND METHODS

Chemicals

All the chemicals were of highest available purity and were procured from E. Merck, Mumbai, India, HiMedia Laboratories, Mumbai, India and SD Fine Chemicals, Mumbai, India.

Procurement of plant material

The leaves and bark of *H. integrifolia* as identified by a qualified Taxonomist, were collected from the wild growing

tree in the Kakatiya University campus, Warangal, India. A specimen was deposited in the institutional herbarium. The collected plant material was made thoroughly free from any foreign organic matter and a part of the material was dried under shade.

Pharmacognostic Evaluation

Organoleptic evaluation

In organoleptic evaluation, various sensory parameters of the plant material, such as size, shape, color, odor, and taste of the leaves, bark and their powders were recorded.

Microscopic evaluation

In this study, stomatal index was determined for fresh leaves and powder analyses were performed for dried leaf and bark powders. The stomatal index was studied by using *camera lucida*, and the type of stomata present in the leaves was also recorded. Various diagnostic characters of leaf and bark powders of *H. integrifolia* were studied by microscopical analyses with or without staining.

1. Powder analysis of leaf and bark

To a little quantity of powder taken onto a microscopic slide, 1-2 drops of 0.1% phloroglucinol solution and a drop of concentrated hydrochloric acid were added and covered with a cover slip. The slide preparation was mounted in glycerol and examined under microscope. Presence of starch grains was detected by the formation of blue color on addition of 2-3 drops of 0.01M iodine solution.

2. Determination of stomatal index

Leaf fragments of about 5 × 5 mm in size were taken in a test tube containing 5 ml of chloral hydrate solution and boiled on water bath until the fragments became transparent (~15 min). These fragments were transferred onto microscopic slide and mounted in glycerol. The slide was examined with 40 × objective and 6 × eye piece to which a *camera lucida* was attached and recorded the epidermal cells and stomata lying within a selected area. Stomatal index was calculated as the percentage of number of stomata present per number of epidermal cells and each stoma was counted as one cell.

Physical evaluation

In physical evaluation, the total ash, alcohol, water, and ether soluble extractive values were determined. The determinations were performed in triplicate and the results are expressed as mean ± SD. The percentage w/w values were calculated with reference to the air-dried drug.

1. Determination of total ash

Accurately weighed powder (2 g) of both leaves and bark were taken separately in a pre-weighed ash-less filter paper and incinerated at 400°C for about 3-4 min or until the vapors completely ceased. The temperature was gradually reduced to come to normal and then the contents/ash was collected and weighed.

2. Determination of alcohol soluble extractive

Accurately weighed powder (5 g) of both leaves and bark were taken separately and macerated with 100 ml of 95% alcohol for 24 h. The contents were frequently shaken during the first 6 h and allowed to remain for 18 h. After 24 h, the extract was filtered and 25 ml of the filtrate was evaporated. The extract was dried at 105°C to a constant weight.

3. Determination of water soluble extractive

Water soluble extractive value was determined using the procedure described for alcohol soluble extractive, except that chloroform water was used for maceration.

4. Determination of ether soluble extractive

Accurately weighed powder (5 g) of both leaves and bark were taken separately and a thimble pack was prepared for each. The crude drug in the pack was extracted with solvent ether in a continuous extraction (Soxhlet) apparatus for 6 h. The extract was filtered; the filtrate was evaporated and dried at 105°C to a constant weight.

Preliminary Phytochemical Screening

The leaf and bark powders separately were subjected to successive extraction in a Soxhlet apparatus using petroleum ether (60-80°C), chloroform, ethyl acetate, and methanol, and the extracts were evaporated to dryness, at room temperature. The dried extracts were weighed, and percentage yields were calculated. The extracts were used for preliminary phytochemical screening with a battery of chemical tests viz., Molisch's, Fehling's, Benedict's and Barfoed's tests for carbohydrates; Biuret and Millon's tests for proteins; Ninhydrin's test for amino acids; Salkowski and Liebermann-Burchard's reactions for steroids; Borntrager's test for anthraquinone glycosides; foam test for saponin glycosides; Shinoda and alkaline tests

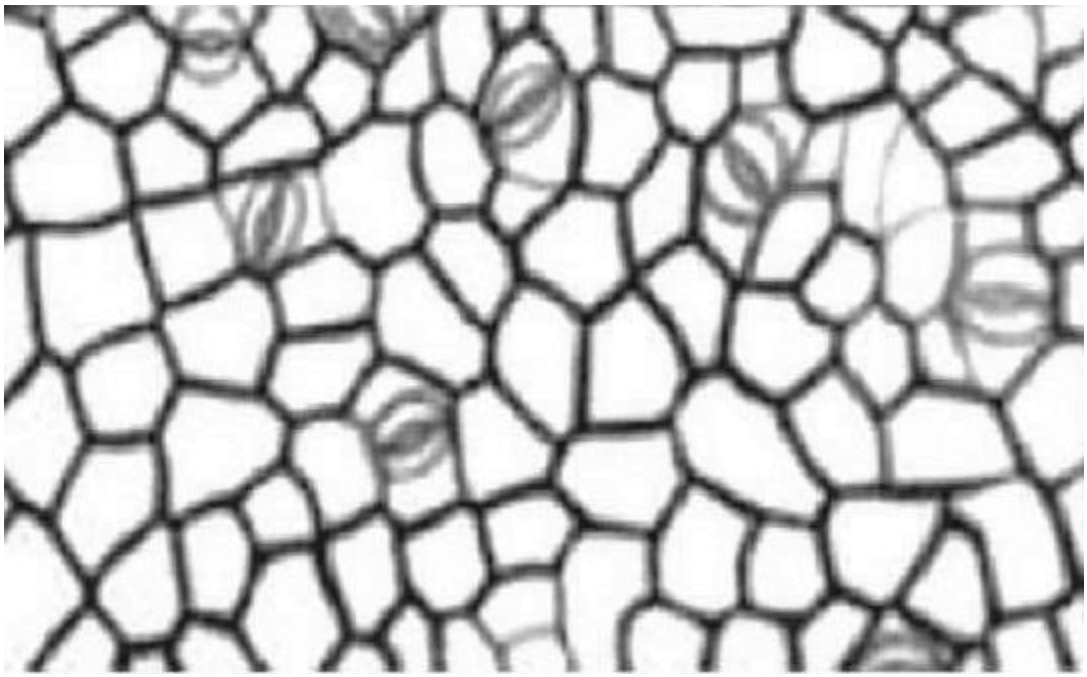

Figure 1. Stomata in lower epidermis of *Holoptelea integrifolia* leaf

for flavonoid glycosides; Dragendorff's, Mayer's, Hager's and Wagner's tests for alkaloids; and ferric chloride, lead acetate, potassium dichromate and dilute iodine tests for tannins and phenolics.

RESULTS AND DISCUSSION

Pharmacognostic Evaluation

Organoleptic and microscopic evaluation

In organoleptic evaluation, appropriate parameters like taste, odor, size, shape and color of the leaves, bark, and their powders were studied. The leaves, bark, and their powders were found to be bitter in taste with unpleasant odor. The leaves were found to be 4-8 cm in length and 2-5 cm in width. The leaves were found to be ovate-elliptic, alternate base rounded or subcordate, entire margin, short acuminate apex, subcoriaceous and glabrous in shape. The bark is of irregular stripes in nature. The leaves and their powder were found to be green in color, whereas the bark and its powder were whitish grey.

Upon microscopic evaluation, the leaves or their powder could show the presence of square, prismatic and rod shaped calcium oxalate crystals; closely packed and straight walled polygonal epidermal cells; palisade cells; thin walled polygonal cells with no intercellular spaces in the parenchyma; anomocytic stomata in lower epidermis (Fig. 1); a stomatal index of 10-15 with lower epidermis; liginified, unicellular, covering trichomes with acute apex and narrow lumen; lignified xylem fibres and scalariform xylem vessels. Similarly bark or its powder has shown the presence of prismatic and rod shaped calcium oxalate crystals; cork cells; mucilaginous parenchyma; lignified phloem fibres and, simple and concentric starch grains.

Physical evaluation of leaves

The results reveal that the leaf and bark powders produced 18.6 ± 0.2 and 7.1 ± 0.2 % w/w of total ash respectively. The ethanol, water and ether soluble extractive values of the leaf powder were found to be 15.2 ± 0.2, 18.2 ± 0.2 and 2.1 ± 0.1 % w/w respectively, and the bark powder produced 6.3 ± 0.2, 7.4 ± 0.2 and 4.1 ± 0.1 of these values respectively. Bhadauria et al. (7) reported the total ash and ether soluble extractive values for leaf powder of *H. integrifolia* as 15.34 and 2% w/w, respectively.

Preliminary Phytochemical Evaluation

The powders of leaves and bark of *H. integrifolia* were extracted with petroleum ether, chloroform, ethyl acetate and methanol and the nature and yield of the extracts were observed. The yield of the extracts was more from leaves than that of the bark. All the solvent extracts of leaves were found to be semisolid in nature. Chloroform

and methanol extracts were of green in color whereas petroleum ether and ethyl acetate produced blackish green extracts. The yields of petroleum ether, chloroform, ethyl acetate and methanol extracts were found to be 3.8, 2.89, 3.2 and 3.1 % w/w respectively. Similarly all the solvents except petroleum ether produced semisolid extracts from bark powder. Petroleum ether, chloroform, ethyl acetate and methanol produced yellow, brownish yellow, brown and reddish brown colored extracts with yields of 1.039, 0.225, 0.432 and 2.315 % w/w respectively.

The leaf and bark extracts obtained from different solvents were tested for the presence of various phytochemicals, such as: carbohydrates, proteins, amino acids, steroids, glycosides, alkaloids, tannins, and phenolics. The results indicate that all these classes of compounds were present in both leaves and bark powders. Carbohydrates were present in chloroform, ethyl acetate and methanol extracts of both leaves and bark. Proteins, amino acids, alkaloids, tannins and phenolics were found to be present in methanol extracts of leaves as well as bark. Petroleum ether, chloroform and ethyl acetate extracts of leaves could show the presence of steroids, whereas the only petroleum ether and ethyl acetate of bark were positive for steroids. Anthraquinone glycosides were present in all the extracts of leaves, and petroleum ether and methanol extracts of bark. Methanol extracts of leaves and bark were found to contain saponin and flavonoid glycosides. Presence of the flavonoid glycosides was also observed in chloroform extract of leaves.

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Pharmacognostical Standardization of Leaves of *Hygrophila spinosa* T. Anders

Patra Arjun[1]*, Jha Shivesh[2], Narasimha Murthy P.[3]

[1] Department of Pharmacognosy and Phytochemistry, College of Pharmacy, IFTM, Moradabad-244 001, U.P., India

[2] Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra-835 215, Ranchi, Jharkhand, India.

[3] Royal College of Pharmacy & Health Sciences, Berhampur-760 002, Orissa, India.

ABSTRACT

The present study deals with pharmacognostical evaluation of leaves of *Hygrophila spinosa* T. Anders (Acanthaceae). Macromorphography and microscopy (transverse section, powder microscopy and quantitative microscopy) were studied to establish the salient diagnostic characters. WHO recommends physicochemical determination and phytochemical evaluation for quality control of medicinal plant materials. The various morphological, microscopical, physicochemical standards developed in this study will help for botanical identification and standardization of the drug in crude form. Further, the authentic plant material can be explored for its pharmacological and phytochemical potential.

Keywords: *Hygrophila spinosa*, Pharmacognostical studies, Physicochemical parameters, Quantitative microscopy, TLC finger printing

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*** Corresponding author:** E-mail: arjun.patra@rediffmail.com

INTRODUCTION

Hygrophila spinosa T. Anders (Acanthaceae) is described in Ayurvedic literature as Ikshura, Ikshugandha and Kokilasha "having eyes like Kokila or Indian cuckoo", common in moist places on the banks of tanks, ditches, paddy fields etc., widely distributed throughout India from Himalayas to Ceylon, Srilanka, Burma, Malaysia and Nepal (1–4). The plant contains various groups of phytoconstituents viz. phytosterols, fatty acids, minerals, polyphenols, proanthocyanins, mucilage, alkaloids, enzymes, amino acids, carbohydrates, hydrocarbons, flavonoids, terpenoids, vitamins, glycosides etc and is useful in the treatment of anasaraca, diseases of urinogenital tract, dropsy of chronic Bright's disease, hyperdipsia, vesical calculi, flatulence, diarrhoea, dysentery, leucorrhoea, gonorrhoea, asthma, blood diseases, gastric diseases, inflammation, cancer, rheumatism, painful micturition, menorrhagea etc (5–8). Insufficient data was found in the literature about the pharmacognostical characteristics of the leaves of *H. spinosa*. Hence, the present study was designed to study the detail pharmacognostic study of the leaves including macroscopy, microscopy, quantitative microscopy, determination of various physicochemical parameters, fluorescence characters of powder, phytochemical screening and TLC profile of different leaf extracts, and determination of inorganic elements etc.

MATERIALS AND METHODS

Collection and authentication of plant material

H. spinosa plants were collected from Berhampur, Orissa, India and botanical identification was done by Dr. N.K. Dhal, scientist, Regional Research Laboratory, Bhubaneswar (Access no. 9999) and also through Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi (Voucher no. BITPcog. 463/07-08). Voucher specimens were preserved in the department of the institutions for further verification.

Morphological and microscopical studies

The macromorphology of the leaves were studied according to standard methods (9–11). Hand section of the leaf was taken, stained and mounted following usual microtechniques (12) and representative diagrams were taken with the help of inverted microscope for photodocumentation (Leitz, Japan). The different powder

characteristics were studied according to standard methods (13–14). Separate slides were prepared for observation of lignified tissues (phloroglucinol + HCl), starch (iodine solution) and non-lignified characters.

Quantitative microscopy

Various leaf constants as stomatal number, stomatal index, veinislet number, veinlet termination number and palisade ratio were determined. Average length of trichome per mg of leaf powder and average epidermal area per g of leaf powder were also determined (11, 15).

Determination of physicochemical parameters

Physico-chemical parameters i.e. percentage of moisture content, percentage of ash values and extractive values were performed according to the official methods (16) and the WHO guidelines on the quality control methods for medicinal plant materials (17). Fluorescence analysis was carried out following reported methods (18–19).

Preliminary phytochemical screening

The shade dried and coarsely powdered leaves were extracted successively with petroleum ether, chloroform and alcohol using soxhlet apparatus. Finally the aqueous extract was prepared by decoction. Different extracts were screened for the presence of various groups of phytoconstituents using different chemical tests (20–24). The ash of the leaf powder was prepared by incinerating the powder in muffle furnace. 50% v/v HNO_3 was added to the ash and kept for 2 hour, then filtered and the filtrate was used for conforming the presence or absence of different inorganic elements (23).

TLC finger print profile

Thin layer chromatography of the petroleum ether, chloroform, alcoholic and aqueous extracts was studied (25) and the R_f values were determined.

RESULTS

Morphological characteristics

Leaves are dark-green to brownish green, fleshy, odourless and slightly bitter in taste, 1–18 cm long, 0.5–2.5 cm wide, sessile, oblong-lanceolate or oblanceolate, two outer leaves are larger in size than the four inner leaves, simple, acute apex, entire margin, sparsely hispid on both sides, pinnate and the lateral veins end at margin, each

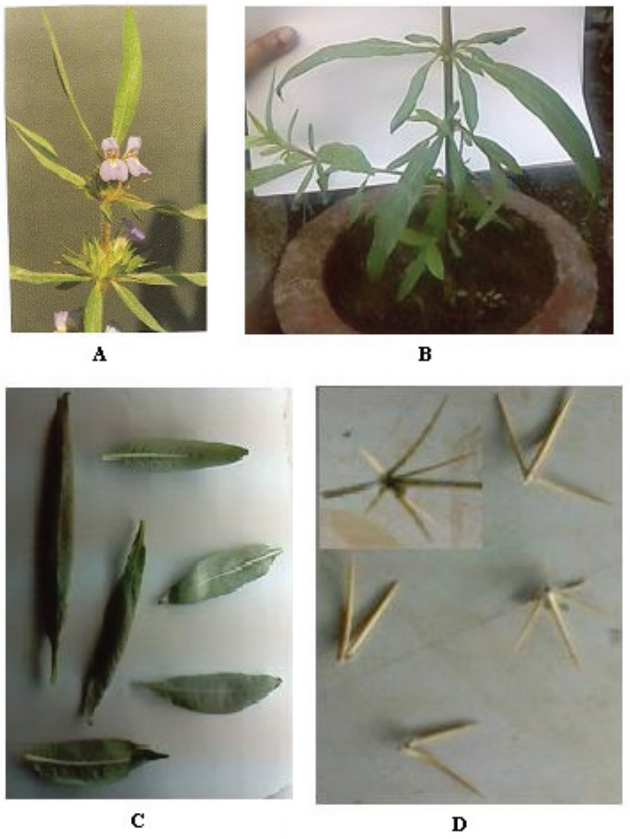

Figure 1. Morphology of *Hygrophila spinosa*
A, flowering shoot; B, Arrangement of leaves at node; C, Leaves; D, Spines of the plant and their arrangement at the node

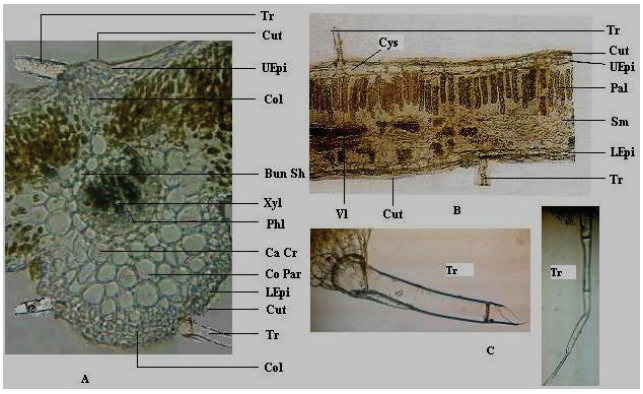

Figure 2. Transverse section of leaf of *Hygrophila spinosa*
A, Transverse section through midrib
B, Transverse section through lamina
C, Trichomes
Abbreviations: Tr, trichome; Cut, cuticle; Col, collenchyma; Pal, palisade parenchyma; UEpi, upper epidermis; Vl, veinlets; Sm, spongy mesophyll; LEpi, lower epidermis; Phl, phloem; Ca Cr, calcium oxalate crystal; Co Par, cortical parenchyma; Cys, cystolith; Bun Sh, bundle sheath; Xyl, xylem

of the six leaves with nearly straight sharp yellow spines, decurrent base and stipules absent (Figure 1).

Microscopical characteristics (Figure 2)

Midrib

Transverse section of the midrib shows concavo-convex outline in the basal and middle region which becomes more or less plano convex in the apical region. Upper and lower epidermis consists of single layer of cells covered with thick cuticle. Covering trichomes are present on both the surfaces, but the number is less in the apical region as compared to the basal and middle region. The trichomes are uniseriate, 1-5 celled with blunt ends. The epidermal cells do not contain cystolith (calcium carbonate crystals) in the midrib region. The upper and lower epidermis is followed collenchymatous cells, but the development and abundance is more in the basal region in comparison to middle and apical region. Collenchymatous cells show well developed angular thickening in the basal and middle region of midrib, whereas the angular thickening reduces considerably in the apical region. Collenchyma on both dorsal and ventral side is followed by a zone of parenchyma consisting round to oval thin walled parenchymatous cells showing small to distinct intercellular spaces. The cortical parenchymatous cells in the ventral side contain acicular crystals of calcium oxalate. Vascular bundle composed of small strands of xylem and phloem is present in the centre. Vascular bundle is covered with a single layer of border parenchyma (bundle sheath). The xylem and phloem of the vascular bundle in the basal region are more developed than the middle and apical region.

Lamina

Lamina has a structure of dorsiventral type of leaf with its mesophyll differentiated into palisade and spongy parenchyma. Lamina shows single layered epidermis on either surface covered with thick cuticle. Diacytic stomata and covering trichomes are present on both the surfaces. Trichomes are uniseriate, 1-5 celled with blunt ends. Large flat bunch shaped or elongated club shaped calcium

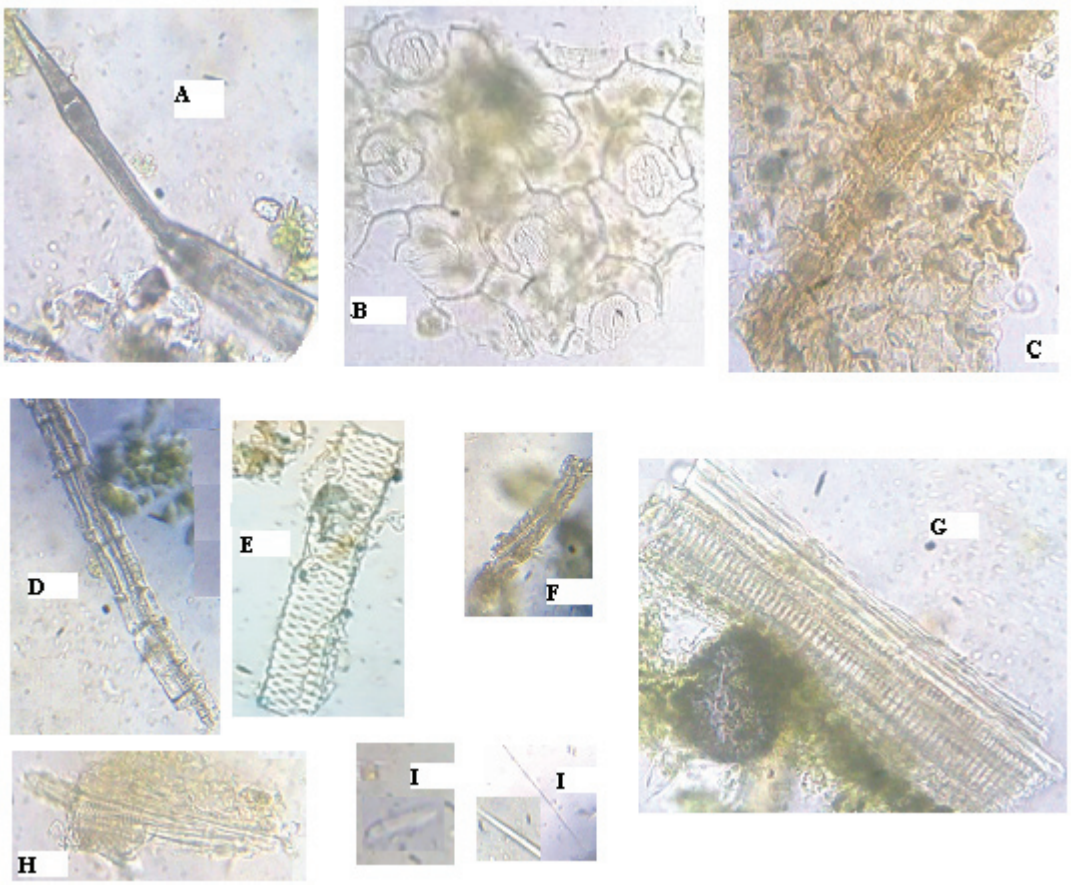

Figure 3. Powder characteristics of *Hygrophola spinosa* leaf
A- covering trichomes; B- epidermal cells with diacytic stomata; C- spongy mesophyll with veinlet; D- fibres; E- reticulate vessel; F- scalarifirm vessel; G- scalariform vessel with fibre; H- fibres with reticulate vessel; I- acicular crystals of calcium oxalate

Table 1. Leaf Constants of *Hygrophila Spinosa*

Leaf Constant	Range	Average
Stomatal No. (Upper surface)	9.0 - 19.0	13.4
Stomatal No. (Lower surface)	10.0 - 21.0	14.6
Stomatal index (Upper surface)	15.38 - 25.80	21.5
Stomatal index (Lower surface)	27.77 - 37.50	32.0
Vein-islet No.	15.0 - 32.0	23.6
Veinlet termination No.	17.0 - 29.0	22.4
Palisade ratio	6.0 - 13.5	8.6
Average length of trichome per mg of leaf powder	269.5 cm	
average epidermal area per g of leaf powder	1490.3 cm^2	

carbonate crystals (cystolith) are present in the epidermal cells. The base of the trichomes in the epidermis look like a swollen mass consisting more amounts of calcium carbonate crystals. Below the upper epidermis 1-2 layered palisade parenchyma is present, which are loosely arranged. The spongy mesophyll is represented by 3-5 layers cells, which are loosely arranged. The mesophyll is traversed by large number of veins and is represented by group of few spiral vessels. In the extreme end of lamina the palisade cells are less or replaced by spongy mesophyll.

Powder characters

The powder is green in colour and contains diacytic stomata, covering trichomes, fibres, epidermal cells with diacytic stomata, spongy parenchyma with veinlet, lignified reticulate and scalariform vessels, and acicular calcium oxalate crystals (Figure 3).

Quantitative microscopy

The results Quantitative microscopy is furnished in Table 1.

Physicochemical parameters

Physico-chemical constants like percentage of moisture content, total ash, acid insoluble ash, water soluble ash, sulphated ash, petroleum ether soluble extractive, chloroform soluble extractive, water soluble extractive and ethanol soluble extractive were determined and depicted in Table 2. The results of fluorescence analysis of the drug powder are presented in Table 3.

Preliminary phytochemical screening

Preliminary phytochemical screening revealed the presence of phytosterols, tannins, carbohydrates, flavonoids, proteins, fats & oils, amino acids, organic acids, glycosides and alkaloids (Table 4). Various inorganic elements present in the leaf are iron, sulphate, phosphate, chloride and sodium.

Table 2. Physicochemical Parameters of *Hygrophila spinosa* Leaf

Parameter	% w/w*
Total ash	17.83
Acid-insoluble ash	7.13
Water-soluble ash	1.87
Sulphated ash	12.53
Petroleum ether soluble extractive	2.03
Chloroform soluble extractive	3.60
Alcohol soluble extractive	16.63
Water soluble extractive	24.33
Moisture content	8.50

* average of three readings

TLC finger print profile

Thin layer chromatography of the petroleum ether, chloroform, alcoholic and aqueous extracts was carried out using Toluene: Ethyl acetate, Toluene: Diethyl ether: Cyclohexane, Toluene: Ethyl acetate: Pyridine and Methanol: Chloroform: Pyridine as mobile phase respectively and the R_f values were recorded (Table 5).

DISCUSSION

Leaves of *H. spinosa* are dark-green to brownish green in colour, oblong-lanceolate or oblanceolate, odourless and slightly bitter in taste. Transverse section of the leaf shows presence of 1-5 celled covering trichome, cystolith and acicular crystals of calcium oxalate in the lamina and midrib region respectively, collenchyma below upper epidermis and above lower epidermis, vascular bundle

Table 3. Fluorescence Analysis of *Hygrophila spinosa* Leaf Powder

Treatment	Normal Light	Under UV Light	
		254 nm	365 nm
Dry Powder	Dark Green	Greenish Brown	Black
Powder + 5% NaOH	Brownish Green	Greenish Brown	Violet
Powder + 5% KOH	Green	Greenish Brown	Black
Powder +Ferric Chloride	Dark Brown	Violet	Black
Powder + Conc. H_2SO_4	Yellowish Brown	Brownish Green	Dark Brown
Powder + Dil. Ammonia	Brownish Green	Dark Green	Dark Brown
Powder + Conc. HCl	Greenish Brown	Brownish Green	Dark Brown
Powder + Conc. HNO_3	Yellowish Red	Greenish Brown	Dark Brown
Powder + Iodine Solution	Greenish Brown	Dark Green	Black
Powder + Dil. HCl	Brownish Green	Brownish Green	Dark Brown
Powder + Dil. H_2SO_4	Brownish Green	Light Green	Black
Powder + Dil. HNO_3	Green	Brownish Green	Black
Powder + Sodium Carbonate	Light Green	Dark Green	Black
Powder + Alcoholic KOH	Brownish Black	Brownish Green	Brownish Green
Powder + NH_4OH	Green	Brown	Brown
Powder + Formaldehyde	Dark Green	Brown	Deep Brown
Powder + Bromine water	Brownish Green	Yellowish Brown	Yellowish Brown
Powder + $KMnO_4$	Green	Brown	Brown
Powder + Silver Nitrate	Green	Yellowish Green	Yellowish Green
Powder + Barium Chloride	Green	Brown	Brown

Table 4. Preliminary Phytochemical Investigation of Different Extracts of *Hygrophila spinosa* Leaf

Group of phytoconstituent	Extract			
	Petroleum ether	Chloroform	Alcoholic	Aqueous
Alkaloids	–	+	+	–
Carbohydrates	–	–	+	+
Gums and mucilage	–	–	–	+
Proteins	+	+	+	+
Fats and oils	+	+	–	–
Amino acids	–	–	–	+
Steroids	–	+	+	–
Glycosides	–	–	+	–
Cardiac glycosides	–	–	–	–
Anthraquinone glycosides	–	–	–	–
Saponin glycosides	–	–	–	–
Coumarins	–	–	–	–
Flavonoids	+	+	+	–
Tannins and phenolic compounds	–	–	+	+
Organic acids	–	–	–	+

+ indicates present and – indicates absent

Table 5. Thin layer chromatography of different extracts of *Hygrophila spinosa* leaf

Extract	Mobile phase	Number of spots and their R_f value
Petroleum ether	Toluene: Ethyl acetate (8:1)	Four spots R_f value- 0.13, 0.54, 0.63 and 0.89
Chloroform	Toluene: Ether: Cyclohexane (5:2:1)	Five spots R_f value- 0.40, 0.58, 0.66, 0.74 and 0.90
Alcoholic	Toluene: Ethyl acetate: Pyridine (17:2:1)	Five spots R_f value- 0.08, 0.44, 0.53, 0.75 and 0.89
Aqueous	Methanol: Chloroform: Pyridine (2:2:1)	Two spots R_f value- 0.37 and 0.67

covered with a single layer of border parenchyma (bundle sheath) is situated in the centre of midrib. The xylem and phloem of the vascular bundle in the basal region are more developed than the middle and apical region; collenchymatous cells show well developed angular thickening in the basal and middle region of midrib than the apical region. In the lamina region upper epidermis is followed by 1-2 layers of palisade parenchyma and in the extreme end of lamina palisade cells are replaced by spongy mesophyll. Average length of trichome per mg of leaf powder and average epidermal area per g of leaf powder are 269.5 cm and 1490.3 cm^2 respectively. Various physicochemical parameters like moisture content, ash values, extractive values, TLC and phytochemical screening of various extracts were established to substantiate standardization data on *H. spinosa*.

The various morphological, microscopical, physicochemical standards developed in this study will help for botanical identification and standardization of the drug in crude form. Further, the authentic plant material can be explored for its pharmacological and phytochemical potential.

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Pharmacognostical studies on the leaf of *Annona squamosa* Linn.

Sharma Abhishek L.*, and Vd. Goray Namrata P.

Herbal Research Lab, Ramnarain Ruia College, Matunga (East), Mumbai-400019.

ABSTRACT

The present communication deals with the macroscopical, microscopical & preliminary phytochemical studies on the leaf of *Annona squamosa* Linn. *Annona squamosa* Linn is used for the treatment of various diseases & ailments viz. stimulant, antispasmodic, sudorific, anthelmintic and insecticidal properties etc. No reports are available on the pharmacognostic nature of the leaf, hence, the present study was undertaken to investigate the same. All the parameters were studied according to the WHO & Pharmacopoeial guidelines. The qualitative phytochemical fingerprint of the methanolic extract revealed the presence of alkaloids, terpenoids and phenolics, fats and waxes. The aqueous leaf slurry was found to be safe at the dose level of 2g/kg body weight of mice.

Keywords: *Annona squamosa* Linn., Pharmacognostic, Acute toxicity.

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Author for Correspondence: abhishek220484@yahoo.com

INTRODUCTION

Annona squamosa Linn. also known as Sitaphala, Custard-Apple, belongs to the family Annonaceae. *Annona squamosa* Linn. is widely cultivated throughout India as an ornamental plant & deciduous tree. Leaves are reported to posses stimulant, antispasmodic, sudorific, anthelmintic and insecticidal properties. The crushed leaves are reported to be applied to the nostrils in hysteria and fits. The poultice of the leaves is used as a cataplasm over boils and ulcers to induce suppuration. It also relieves pain and swellings [1]. The ethanolic extract of the leaves has been found to show activity against L-1210 lymphoid leukemia, sarcoma 180, Walker carcinoma 256, and epidermoid carcinoma of human nasopharynx in tissue culture. The extracts with acid, ether and acetate buffer have shown antibiotic activity against Micrococcus pyogenes var. aureus. The water-soluble portion of the alcoholic extract of leaf was found to stimulate the isolated heart and relax the isolated duodenum of rabbit, exert spasmogenic effect on the ileum of guinea pig, and increase the blood pressure of the anaesthetized animals; the extract also possessed oxytocic activity on the uterus of rat. The active principle is very similar to adrenaline in its pharmacological action [2].

Herbal medicine is a triumph of popular therapeutic diversity. Almost in all the traditional medicine, the medicinal plants play the major role & constitute the backbone for the same. In order to make sure the safe use of these medicines, a necessary first step is the establishment of standards of quality, safety & efficacy [3]. Keeping these facts into consideration, attempts are made to establish pharmacognostic standards of the plant leaf. *Annona squamosa* Linn. is a large evergreen, straggling shrub or small tree, 7 m in height, introduced into India, found wild and cultivated in various parts, up to an altitude of 900m. Bark thin, grey; leaves oblong-lanceolate or elliptic, pellucid-dotted, peculiarly scented, 5.0–15.0 cm × 1.9- 3.8 cm; flower 1–4, greenish, fleshy, drooping, extra- axillary, more on the leaf shoot than on the older wood, tending to open as the shoot enlongates; carpels many, lozenge-shaped, on a central torus, fused into am irregularly globose or heart shaped, tubercled, yellowish green syncarpium, 5–10 cm in diameter; seeds oblong, deep brownish black, aril shining, covered with whitish pulp [2–4].

The present study investigates the macroscopical, microscopical & preliminary phytochemical nature of the leaf of *Annona squamosa* Linn.

MATERIALS AND METHODS

The leaves of *Annona squamosa* Linn. was collected from the local area of Karjat, Maharashtra (India). It was authenticated by Agharkar Research Institute, Pune. The leaves were stored under the normal environmental condition. The macroscopical characters of the leaf were studied as per the procedure given in WHO guidelines & Indian herbal Pharmacopoeia. Physico-chemical parameters such as extractive values, ash values, loss on drying were performed as per the official standard procedures [5–6]. Microscopical investigations were made with transverse section & powder microscopy of the leaf. Transverse section of the leaf was taken for microscopic examination and the powder microscopy was performed according to the prescribed procedure [7–8]. For phytochemical screening 5 gram powdered leaf was subjected for Soxhlets extraction as described by Harborne [9]. To evaluate safety of leaf powder acute toxicity study on Swiss Albino mice was performed as per the detail laid down in the OECD guidelines 420 viz., Fixed Dose Procedure (Evident Toxicity) [10].

RESULT AND DISCUSSION

Macroscopically the leaf was found to be leaves oblong-lanceolate or elliptic, pellucid-dotted, peculiarly scented [Figure 1, Table 1]. The T.S. of lamina of leaf showed the presence of single layered epidermal cells, mesophyll differentiated into palisade tissues and spongy parenchyma of 3 to 5 layers, stomata anomocytic, present on lower surfaces only [Figure 2]. T.S. of midrib showed single layer epidermis on both surfaces, collenchymatous cell, followed by thin walled, round or oval parenchymatous cells, an arc shaped vascular bundle consisting of xylem and phloem, present in centre, beneath the vascular bundle lies a layer of cortical parenchyma cell followed by lower epidermis [Figure 3]. Powder microscopy shows the presence of spiral vessel, epidermal cell, trichome with leaf attachment, trichome, parenchyma cells [Figure 4]. The values of the physical constant like ash values, extractive values, loss on drying were determined [Table 2]. Preliminary qualitative phytochemical screening of the methanolic extract revealed the presence fats and waxes, terpenoids and phenolics, Basic extract-Most alkaloids and Polar extract-Quaternary alkaloids and N-oxide. Chromatographic studies of methanolic extract showed three distinct spots after detection in UV mode at 254 nm with the phytoconstituents separated [Figure 5 and 6, Table 3]. From acute toxicity study carried out on Swiss albino mice the aqueous leaf slurry was found to be non-toxic at dose level of 2g/kg body weight of mice [Table 4].

Table 1: Macroscopical Evaluation of *Cassia fistula* L bark

Sr. No	Features	Observations
1	Color	Upper surface - deep green, Lower surface - paler
2	Odour	Bitter
3	Taste	Bitter
4	Shape	Acute
6	Size	Length: 10 – 15 cm, Width: 3 – 6 cm.

Table 2: Physio-chemical Parameter for *Annona squamosa* Linn.

Sr. No.	Parameters	% Content*
1	Foreign organic matter	0.252 ± 0.014
2	Ethanol soluble extractive	20.2698 ± 0.3326
3	Water soluble extractive	31.3172 ± 0.9060
4	Total ash	6.8467 ± 0.1258
5	Acid-insoluble ash	0.9576 ± 0.0075
6	Water soluble ash	4.4476 ± 0.0712
7	Loss on drying	7.74 ± 0.6863

* Each result is expressed as Mean ± Standard deviation of three readings

Table 3: Percentage of phytochemical Constituents of *Annona squamosa* Linn.

Phytochemical Constituents	% Extract*
Neutral extract - Fats & waxes	2.5033 ± 0.015
Moderately polar extract-Terpenoids and Phenolics	4.19 ± 0.020
Basic extract-Most alkaloids	26.78 ± 0.020
Polar extract-Quaternary alkaloids and N-oxides	1.6226 ± 0.0023
Fibers	60.66 ± 1.5275

* Each result is expressed as Mean ± Standard deviation of three readings.

CONCLUSION

The microscopic characteristics of the leaf powder have been presented for quality assurance at the collection and production stages. A key (anatomical marker) has been developed for leaf powders of *Annona squamosa* Linn. This may enable the detection of adulterants from the genuine raw materials of *Annona squamosa* Linn. Phytochemical profile, foreign organic matter, total ash, acid insoluble ash, water soluble ash, ethanol soluble extractive, water soluble extractive, loss on drying, have also been determined. Acute toxicity study of slurry of leaf powder

Table 4: Cage side observations for Acute Toxicity study

Parameters Observed	Observation
Condition of the fur	Normal
Skin	Normal
Subcutaneous slug	Nil
Abdominal distension	Nil
Dullness of the eyes	Nil
Opacity of the eyes	Nil
Discharge from the eyes	Nil
Ptosis of the eyes	Nil
Pupil diameter	Normal
Colour and consistency of faeces	Normal
Condition of teeth	Normal
Breathing abnormalities	Nil
Gait	Normal

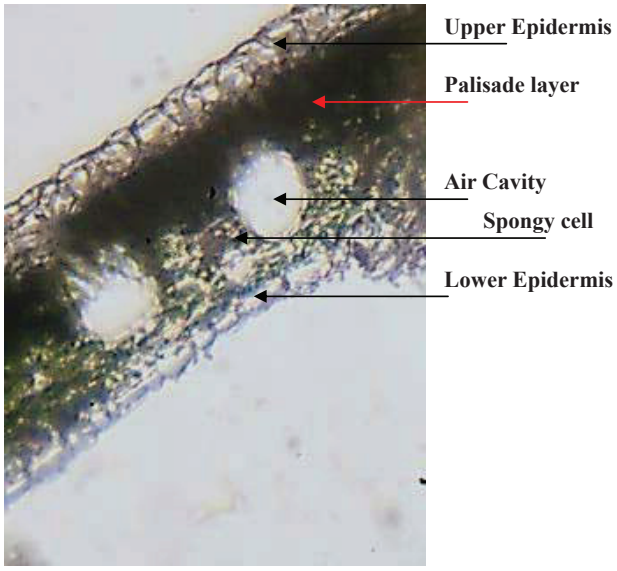
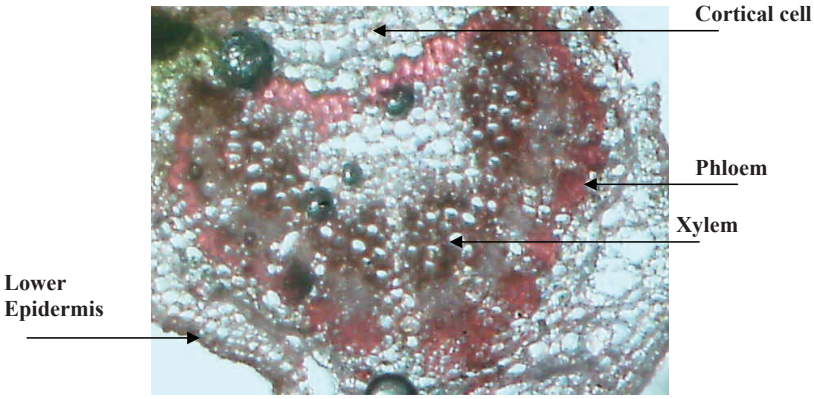


Figure 2: Transverse section of leaf of *Annona squamosa* Linn. (under 10x).



Lower epidermal peel showing epidermal cells and stomata (under 10x).

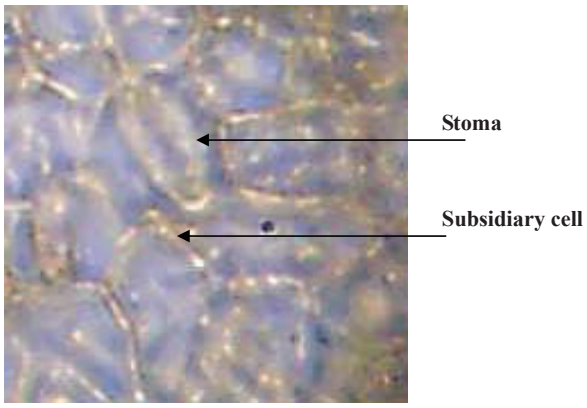
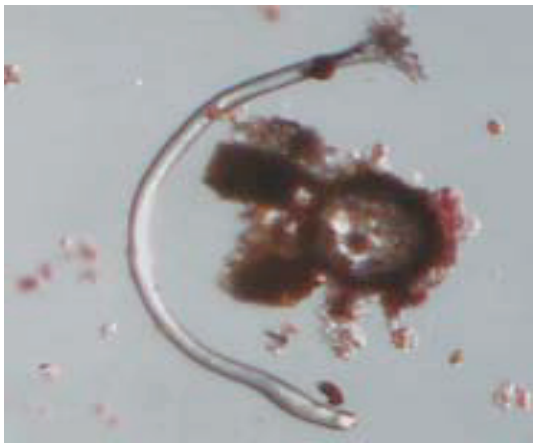


Figure 3: Transverse section leaf of *Annona squamosa* Linn. passing through mid-rib region of (under 10x).

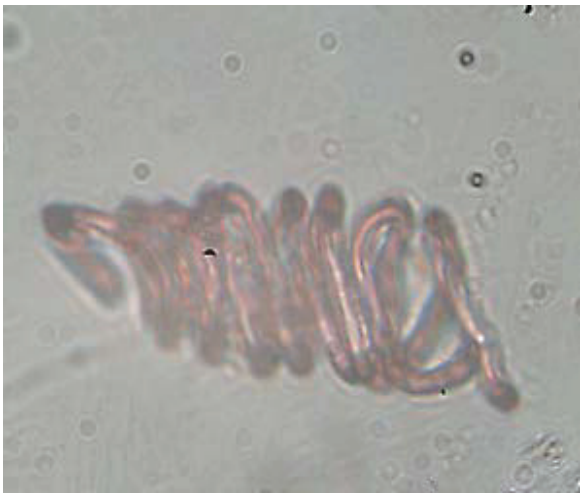
Trichome

Parenchyma Cells

Epidermal cell

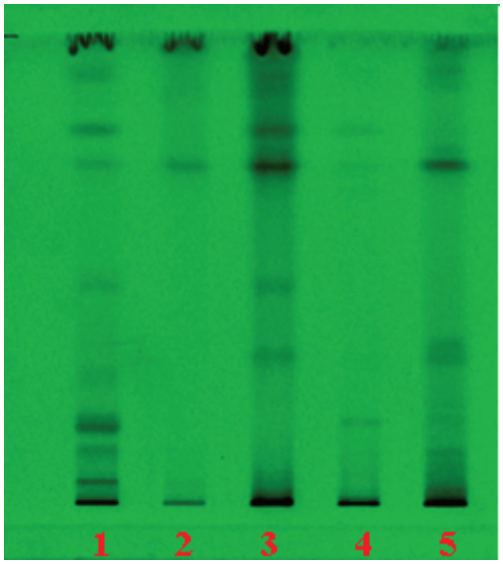


Trichome with leaf attachment



Spiral vessel

Figure 4: Microscopic Analysis of leaf powder of *Annona squamosa* Linn (under 10x).



Track 1 – Methanolic extract of *Annona squamosa* Linn. (Leaf)

Track 2 – Methanolic extract of Fats & waxes

Track 3 – Methanolic extract of Terpenoids and Phenolics

Track 4 – Methanolic extract of BasicAlkaloids

Track 5 – Methanolic extract of Quaternary alkaloids and N-oxides

Figure 5: Chromatographic plate of *Annona squamosa* Linn. with its phytoconstituents.

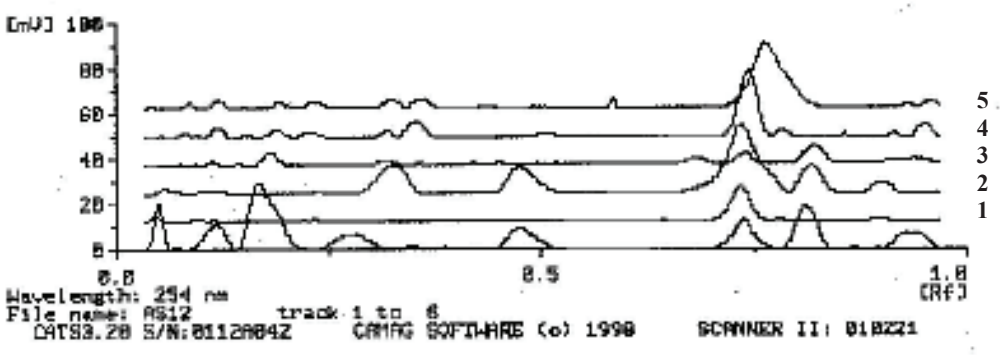


Figure 6: Chromatographic overlay of *Annona squamosa* Linn. with its phytoconstituents.

was evaluated on the basis of mortality, daily food, water intake, body weight and general behavioral changes.

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Pharmacognostical and Phytochemical Evaluation of Rare and Endangered *Habenaria spp.* (Riddhi and Vriddhi)

Rath Chinmay[#], Suman Kumari*, Dhar Bishnupriya[#], Mohanty RC[##], Singh Arjun[#], Padhi MM[#], Lavekar GS[#]

[#]Central Council For Research in Ayurveda & Siddha, Dept. of Ayush, Ministry of Health & F.W., Janakpuri, New Delhi-58

[##] Dept. of Botany, Utkal University, Vani Bihar, Bhubaneswar, Orissa

ABSTRACT

Genus Habenaria belongs to the family Orchidaceae and *H. intermedia* & *H. edgeworthii* are two species mentioned as drugs of Astavargha group of having antioxidant and anti ageing activity for a long time in ancient text of Ayurvedic literature. The increasing demand has resulted in commercial harvesting pressure on wild populations and that were already at risk of extinction and now become rare species due to deforestation. The botanical identities of both the species are controversial. As a result, people are using their adulterants and their substitutes instead of genuine drug. The present study is to carryout and verifies its botanical / pharmacognostical identity and their phyto-chemical difference of market samples used in the name of Riddhi and Vriddhi in several parts of India.

Keywords: Rare and endangered, Pharmacognosy, Standardization and Quality control.

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*** Author for correspondence :** *Senior Research Fellow, Central Council for Research in Ayurveda & Siddha, Dept. of Ayush, Ministry of Health & Family Welfare, Janakpuri, New delhi-58, +919968532138 (m), E-mail- sumanmaurya82@yahoo.co.in

INTRODUCTION

Despite impressive accomplishment of modern medicines or products (or many) disease remains uncured or leads to iatrogenic disorders as a result people are diverging towards the alternative system of medicine. The increasing annual demand and poor cultivation leads to destructive harvesting system to fulfill the market demand that reduces the affluence and prosperousness of plant species in nature. The continuous exploitation of several medicinal plant species from wild and considerable loss of their habitats during past few years have resulted in population decline of many high yield value of medicinal plant species. In India out of 44% of medicinal plants 22% are found in himalayan region[1]. Himalayan region have been found rich source of varied species of medicinal plants. Habenaria is one of the important genus of family orchidaceae and found in the habitat of Himalaya.

Habenaria intermedia D. Don. and Habenaria edgeworthii Hook. f. are two plant known as Riddhi and Vriddhi in Ayurveda. Acharya Charaka and Sushruta mentioned these drugs under Astavarga group[2]. These are one of the endangered species which are going to be extinct due to heavy exploitation for medicinal purpose, poor regeneration, low seed germination and seedling establishment, habitat loss, grazing, forest fire, competition with other dominant species of community[3]. So due to scarcity of these plant drugs in market some drugs are sold to be adulterated or substituted which leads to deteriorate the quality and efficacy of drug. Some times shows toxic effect on body. Quality control of herbs and formulations is a major problem which effects the efficacy and reproducibility of the results in formulation[4].

The present study will be helpful to the ayurvedic practitioners, researchers and industries to identify and standardize the drug.

MATERIAL AND METHODS

The adventitious roots and tubers of authenticated plants of H. intermedia (R5) and H. edgeworthii (V5) were collected from Medicinal Plants Garden of Regional Research Institute (Ayu.), Tarikhet and Indian Medicines Pharmaceutical Corporation Limited, Mohan (Almora).

Some market samples were collected in the name of Riddhi viz. R1, R2, R3, R4 and vriddhi viz. V1, V2, V3, V4 from Delhi, Lucknow, Mandi and Jaipur respectively. Raw ingredients were washed, cut into pieces and preserved in Formalo-Acetyl-Alcohol (FAA) for pharmacognostical study and some were shade dried and coarse (20 - 30 #) powdered for qualitative tests and standardization as per IP/ API / WHO Guidelines.

The thin layer chromatography of 90 % ethanolic extract of all samples were performed on pre-coated silica gel 60 F_{254} aluminum plates and the plates were developed using suitable solvent system. The developed plates were observed under UV 254 nm and 366 nm and after derivatization under white light.

RESULTS

Pharmacognosy of Habenaria intermedia D. Don.

Macroscopic Characters

The fresh tubers are 15-35 mm long and 10-25 mm thick, oval, obovate, or oblong in shape, buff to yellowish brown in colour, sometimes with shrunken surface, covered with numerous fine white hairs; internally they are white to creamish in colour and mucilaginous to touch. The dried tubers of the market are hard, difficult to break, rough with fine reticulate surface and creamish brown to light brown in colour; the brown to light brown in colour; the broken surface is uneven exhibiting creamy interior. Both fresh and dry samples show scars of the aerial portion at the apex and beaked or sometimes round base. The tubers are odourless and taste is palatable and mucilaginous.

Microscopic Characters

The T.S. of adventitious roots (Figure 1a-1b) shows a single layered epidermis, some of the cells of which elongate and form unicellular hairs (380-890-1250-1720) µm. The hairs are either straight with tapering to blunt ends, or broad, conical at the base and abruptly tapering to tail like ends or tortuous showing helical bendings (Figure 1e). The epidermis is followed by 8-12 layers of parenchymatous cortex. Some of the outer cortical cells of varying size (30-90-150-240 X 60-105-150-180) µm exhibit presence of bundles of raphides of calcium oxalate (36-45-90 X 75-90-140) µm. Below the endodermis is a layer of thin walled pericycle which encloses a polyarch stele.

The T.S. of a mature tuber, about 22 mm thick (Fig. 1c-1d) shows 2-3 layered epidermis bound proximally by a distinct exodermis. A number of outer epidermal cells (15-21-45-60 X 60-90-150-210) µm elongate to form unicellular hairs (540-930-1840-2100 µm) similar to as described earlier. The exodermis is followed by 15-20 layers of cortical parenchyma. The cells of which is proximity of the cortex. Below the cortex a typical polystelic condition is found and it consists of 14-16 steles arranged in a single and 7-10 steles distributed within the parenchyma in the central region (Figure 1c). Each stele of the tuber itself represents a clear diarch condition (Figure 1c, 1d).

A few parenchymatous cells of outer cortex contain bundles of raphides of calcium oxalate (45-60-90 X 60-120-150)µm (Figure 1d). The schizogenous mucilage canals (180-240-360 µm) lined by an epithelium of usually 7-9 cells are found distributed throughout the parenchymatous tissue. The starch grains, mostly of simple type, consisting of both small (3-6-12µm) and (30-45-60-90µm) large grains are found distributed in abundance throughout the parenchyma as well as in the epithelial cells of mucilage canals. The small starch grains are mostly round with helium as point or cleft and larger ones are round to oval in shape with helium in form of a point or cleft or central triangular or 2-3 stellate clefts (Figure 1f).

Pharmacognosy of Habenaria edgeworthii Hook. f.

Macroscopic Characters

The fresh tubers are 12-28 mm. long and 9-12 mm. thick, oval, obovate or oblong in shape, fleshy, buff in colour with shrunken surface and covered with a large number of fine white hairs; internally the tubers are white and slimy to touch. The completely dried tubers of the market are hard, difficult to break, rough with fine reticulate markings, grayish brown to brown in colour; broken uneven surface shows creamy-yellow central area. Both fresh and dry samples show scar or remains of the aerial stem at the apex and finely beaked base. The tubers are odourless and the taste is bland and mucilaginous.

Microscopic Characters

Adventitous root in T.S. shows a single layered epidermis, some of the cells of which elongate to form unicellular hairs (482-958-1375-1665) µm. It is followed by 3-15 layers of parenchymatous cortex. A few cortical cells contain bundles of raphides of calcium oxalate (45-60-96 X 66-81-124)µm. The endodermis is distinct. Below the endodermis is a layer of thin walled pericycle which encloses a polyarch stele (Figure 2a-2b).

The T.S. of tuber, about 10 mm thick (Figure 2c-2d) shows 2 layered epidermis bounded proximally by a distinct exodermis. A number of cells of outer layer of epidermis elongate to form unicellular hairs (380-860-1230-1540)µm similar in shape to those described for

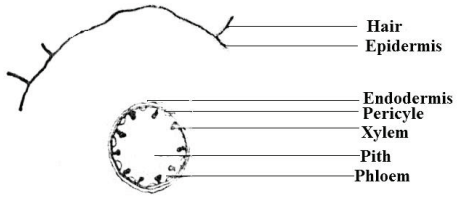


Figure 1a

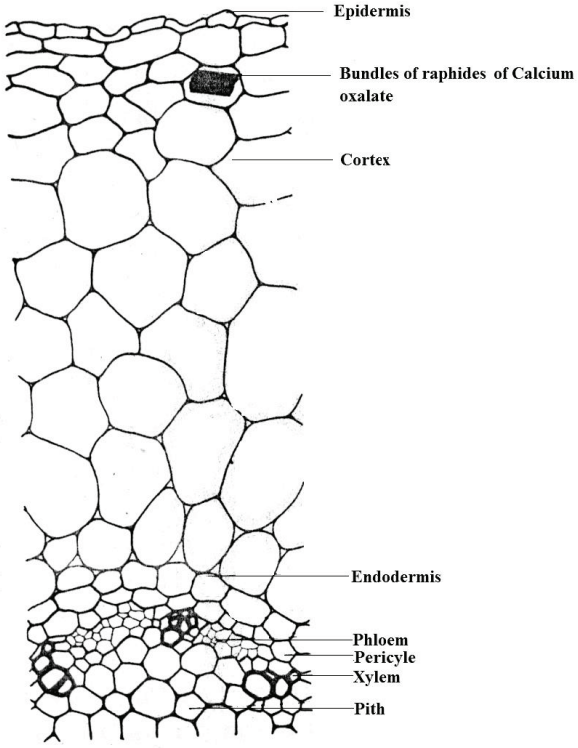


Figure 1b

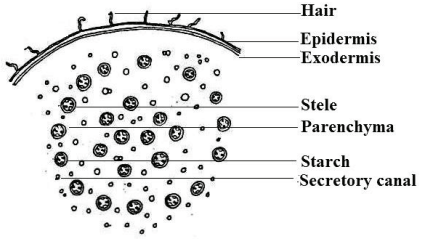


Figure 1c

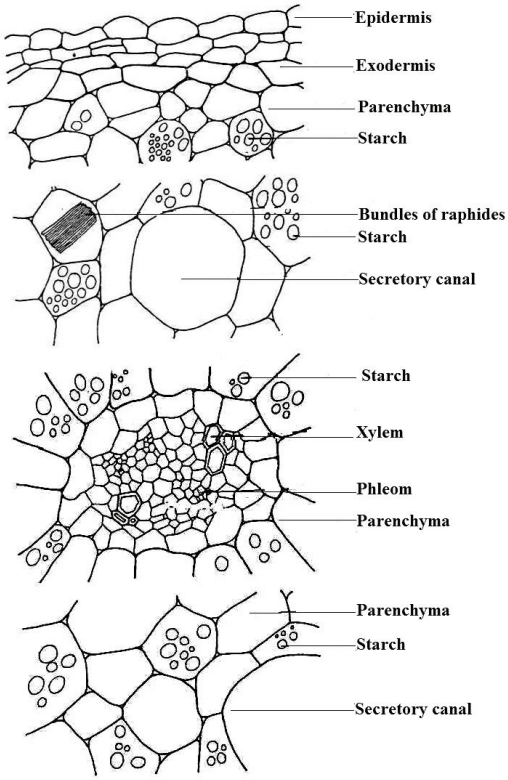


Figure 1d

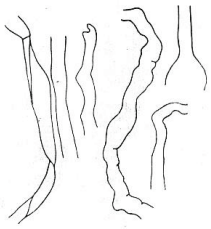


Figure 1e

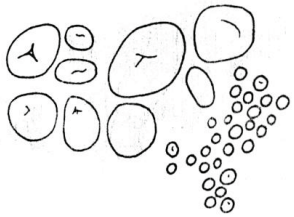


Figure 1f

Legend :

Figure 1a: T.S. of a portion of adventitious root of Habenaria intermedia* D.Don.*showing poly-arch condition (diagrammatic)

Figure 1b: T.S. of a portion of adventitious root of Habenaria intermedia* D.Don. *showing cellular details

Figure 1c: T.S. of a portion of tuber of Habenaria intermedia* D.Don. *showing polystelic condition

Figure 1d: T.S. of a portion of tuber of Habenaria intermedia* D.Don. *showing cellular details

Figure 1e: Fragments of tracheids

Figure 1f: Starch grains

Figure 1.

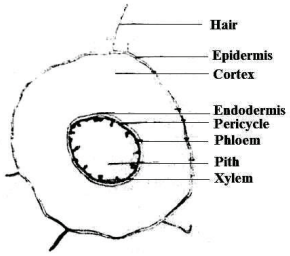


Figure 2a

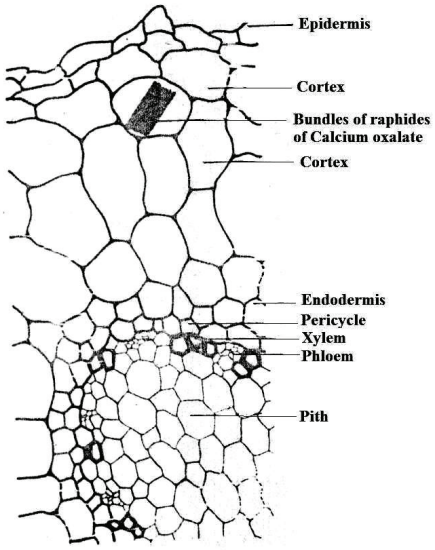


Figure 2b

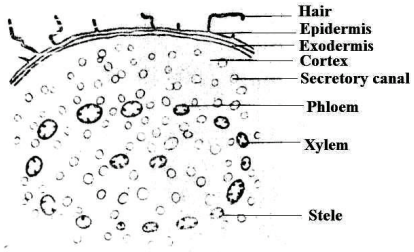


Figure 2c

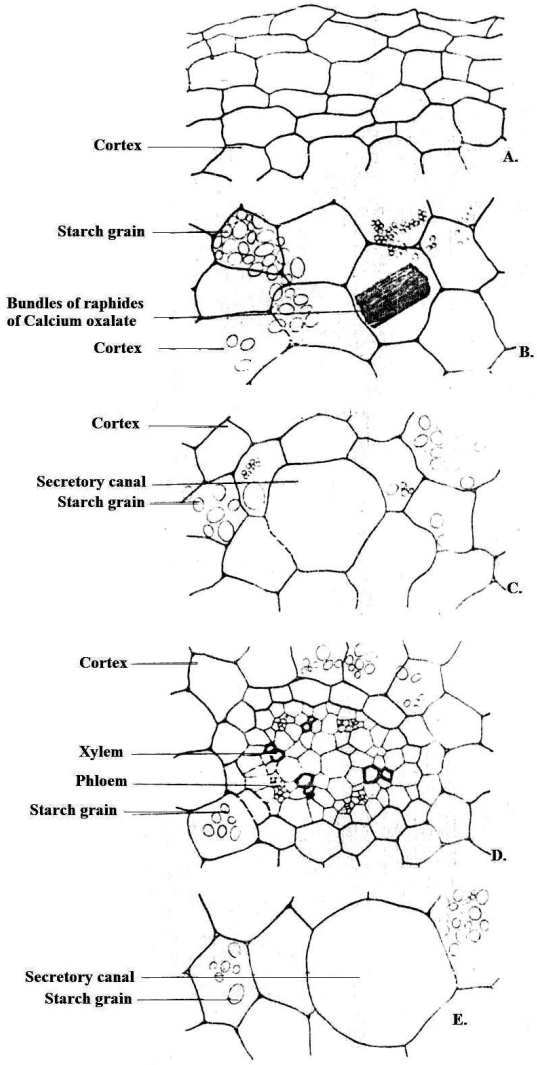


Figure 2d

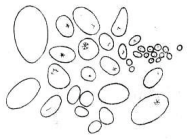


Figure 2e

Legend:

Figure 2a: T.S. of a portion of adventitous root of Habenaria edgeworthii* Hook.f. *showing poly -arch stele (diagrammatic)

Figure 2b: T.S. of a portion of adventitous root of Habenaria edgeworthii* Hook.f. *showing cellular details

Figure 2c: T.S. of a portion of tuber of Habenaria edgeworthii* Hook.f. *showing poly-stelic condition (diagrammatic)

Figure 2d: T.S.of a portion of tuber of Habenaria edgeworthii* Hook.f. *showing cellular details-

A : Epidermis to outer cortex region
B-C: Cortical region
D : A stele showing tetra-arch condition
E : Central region

Figure 2e: Starch grains

Figure 2.

H. intermedia. The exodermis is followed by 10-12 layers of cortical parenchymatous cells of sizes (60-120-165 X 45-90-160)µm, the cells of which in proximity of exodermis are smaller as compared to the remaining cells of cortex. Below the cortex a typical polystellic condition is observed and this consists of 8-19 steles arranged in a ring and 1-8 steles distributed within the parenchyma in the central region. Each stele in itself represents a clear mono to pentarch condition (Figure 2c-2d.D).

A few paranchymatous cells of outer cortex contain bundle of raphides of calcium oxalate. The schizogenous mucilage canals (180-240-330) µm lined by an epithelium of usually 6-9 cells are found distributed throughout the parenchymatous tissue (Figure 2c, 2d, C, E). Starch grains, mostly of simple type are found in abundance and are distributed throughout the parenchyma including the epithelial cells of mucilage canals. The smaller starch grains (6-9-16-24) µm are mostly round with hilum as point or cleft and larger ones (30-65-135-150) µm are round to oval in shape with hilum in form of a point or cleft or central triangular or 2-7 stellate cleft (Figure 2e).

Comparative pharmacognostic characters of market samples

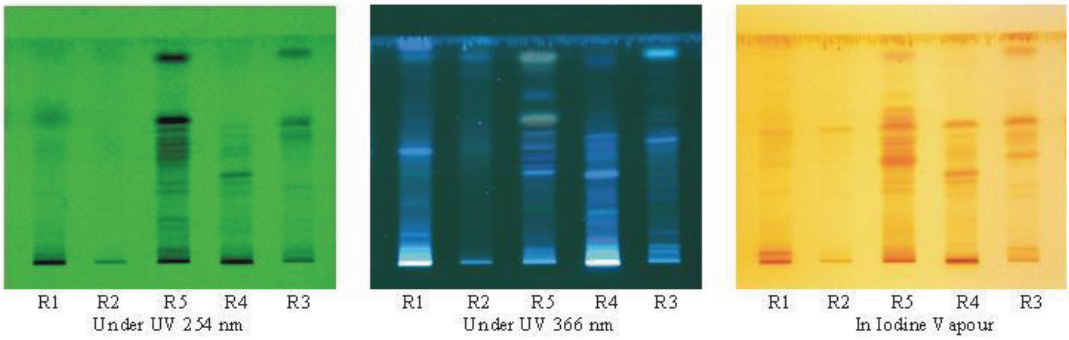
The detail macro and microscopical characters of all the market samples V1-V4 & R1-R4 were studied and compared with both Habenaria species and it was observed that only sample V1 tubers are found very hard to break which shows more or less similar macro and microscopical details with that of H. edgeworthii however the characters of market samples V2 and V3 were 1- 6 × 0.5- 1 cm pseudo-bulb, conical, translucent, reddish brown in colour, covered with light brown sheathing leaves, membranous with parallel venation, rough surface and slightly mucilaginous in taste. The transverse section passing through bulb shows a single layer of cuticle and a layer of thick walled sclerified epidermal cells followed by 1 or 2 layers of large sclerified cells and these extend unevenly into irregular ground parenchymatous tissue, with large air spaces at some places, prominent vascular bundles scattered throughout the ground tissue surrounded by thick walled sclerenchymatous cells which resembles to the characters of Microstylis wallichii Lindl. (Figure 6a-6c)(Family Orchidaceae).

The physicochemical analysis (Table 2) and TLC of 90 % ethanolic extract (Figure 4) of powders of all the samples were carried out using solvent system Toluene: Chloroform: Methanol (2.7 : 6.0 : 1.3) and the observations were compared.

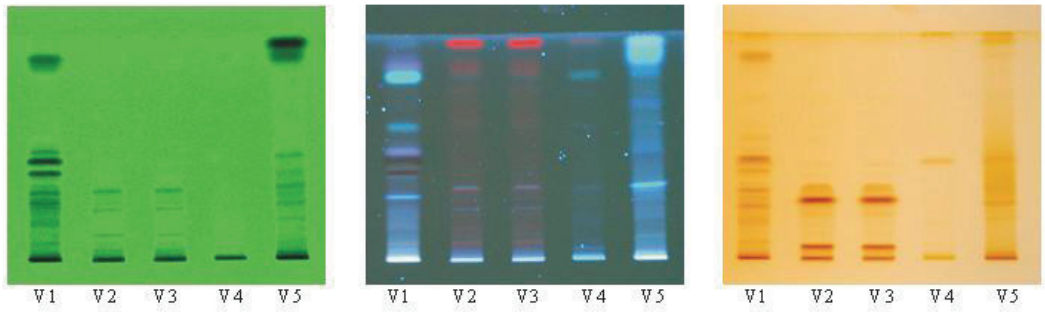
The pharmacognostic characters of R1 and R3 were identified same as H. intermedia while charecters of sample R4 was found similar to the Dioscorea bulbifera L. (Figure 5a-5f) (Family Orchidaceae) showing 9-40 mm

Table 2: Observations of physicochemical parameters of powdered samples of Vriddhi

S. No.	parameters %		Habenaria edgeworthii	V1	V2	V3	V4
1.	Total ash (% w/w)		3.1	4.2	3.5	2.9	4.2
2.	Acid insoluble ash (% w/w)		0.6	0.98	1.2	1.0	1.5
3.	Water soluble extractive (% w/w)		29.9	20.3	15.3	16.2	12.7
3.	Alcohol soluble extractive (% w/w)		21.2	17.43	6.2	6.8	10.2
5.	TLC(Figure 4)	Under UV 254 nm (R_f Values)	0.18, 0.25, 0.32, 0.41, 0.45, 0.89, 0.96	0.07, 0.11, 0.15, 0.25, 0.29, 0.38, 0.43, 0.47, 0.85, 0.89	0.04, 0.07, 0.10, 0.22, 0.30	0.04, 0.07, 0.10, 0.22, 0.30, 0.37	0.05
		Under UV 366 nm (R_f Values)	0.10, 0.19, 0.30, 0.32, 0.48, 0.67, 0.81, 0.89, 0.95	0.03, 0.06, 0.27, 0.36, 0.38, 0.43, 0.47, 0.57, 0.80, 0.85	0.06, 0.13, 0.21, 0.30, 0.31, 0.80, 0.95	0.06, 0.13, 0.21, 0.30, 0.31, 0.80, 0.83, 0.95	0.05, 0.31, 0.80, 0.95
		In Iodine vapors under white light (R_f Values)	0.17, 0.24, 0.43, 0.95	0.07, 0.17, 0.23, 0.30, 0.38, 0.44, 0.88	0.05, 0.26, 0.31	0.05, 0.26, 0.31	0.43



Mobile Phase : Toluene : Chloroform : Methanol (2.0:6.0:2.0)
Figure3 : TLC Fingerprint of 90% ethanolic extract of samples



Mobile Phase : Toluene : Chloroform : Methanol (2.7:6.0:1.3)
Figure 4 : TLC Fingerprint of 90% ethanolic extract of samples

Legends:
Samples of *Habenaria intermedia* from
R1: New Delhi, R2: Lucknow (Uttar pradesh), R3: Mandi (Himachal Pradesh), R4: Jaipur (Rajasthan), R5 : Tarikhet (Uttrakhand)
Samples of *Habenaria edgeworthii* from
V1: New Delhi, V2: Lucknow (Uttar pradesh), V3: Mandi (Himachal Pradesh), V4: Jaipur (Rajasthan), V5 : Tarikhet (Uttrakhand)

Figure 3 and Figure 4.

Table 1: Observations of physicochemical parameters of powdered samples of Riddhi

S. No.	parameters %		Habenaria intermedia	R1	R2	R3	R4
	Total ash (% w/w)		4.5	6.4	3.2	5.2	6.96
	Acid insoluble ash (% w/w)		0.81	1.2	0.9	0.98	2.11
	Water soluble extractive (% w/w)		32.2	23.7	22.3	28.3	18.34
	Alcohol soluble extractive (% w/w)		15.2	9.5	18.9	9.8	5.18
	TLC(Figure 3)	Under UV 254 nm (R_f Values)	0.06, 0.11, 0.16, 0.19, 0.31, 0.35, 0.45, 0.50, 0.52, 0.55, 0.59, 0.63, 0.90	0.30, 0.49, 0.63	0.90	0.05, 0.33, 0.50, 0.61, 0.92	0.14, 0.30, 0.35, 0.39, 0.49, 0.55, 0.59
		Under UV 366 nm (R_f Values)	0.05, 0.11, 0.24, 0.39, 0.42, 0.46, 0.50, 0.56, 0.62, 0.73, 0.89	0.06, 0.12, 0.26, 0.39, 0.48, 0.89, 0.94	0.90	0.05, 0.08, 0.13, 0.19, 0.29, 0.39, 0.49, 0.53, 0.61, 0.66, 0.91	0.08, 0.11, 0.18, 0.23, 0.27, 0.38, 0.46, 0.52, 0.55, 0.87
		In Iodine vapors under white light (R_f Values)	0.31, 0.35, 0.44, 0.53, 0.59, 0.65, 0.73, 0.91	0.03, 0.30, 0.42, 0.49, 0.57, 0.80	0.58	0.06, 0.29, 0.33, 0.47, 0.56, 0.62, 0.93	0.08, 0.30, 0.35, 0.39, 0.48, 0.55, 0.60

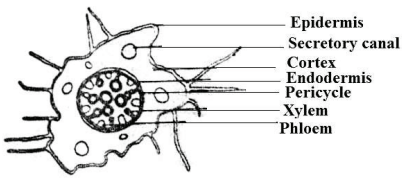


Figure 5a

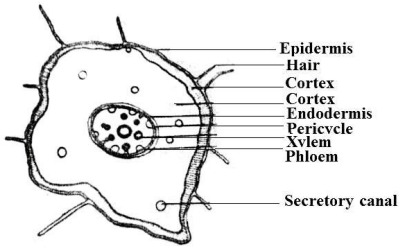


Figure 5b

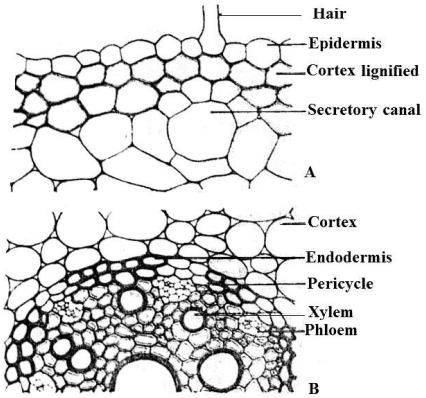


Figure 5c

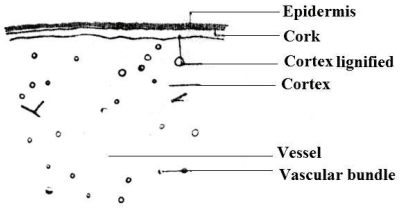
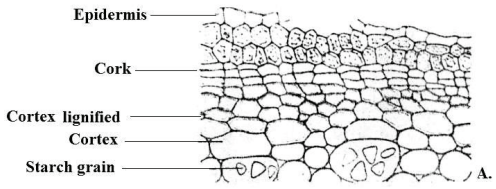
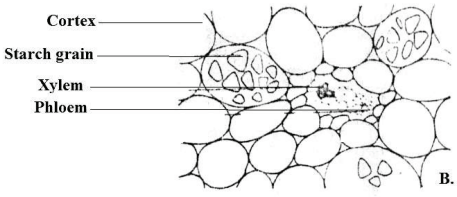
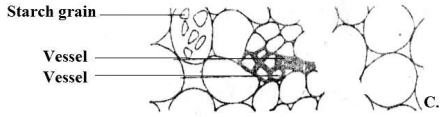


Figure 5d







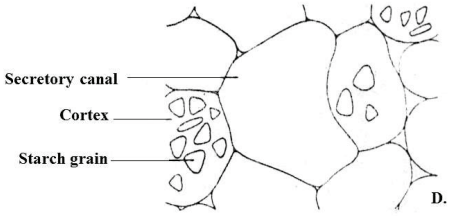


Figure 5e

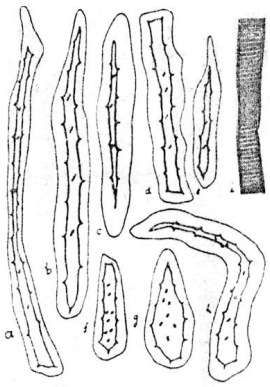


Figure 5f

Legend:

Figure 5a: T.S. of an adventitious root showing poly-arch condition (diagrammatic)

Figure 5b: T.S. of advanced stage of an adventitious root (diagrammatic)

Figure 5c: T.S. of a portion of an adventitious root showing cellular details

A. Epidermis to inner cortical region

B. Cortex to central region showing details of stele

Figure 5d: T.S. of a portion of tuber (diagrammatic)

Figure 5e: T.S. of a portion tuber showing cellular details

A. Epidermis to outer cortical region

B-C. Cortical region showing vascular bundle and group of vessels

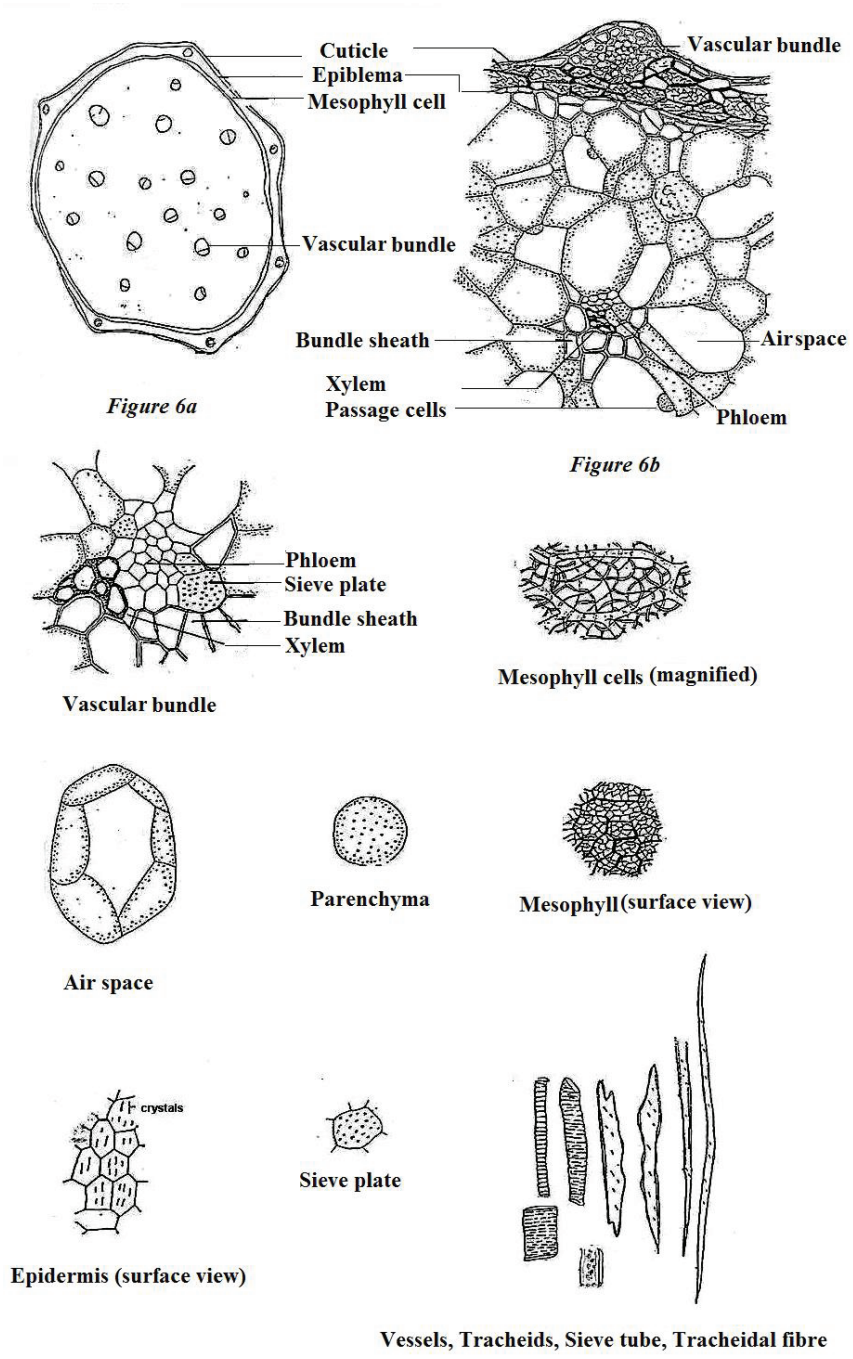
D. Central region

Figure 5f: a-i. Isolated elements of the tuber

a-h. Stone cells

i. Vessels

Figure 5.



Legend:

Figure 6a: T.S. pseudo bulb of Microstylis wallichii* Lindl. *(diagrammatic)

Figure 6b: T.S. pseudo bulb of Microstylis wallichii* Lindl. *(portion enlarged)

***Figure 6c: Powder characteristics of Microstylis wallichi* Lindl.**

Figure 6.

thick, globose to pyriform light brown to brown colored tuber having rough surface usually covered with abundant small adventitious roots. The transverse section showed single layered epidermis followed by a narrow strand of stone cells of varying size (150-270-390-660) µm, 2-3 layers thick. The stone cells were mostly elongated with tapering to blunt ends and their walls were thick, lignified and bear simple pits on them (Figure 5f, a-h). It was followed by 2-4 layers of cork cells (12-21-30 X 18-36-60) µm below which was the ground tissue consisting of thin walled parenchymatous cells (45-75-180 X 54-90-180) µm. However, outer 2-5 layers of cortical cells become thick walled and lignified of sizes (21-45-66 X 27-54-90) µm (Figure 5e.A). Within the ground tissue a large number of vascular bundles consisting of lignified scalariform vessels and soft phloem tissues were distributed. A large number of schizogenous mucilage canals (127-210-320) µm lined by epithelium of 7-9 cells and numerous starch grains of varying sizes (15-27-45-60) µm were also distributed in the ground tissue.

The pharmacognostic characters of sample R2 resembles to H. edgeworthii. The physicochemical analysis (Table 1) and TLC (Figure 3) of 90 % ethanolic extract of powders of all the samples were carried out using solvent system Toluene : Chloroform : Methanol (2.0 : 6.0 : 2.0) and the results compared to the authentic sample.

DISCUSSION AND CONCLUSION

From the foregoing observations, it has been seen that both species of Habenaria viz. H. intermedia and H. edgeworthii resemble to a great extent in their morphological and histological characters like in adventitious roots , presence of unicellular hairs with tapering or blunt ends or tortuous showing helical bendings, polyarch primary stele, bundles of raphides of calcium oxalate present in outer cortex and both species are devoid of starch grains.

Similarly in tubers of both species, epidermis are 2-3 layered, single layered exodermis, scalariform thickening on vessels and each stele is diarch but the no. of starch grains per mg. powder is 56,210 in case of H. intermedia while 61,319 in case of H. edgeworthii. Like the anatomical structure of tubers of D. bulbifera are quite characteristic and can be differentitated by having single layered epidermis ruptured at places, stone cells either elongated or rectangular with simple pits on their walls forming continuous ring below the epidermis, ground tissue consists of thin walled parenchymatous cells excent a few layers of outer cells which are thick walled & lignified, and no. of starch grains per mg powder is 72,516 from both Habenaria species.

Similarly the anatomical structure of M. wallichii showing a single layered, thick walled, sclerified epidermis having acicular crystals of calcium oxalate, followed by mesophyll adjacent to the upper epidermis composed of 2 to 4 layers of elongated cells with lignified reticulate thickening, devoid of chloroplast; vascular bundles prominent, phloem well developed with large sieve plates, surrounded by sclerenchymatous bundle sheath; single layer cuticle and a layer of thick walled sclerified epidermal cells; below this lie 1 or 2 layers of large sclerified cells and these extend unevenly into ground parenchymatous tissue; ground parenchyma irregular, with large air spaces with passage cells in the form of small protuberances at some places; vascular bundles scattered throughout the ground tissue surrounded by thick walled sclerenchymatous cells, which occasionally extend into intercellular spaces.

The Drugs under study are not easily available in market and which are available the observation of Physico-chemical evaluation of the same indicates that the drugs are not genuine. Hence the species are considered to be rare and endangered.

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Pharmacognostical Investigation of *Hiptage benghalensis* (L.) Kurz. (Malpighiaceae)

Chenthurpandy P.[1], Kalidass C.[2], Mohan V.R.[2]*

[1] Department of Botany, Kamaraj College, Tuticorin.
[2] Ethnopharmacology unit, Research Department of Botany, V.O.Chidambaram College, Tuticorin-628008, Tamil Nadu, India.

ABSTRACT

Hiptage benghalensis is used in the traditional system of medicine. The leaf is considered one of the important plant organs for the treatment of various diseases such as burning sensation, wounds, ulcers, inflammations, leprosy, scabies, cough and rheumatism. The aim of present research was focused on the pharmacognostical, physico-chemical and phytochemical properties of *H. benghalensis*. Various parameters like microscopy, physico-chemical (ash & extractive values), fluorescence analyses and phytochemical profile for leaf part was studied and the salient diagnostic features were documented.

Keywords: *Hiptage benghalensis*, Pharmacognostical evaluation, Phytochemical screening
Editor: Mueen Ahmed KK, Phcog.Net

*** Corresponding author e-mail:** vrmohan_2005@yahoo.com

INTRODUCTION

Hiptage benghalensis (L.) Kurz. belong to the family Malpighiaceae and also found almost throughout India and North of South America (1). The genus *Hiptage* (syn: *Banisteriopsis*) and *Diploterys* include some species with hallucinogenic compounds (2). The bark, leaves and flower of *H. benghalensis* are aromatic. They are useful in conditions of burning sensation, wounds, ulcers, inflammations, leprosy, scabies, cough and rheumatism (3). In view of its important of *H. benghalensis* in traditional and modern system of medicine, it was though worthwhile of develop quality standard for the same. As for as botany and chemistry of this plant is concerned, large number of scientific data is available but a systematic standardization study is still lacking. Hence, in the present investigation an attempt has been made to standardize the *H. benghalensis* leaves using microscopic characters and physico-chemical parameters.

MATERIAL AND METHODS

The leaves of *H. benghalensis* were collected from the well grown healthy plants inhabiting the natural forests of Petchipparai reserve forest, Kanyakumari District, Southern Western Ghats, Tamil Nadu, India. For anatomical investigations standard microtome techniques (4) were followed. Take T.S. of 10 to 12 µm thickness were prepared. These microtome sections were stained with 0.25% aqueous Toluidine blue (Metachromatic stain) adjusted to pH 4.7 (5). Photomicrographs were taken with NIKON trinocular photo micrographic unit. The most accepted descriptive terms were being used to describe the leaf anatomy (6,7).

Physico-chemical constant and fluorescence analyses

These studies were carried out as per the standard procedures (8). In the present study, the leaf powder was treated with 1N aqueous sodium hydroxide and 1N alcoholic sodium hydroxide, acids like 1N hydrochloric acid and 50% sulphuric acid. These extracts were subjected to fluorescence analysis in visible/daylight and UV light (254nm & 365nm). Various ash types and extractive values were determined by following standard method (9, 10).

Preliminary phytochemical analysis

Shaded dried and powdered leaf samples were successively extracted with petroleum, benzene, chloroform and alcohol. The extracts were filtered and concentrated using vaccum distillation. The different extracts were subjected to qualitative tests for the identification of various phytochemical constituents as per standard procedure (8, 11).

RESULTS AND DISCUSSION

Transverse section of the leaf is dorsiventral and transcurrent. Lamina portion shows upper epidermis which is singled layered having rectangular cells and cuticularized (Fig 1). Surface framing uniseriate multicellular 2 – 3 trichomes. Mesophyll consists of double layers of palisade parenchyma and 4 – 5 layers of loosely bound spongy parenchyma. Midrib portion shows both upper and lower epidermis which is continuous. Upper convex surface is grooved and below each groove, it shows 6 – 8 layers of collenchymas which are followed by double layers of embedded palisade parenchyma. Vascular bundles are arc shaped. Xylem is lignified and phloem is non-lignified. Calcium oxalate druses are occasionally seen in the ground parenchyma cells (Fig 2).

(Abe: Abaxial epidermis; Abvb: Abaxial vascular bundle; Ade: Adaxial epidermis; Ads: Adaxial side; Advb: Adaxial vascular bundle; Gt: Ground tissue; La: Lamina; Mr: Midrib; Ph: Phloem; Pm: Palisade mesophyll; Sc: Sclereids; Sm: Spongy mesophyll; X: Xylem).

The detailed and systematic pharmacognostical evaluation would give valuable information for the future studies. The physico-chemical constant like ash and extractive values were determined (Table 1). The results of various types of ash may provide a basis to identify the quality and purity of the drug. In fluorescence analysis revealed that the powdered leaves of *H. benghalensis* was treated various chemical reagents to give different colours (Table 2). Fluorescence is the phenomenon exhibited by various chemical constituents present in the plant material. Many phytocompounds fluoresce when suitably illuminated. The fluorescence colour is specific for each compound. A non fluorescent compound may fluoresce if mixed with impurities that are fluorescent. The fluorescent method is adequately sensitive and enables the precise and accurate determination of the analyze over a satisfactory concentration range without several time consuming dilution steps prior to analysis of pharmaceutical samples (12). The phytochemical analysis of the leaf extract revealed the presence of steroid, tannin, phenol, coumarin, flavonoid, saponin, terpene and sugar. While alkaloid, anthraquinone and xanthoprotein compound were not found in all crude extracts (Table 3). From the phytochemical analysis noted that steroid and sugar compounds were present in all crude extracts. The observation also indicated that only on chloroform extract shows a presence of phenol compounds. The phytochemical compounds are known to play an important role to identify the bioactivity of medicinal plants. It should be noted that steroidal compounds are of such compounds as sex hormones (13). The presence of the saponin compound in this plant which is supported the usefulness of this plant in the managing inflammation.

Table 1. Extractive and ash values of Hiptage benghalensis.

Parameter	Determined value (%w/w)
Extractive values	
Ethanol soluble	19.23%±1.21
Water soluble	23.54%±1.31
Ash value	
Total ash	7.93%±0.34
Acid insoluble	2.98%±0.43
Water insoluble	1.46%±0.21
Sulfated ash	12.85%±0.69

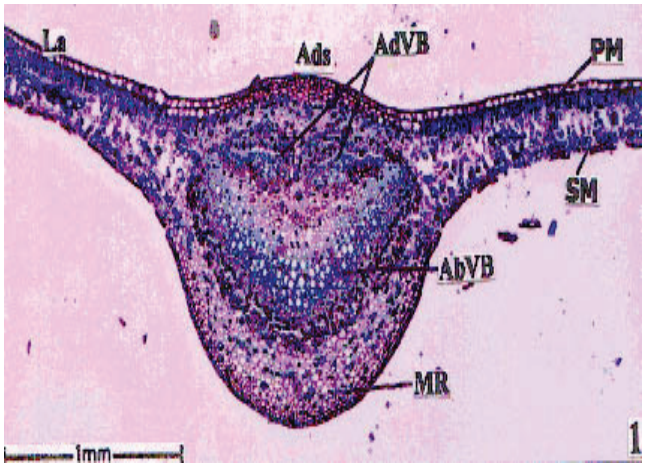

Figure 1. T.S. of midrib and lamina.

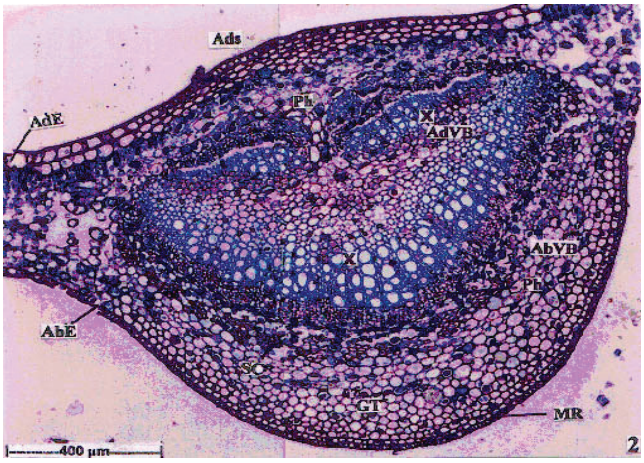

Figure 2. Midrib enlarged.

Table 2. Fluorescence analysis of the powdered leaf of Hiptage benghalensis.

Powdered drug	Visible/Day light	Short UV light (254nm)	Long UV light (365nm)
Powder as such	Light yellow	Yellowish green	Yellow
Powder + 1N NaOH(aqueous)	Wine red	Greenish yellow	Greenish yellow
Powder + 1N NaOH(alcoholic)	Green	Light green	Dark green
Powder + 1N HCL	Yellowish pink	Light yellow	Golden yellow
Powder + 50% H2SO4	Wine red	Green	Dark green

Table 3. Qualitative chemical analysis of leaf extracts of Hiptage benghalensis.

Test	Benzene	Chloroform	Methanol
Alkaloid	–	–	–
Anthraquinone	–	–	–
Coumarin	–	–	+
Flavonoid	–	+	+
Phenol	–	+	–
Saponin	–	+	+
Steroid	+	+	+
Tannin	–	–	+
Terpenoid	–	–	+
Xanthoprotein	–	–	–
Sugar	+	+	+

Note: + Present; – Absent.

CONCLUSION

Pharmacognostical studies and phytochemical screening can serve as a basis for proper identification, collection and investigation of the plant. Since *Heptage benghalensis* leaf is known for its various medicinal properties hence the present study may be useful to supplement information in respect to its identification, authentication and standardization, since no such data is available for the same.

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Pharmacognostical studies of anti-leprosy plant *Aristolochia bracteolate* retz (aristolochiaceae)

Subramanian M.S.* and SathishKumar N.H.

PG and Research Department of Botany, Kongunadu Arts and Science College, G.N. Mills Post, Coimbatore, 641 029

ABSTRACT

Aristolochia bracteolate Retz. (Family: Aristolochiaceae) are important constituents among 22 plants/ parts used in the perpetration of an anti-leprosy drug "SULAK". The latter is also used in the preparation of "KALMEGH"- an Ayurvedic drug. Pharmaco-chemical data available on *A. bracteolate* are scanty and totally lacking on the other. The present paper deals with pharmaco-chemical evaluation of the species with the following parameters: organoleptic, microscopic and fluorescence evaluation, estimation of biochemical and geochemical and determination of active principle and physical constants. Since the above species are used in the perpetration of the anti-leprosy drug, their chemical identity is compared with that of standard allopathic anti-loprosy agents like clofazimine, dapsone and riampicin by chemical reaction (IP method) to assess their activity equivalence.

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*** Corresponding Author Email:** mssr6000@rediffmail.com

INTRODUCTION

Aristolochia bracteolate Retz. belongs to the Family Aristolochiaceae are the important constitutes among 22 plants used in the preparation of an anti-leprosy drug "SULAK" (1–3). The pharmacognostical characters of *A. bracteolate*, used in the preparation of an Ayurvedic drug "KALMEGH" (4–7) have been to certain extent studies by (8), but no information is available on the other species.

The present study deals with the pharmaco-chemical characterisation of *A. bracteolate*. A chemical identity comparison is made with standard allopathic anti-leprosy agents like clofazimine, dapsone and rifampicin to assess their activity equivalence to treat leprosy by adopting Indian pharmacopoeal (IP) methods.

MATERIALS AND METHODS

The plant of *A. bracteolata* were collected at their flowering from Coimbatore. Herbaria were deposited in the botany department herbarium, Bahrathiar University, Coimbatore. Whole plant species are fixed in FAA for free hand sections. The sections are stained in safranin and fast green (9) for anatomical studies. The plants were powdered with electric pulvarizer and sieved through 40mesh sieves for powder analysis.

The fluorescence studies are made according to (10–11) the procedure recommended by (12) and Indian pharmacocopia are followed for the determination of ash values and extractive values. For estimation of protein and free sugar, the methods describes by (13–14) are followed. For geochemical studies triple acid digestion of the powder and atomic analyzer are used. Histochemical location is done according to the methods described by (15–18). For preliminary phytochemical studies were made as by describes in experiments in pharmaceutical science by (19) are followed.

OBSERVATION AND RESULTS

Macroscopic characters

Leaves alternate, cordaterniform, 1.2–4.1 × 1.5–3.7cm. cordate to sagittate at base, entire, obfuse, glaucous beneath. Bracts orbicular. Flowers solitary. Perianth tube green; lipped, dark purple, 1 lipped, stamens 6, stigmatic lobes 6, glandular hairy. Capsules glabose, 10 ribbed. Seeds many cordate with rounded glands.

Vegetative features

Preparation from whole part: Cylinderical; colour dark brown; fissures absent; highly flexible; more fibrous; aromatic.

Microscopic characters

A. bracteolate (stem) it is angled in outline. Epidermis is single layered and formed of elongated cells. Primary

corex is formed of parenchymatous cells. The cells are rich in starch grains. Cork orginates from sub-epidermal region. It has alternate layers of thin walled cubical cells. Endodermis is less prominent. Large gabs are seen in the pericyclic sclerenchyma. Phloem are crystals. Xylem has large vessels and pitted. The pith is formed of thin walled parenchymatous cells. Medullary rays are broad. Secretory cells with oil and crystals in cluster are seen.

Leaf

Epidermal cells are polygonal silicified cells are seen in upper epidermis stomata is of paracytic type and its index is 5.13–7.25. small group of palisade cells are horizontally divided by transverse wall. Veins are provided with greenish sheath secretory cells in mesophyll show yellowish contents. Crystals occur in mesophyll and epidermil cells.

Fluorescence evaluation

The fluorescence property of the powders and their extracts of A. bracteolate as such and after treatment with different chemical reagents under day and UV light are summarized in Tables 1, 2, and 3.

Biochemical evaluation

The biochemical are estimated in percentage on dry weight basis. The values obtained are average of three explicative (Tables 4 and 5).

Table 1. Behaviour of *A. bracteolata* (whole plant) with chemical reagents.

S.No	Treatment of powder with under day light	Colour
1	Powder such as	Deep grey
2	N NaoH (aqueous)	Copper leaf
3	N NaoH (in methanol)	Copper leaf
4	Picric acid saturated solution	Pistachio green
5	Acetic acid (Conc)	Truck brown
6	Hcl (Conc)	Oliver green
7	HNO_3 (Conc)	Copper leaf
8	H_2So_4 (Conc)	Deep green
9	Soliwanoff's reagent	Sand stone
10	Ferric chloride 5%solution	Deep green
11	40% NaoH (aqueous) + 10% lead acetate	New oliver green
12	Iodine solution (5%)	Deep green
13	Sadan III (in ethanol)	Brown
14	HNO_3 (Conc) + Ammonia solution	Gold brown

Geochemical evaluation

The minerals are estimated in parts per million (ppm) from the plant powders (Table 6)

Phytochemical test

The inference obtained from screening the plant powders for their active principales are presented in the table 8.

Table 2. Behaviour of powder of *A. bracteolata* (whole plant) with chemical reagent under UV light

S.No	Treatment of powder with	Colour under UV light		
		200–280nm	280–320nm	320–400nm
1	Powder such as	Lt. adm. Grey	Sand stone	Dark bs grey
2	Nitrocellulose in amylacetate	New oliver green	New oliver green	Deep green
3	N naoH (aqueous)	Jede green	Oliver green	Deep green
4	N HaoH (aqu)+ Nitrocellulose (in amylacetate)	New oliver green	New oliver green	Copper leaf
5	N NaoH (in Methanol)	Dark bs grey	Deep green	Deep green
6	NaoH (in met) + Nitrocellulose (in amylacetate)	New oliver green	New oliver green	Deep green
7	N Hcl	Smoke grey	Dark bs grey	Sand stone
8	N Hcl + Nitrocellulose (in amylacetate)	New oliver green	New oliver green	Deep green
9	50% HNO_3	Oliver green	Oliver green	Sand stone
10	50% H_2So_4	New oliver green	Bus green	Deep green
11	Methanol	T.A. grey	Air gruft grey	Lt bus grey
12	Saliwanoff's reagent	Brown	Deep green	brown

Table 3. Behaviour of powder extractive of *A. bracteolata* (whole plant) under day and UV light

Name of the extractives	Day light	Colour under UV light		
		200–280nm	280–320nm	320–400nm
Petroleum ether	Mid buff	Pistachio green	Opaline yellow	Golden green
Solvent ether	Deep green	Crimson red	Crimson red	Crimson red
Benzene	Copper leaf	Peal green	Copper leaf	Brown
Chloroform	Deep green	Crimson red	Crimson red	Crimson red
Acetone	Deep green	Opaline green	Opaline green	Crimson red
Ethyl acetate	Deep green	Deep green	Deep green	Deep green
Ethanol	Deep green	Deep green	Oliver green	Crimson red
Water pH 5	Brown	Copper leaf	Copper leaf	Brown
Water pH7	Brown	Brown	Brown	Brown
Water pH9	Deep green	Truck brown	New oliver	Truck brown

Table 4. Estimation of biochemical from *A. bracteolata* (whole plant)

Compound	Amount in % on dry weight basis
Sugar	37
Protein	11.7
Total free amino acid	11.0
Oil	3.1

Table 5. Distribution of free amino acids *A. bracteolata* (whole plant) in powder

Name of amino acid	Intensity of spot
Histidine	–
Serine	+
Lysine	–
Arginine	–
Aspartic acid	–
Glumatic acid	–
Glycine	+
Alanine	+
Theronine	–
Praline	4+
Thyrosine	+
Methionine	–
Valine	+
Tryptophane	+
isoleucine	+
Phenylalanine	–
Leucine	+
Hydroxyproline	–

Table 6. Mineral estimation from *A. bracteolata* (whole plant) in powder

Minerals name	Amount in ppm
Copper	25.01
Ferrous	2025.00
Manganese	124.95
Potassium	84.00
Sodium	20.00
Zinc	30.08
Phosphorus	7.90
Sulphur	400.35

Table 7. Phytochemical evaluation of *A. bracteolata* (whole plant) in powder

Active principle	Name of the test	Degree of precipitation / colouration
Leuco anithocyanins	–	4+
Flavones	–	+
Glycones	–	+
Aglycones	–	7+
Sterols and triterpenes	Liebermann burchard salkowski	+–
Anthraquinones heterosides	Borntrager BZ AZ Modified	3+3+3+2+
Saponin	BZBorntrager AZ FrothLiebermann Burchard	–7+
2–deoxy sugar	Killer–kiliani	3+
Tannin	–	–
Phenol	–	7+
Cardiac glycosides	Keddi reagentModifies keddi reagent	-–

Table 9. Physical constants of *A. bracteolata* (whole plant) in powder

Percentage of			Percentage of insoluble ash in			
Total solid	**Total ash**	**Total sulphated ash**	**Ethanol**	**Water**	**N NaoH**	**5% Hcl**
92	16.7	17.25	7.25	6.25	5.70	1.21

Table 8. Results of alkaloid screening of *A. bracteolata* (whole plant) in powder

Extract	Degree of precipitation				
	DROG	**HAG**	**SCH**	**VAL**	**WAG**
Petroleum ether	+	+	–	–	+
Solvent ether	+	+	–	–	–
Benzene	+	+	–	–	–
Chloroform	+	+	–	–	–
Acetone	2+	+	–	–	+
Ethyl acetate	+	+	–	–	–
Ethanol	+	+	+	+	+
Water	+	+	–	+	+

+ = indicates positive; – = indicates negative; No. + = indicates intensity of precipitation

Table 10. Extractive values of *A. bracteolata* (whole plant) in powder

Name of the extract	Value in percentage
Petroleum ether	12.33
Solvent ether	12.79
Benzene	8.73
Chloroform	14.47
Acetone	14.92
Ethyl acetate	7.77
Ethanol	16.95
Water	60.00

Extractive values

The ash values and extractive values were tabulated in 9 and 10.

Chemical identity comparison

The results of chemical identity of plant powders with that of standard sntileprosy agents like clofazimine, dapsone and rifampicin for their chemical activity equivalence by chemical reaction method (IP) are summarized in Table 11.

DISCUSSION

The plant has high content of sugar (37%). Free amino acids like serine, glycine, alanine, prolone, tyrosine, valine, isoleucine and leucine are located. Among them praline is rich. Geochemical analysis reveals the richness of ferrous (2025ppm) and sulpher (400.35ppm) in the plant. The maximum alkaloids precipitation reaction with its precipitating reagent is observed from water and ethanol extractive.

Total solid and ash content of the sample is 92% and 16.70% respectively. The ash insolubility is less in 50% Hcl and light in ethanol. Sample gives high extractive values (60%) in water.

The chemical reaction comparison with anti leprosy agents showes activity equivalent to the presence of clofozimine (4.1%), dapsone (9.3%) and rifampicin (2.1%). When chemical identity of plant powders are compared with that of standard anti-loprosy agents, it is

Table 11. Chemical comparison of *A. bracteolata* (whole plant) powder with antileprosy agents.

Antileprosy agents	Preliminary test				Confirmatory test by titration% ob=n dry wt. basis
	A	**B**	**C**	**D**	
Clofazimine	4+	3+	0	0	4.1
Dapsone	3+	4+	4+	5+	9.3
Rifampicine	3+	–	–	–	2.1

* the values are average of three replicatives
ABCD - colorations/precipitation reaction as per ID method
Dapsone - have ABCD reactions/tests.
Rifampicin - have a only

found that the powders follow similar reaction to that of the standard. The chemical activity equivalence by chemical reaction reveals that *A. bracteolate* is slightly more close to the anti-leprosy agents. However, their efficacy in leprosy treatment will be confirmed by our pharmacological studies.

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Pharmacognostical and Phytochemical Studies of *Mirabilis Jalapa* Linn. Leaves

Nath Lekshmi R.*, Manjunath KP, Savadi RV and Akki KS

Dept of Pharmacognosy and Phytochemistry, K.L.E.S. College of Pharmacy, Vidyanagar, Hubli - 580031, Karnataka, India.

ABSTRACT

Mirabilis Jalapa Linn. is a widely used traditional medicine in many parts of the world for the treatment of various diseases viz. virus inhibitory activity, anti tumour activity. No reports are available on the pharmacognostic and phytochemical nature of the leaves of *Mirabilis Jalapa* Linn. The present study deals with the macroscopic, microscopic and preliminary physico-chemical investigation. All the parameters were studied according to WHO and Pharmacopoeial guidelines. The qualitative chemical tests of the total alcoholic extract revealed the presence of triterpeniods, alkaloids, glycosides and flavonoids. Some of the diagnostic features of the leaf are the presence of multicellular trichomes, anisocytic stomata and calcium oxalate.

Keywords: *Mirabilis Jalapa* Linn; Phytochemical; Pharmacognostic; Microscopic; Traditional medicine.

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***Author for correspondence:** + 91-9495867759, lrnvaishnav@gmail.com

INTRODUCTION

Mirabilis Jalapa Linn. (Nyctaginaceae) is a perennial herb that reaches a height of 50-100 cm from a tuberous root (1). It produces beautiful flowers that usually open around 4o'clock in the afternoon-hence its common name, *four o'clocks* (2). Leaves and roots are used medicinally in Ayurveda, Siddha and other traditional system of medicine for curing various ailments (3). In ayurveda, it is known by the name "Gulambasa". The plant is reported to possess tonic, diuretic and anti bacterial properties (4–5). The leaves are used traditionally in the treatment of inflammation (6). Adverse side effects and high cost of modern medicine has intensified the search for other effective alternatives. Natural products in general and medicinal plants in particular, are believed to be an important source of new chemical substances with potential therapeutic efficacy (7). The pharmaceutical use of traditionally used medicinal plants is hampered due to the lack of standards of quality, safety and efficacy (8). The present study comprises the macroscopical, microscopical and photochemical nature of the leaves of *Mirabilis Jalapa* Linn.

MATERIALS AND METHODS

The leaves of *Mirabilis Jalapa Linn.* were collected from the local areas of Hubli, Karnataka, and authenticated by Dr. B.D. Huddar, Head, Department of Botany, H.S.K. Science Institute, Hubli, India. The anatomical studies were carried out as per standard methods given in WHO guidelines and Indian Herbal Pharmacopoeia. For anatomical studies, sections of 10–12µm thick were prepared and stained with phloroglucinol-hcl (1:1). Stomatal index was calculated as per standard methods (9). Clearing of leaf was done to study the venation pattern (10). Microphotographs at different magnification were taken. The physico-chemical characters such as extractive values, ash values, loss on drying were performed as per the official standard procedures (11–12). Coarsely powdered leaves were subjected to soxhlet extraction and the alcoholic extract obtained was subjected to successive fractionation with pet ether, n-butanol, and chloroform in the increasing order of polarity. All fractions were further subjected to qualitative test for identification of various phytoconstituents (13).

RESULTS AND DISCUSSION

a. Morphological Evaluation

Leaves are green, bitter and having characteristic odour. These are ovate shape, pinnatified, acuminate apex, crenate, cordate base and 6–11cm in length, 5–7cm in width.

Table 1: Description of Transverse section of *Mirabilis Jalapa* Linn Fresh leaf

Sl.No	Features	Observation
1	Upper epidermis	Single layered-oval shaped parenchyma. Cells covered with cuticle having multicellular covering trichomes, but no stomata.
2	Lamina	Being dorsiventral leaf, it is differentiated into upper palisade cell and lower spongy parenchyma.
3	Vascular bundles (Mid rib)	Possess vascular bundles Xylem and phloemin collateral open arrangement.
4	Lower epidermis	Possess multicellular glandular trichomes with anisocytic stomata

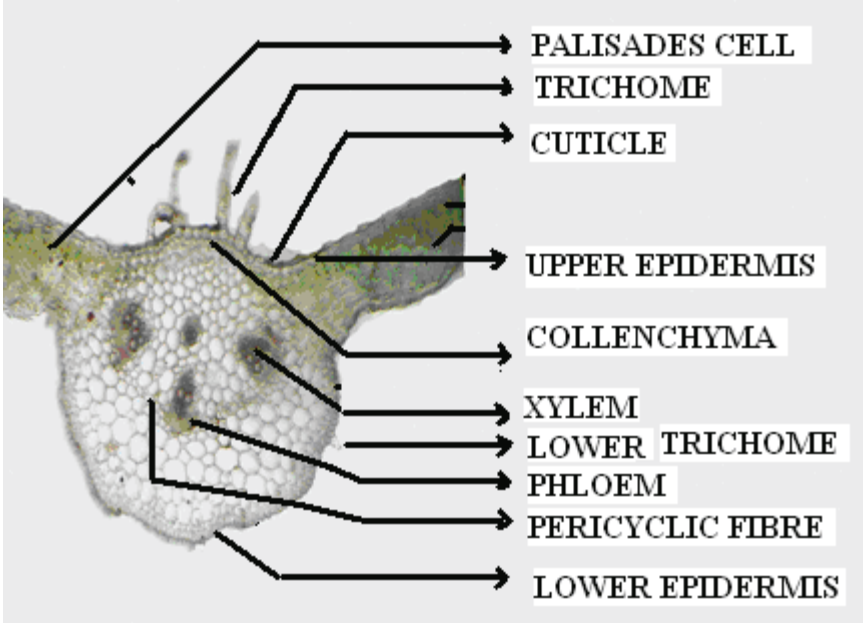

Figure 1: T.S of *Mirabilis Jalapa* L. leaf

b. Microscopical Evaluation

Transverse section of *Mirabilis Jalapa* Linn leaf shows presence of multicellular trichomes on both surfaces (Figure 1, Table 1). The upper and lower epidermis consists of oval shaped parenchyma cells in single layer (Figure 2). Upper epidermis shows absence of stomata, but lower epidermis shows presence of anisocytic stomata. The vascular bundles are composed of both xylem and phloem in collateral open arrangements. *Mirabilis Jalapa* Linn. leaves also show the presence of cubuoidal calcium oxalate crystal and starch grains (Figure 2).

c. Physical Constants

Various physical constants namely Extractive values (Absolute alcohol and water soluble extractive values; 11% w/w and 19.0% w/w respectively) Moisture content (9.4% w/w), Total ash value (9.8% w/w), Acid insoluble ash (2.2% w/w), Water soluble ash (5.6% w/w) and Sulphated ash (9.1% w/w) were determined.

d. Phytochemical Investigations

Qualitative chemical examinations of various extracts revealed the presence of triterpenoids, alkaloids,

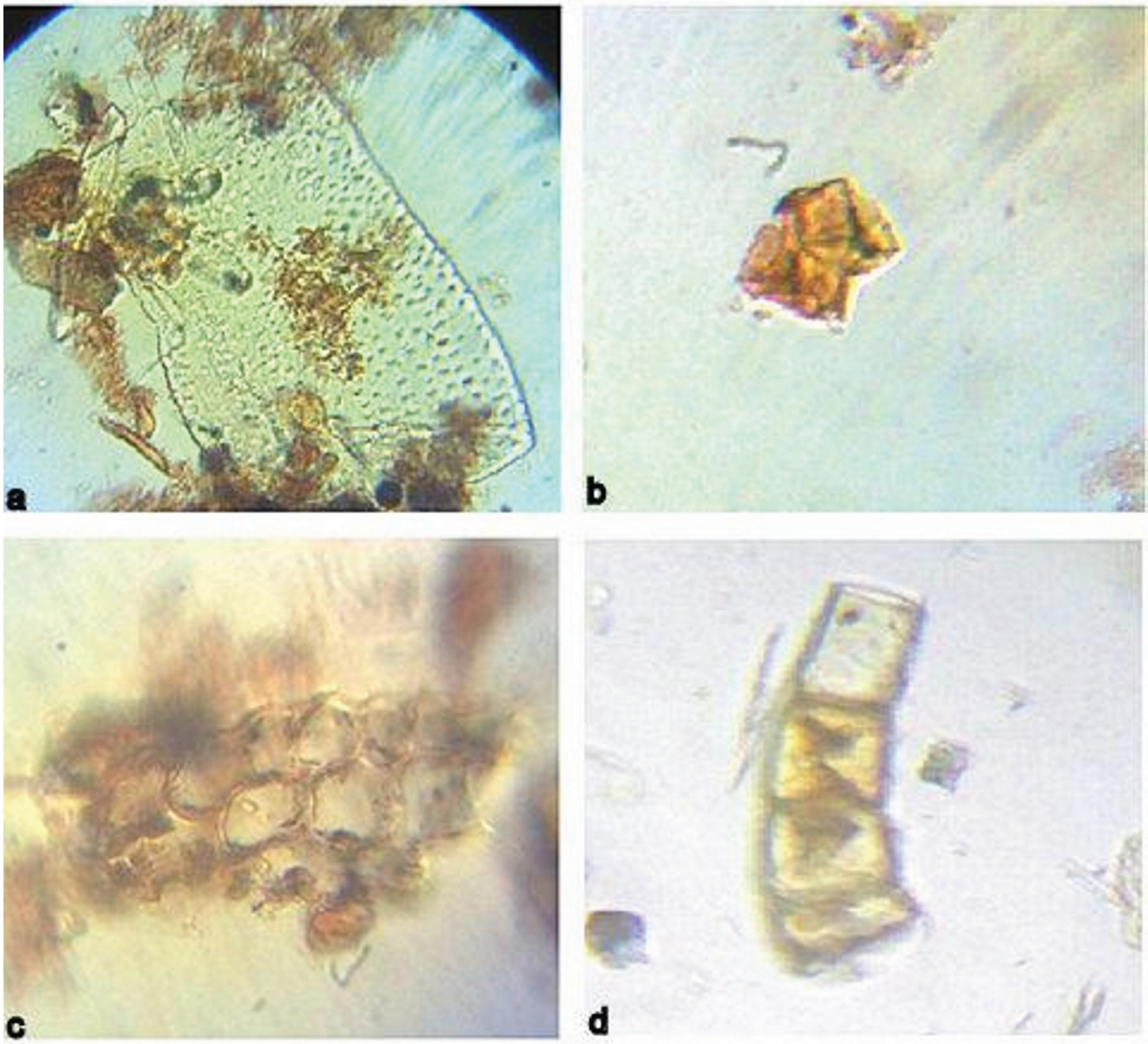

Figure 2: Photographs of cellular components of powdered drugs.
Phloem with sieve plates (a),
wavy type epidermal cells (b),
Rhombohedral type calcium oxalate crystals (c),
Multicellular surface trichome (d).

glycosides, carbohydrates and flavonoids, Further, TLC studies for various extracts substantiated the presence of triterpenoids in total alcoholic, pet.ether fraction and successive butanolic fraction, presence of alkaloid in total alcoholic and successive chloroform fraction, glycoside in total alcoholic, successive butanolic fraction and aqueous fraction. Carbohydrates in total alcoholic and successive aqueous fraction were found. Flavonoids were also found in the total alcoholic and successive butanolic fraction and aqueous fraction.

CONCLUSION

Herbal drugs are derived from heterogeneous sources leading to variations. This makes the standardization of herbal medicines all the more important as erroneous results can cause variations in pharmacological and phytochemical studies. The pharmacognostic characters and phytochemical values reported in this paper could be used as a diagnostic tool for the standardization of this medicinal plant. Presence of adulterants can be

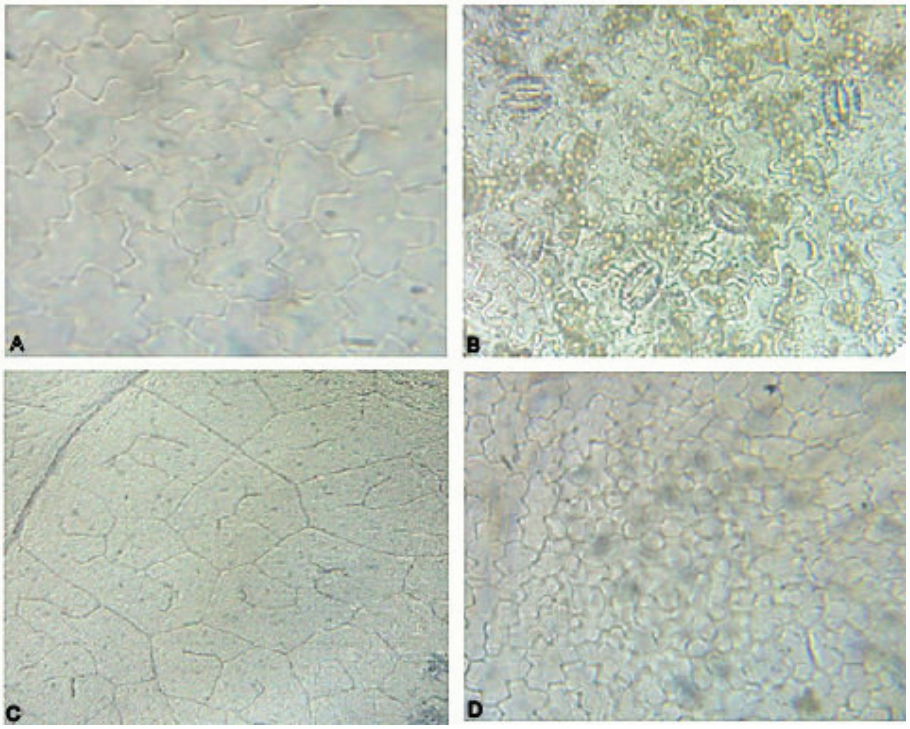

Figure 3: Photographs of *Mirabilis Jalapa* Linn leaves. Upper epidermis without stomata (A), Lower epidermis with anisocytic stomata (B), Vein islet and vein termination (C), Palisade cells below the epidermal layer (D).

Table 2: Description of powder characteristics of *Mirabilis Jalapa* Linn leaf

Sl.No	Features	Observation
1	Nature	Coarse powder
2	Color	Light Green
3	Odour	Characteristic
4	Taste	Acrid
	Microscopic	
5	Xylem vessel	Sieve plate type
6	Trichome	Multicellular glandular type
7	Stomata	Anisocytic stomata with wavy walled surrounding the epidermal cells
8	Ca oxalate crystals	Rhombohedric crystal

Table 3: Details of Leaf surface data

Sl.No	Leaf surface data	Value
1	Stomatal Index of Upper Epidermis	---
2	Stomatal Index of Lower Epidermis	12.5–13.5
3	Vein Islet Number	8–10
4	Vein termination number	6–8
5	Palisade Ratio (Upper epidermis)	5–7
	(Lower epidermis)	3–5

Table 4: Details of physical constants

Sl.No.		Parameter	Determined Value % w/w
A		Extractive value	
	1	Alcohol soluble extractive value	11.00
	2	Water soluble extractive value	19.00
B		Moisture Content	
	1	Total Moisture content	9.40
C		Ash Values	
	1	Total ash	9.80
	2	Acid insoluble ash	2.20
	3	Water soluble ash	5.60
	4	Sulfated ash	9.10

easily identified using these parameters. The microscopic features could help in laying down micro morphological standards as per WHO guidelines for authentication of the drug.

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Table 5: Details of preliminary phytochemical studies

Sl.No	Extract and consistency	% Dry weight in gm	Colour
1	Alcoholic Characteristic Sticky	14	Blackish green
	Successive fractionation		
2	Petroleum ether (40–60°C) Characteristic Waxy	18.9	Pale yellow
3	Chloroform Characteristic Sticky	29.1	Dark green
4	Butanol Characteristic Sticky	33.7	Dark brown
5	Water Characteristic Powder	18.3	Dark brown

Table 6: Details of chemical evaluation

Sr.No	Phyto-Constituent	Alcoholic extract	Successive fractions			
			PE	CL	BU	AQ
1	Carbohydrate	+	–	–	+	+
2	Glycosides	+	–	–	+	+
3	Phytosterol Steroids	+	+	–	–	–
4	Triterpenoids	+	+	–	+	–
5	Tannins and Phenolic Group	+	–	–	+	+
6	Alkaloids	+	–	+	–	–
7	Flavonoids	+	–	–	+	+

for providing the necessary facilities to carry out this study.

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Pharmacognostical, phytochemical screening and acute toxicity study of *Crateva nurvala* stem bark

Sikarwar Mukesh S.[1]*, Patil M.B.[1], Kokate C.K.[2], Sharma Shalini[3], Bhat Vishnu[1]

[1]K. L. E. S College of Pharmacy, Ankola, Karnataka, India
[2]Vice Chancellor, K L E University, Belgaum, Karnataka, India
[3]Dhanwantari Ayurveda Medical College, Hospital & Research centre, Siddapur, Karnataka

ABSTRACT

The aim of this research was to study the pharmacognostic, phytochemical parameters & acute toxicity of *Crateva nurvala* stem bark (family: Capparidaceae). Its stem bark was extracted with non-polar and polar solvents by simple maceration method.TLC were also carried out to identify the chief chemical constituent. The acute toxicity study of these extracts was also carried out in female albino rats (50mg to 5000 mg/kg body weight) as per OECD guidelines. Macroscopic study showed that it was yellowish brown powder with slight characteristic smell and bitter taste. Powder microscopy showed the presence of collenchyma, starch grains, pericyclic fibres, cork cells, stone cells, calcium oxalate crystals and phloem fibres. Phytochemical screening reported the presence of triterpenoids, Flavonoids, tannins & steroidal compounds. In the acute toxicity study, oral administration of 5g/kg of *Crateva nurvala* stem bark various extracts produced neither mortality nor changes in behaviour or any other physiological activities.

Keywords: *Crateva nurvala*, phytochemical, microscopy, acute toxicity.

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***For Correspondence:** Ph. D. Scholar, K L E University, K. L. E. S College of Pharmacy, Ankola, Karnataka, India E-mail: mukeshsikarwar@gmail.com Phone: +91-9343260056

INTRODUCTION

Since ancient times, plants and herbal preparations have been used as medicine. During the past decade, traditional systems of medicine have become a topic of global importance. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs (1).

Crateva nurvala (Family: Capparidaceae) is a small tree with a much branched head. Leaves are deciduous 3 foliolate; petioles 3.8–7.6 cm long; leaflets 5–15 ovate, lanceolate or obovate, acute or acuminate, attenuate at the base, entire, glabrous on both surfaces, pale beneath and reticulately veined. It is usually cultivated in the vicinity of temples in Central India, Bengal & Assam. Its bark is hot, bitter at first and then sweet sharp taste, easy to digest, stomachic, laxative, antilithic, anthelmintic, expectorant and antipyretic. Researches shown that it bark contains saponins and especially useful in urinary complaints such as kidney and bladder stones (2–3).

MATERIALS

Plant material

Crateva nurvala stem bark was collected from local forest area of sirsi in Western Ghats, Karnataka and authenticated by Prof. G. S. Naik (Botanist) of Department of Botany, G. C. Science and Art College, Ankola. A voucher herbarium specimen number GCSAC/CN/01 was also preserved in the same college. The collected material was chopped into small pieces and powdered to coarse consistency in cutter and grinder mill respectively. The powder passed through .40 # mesh particle size and stored in an airtight container at room temperature.

Animals

Healthy young female albino rats (Sprague-Dawley Strain) of weighing 150 to 250 g were selected for experiment & were obtained from animal house of KLES College of Pharmacy, Belgaum and ethical clearance was granted by institutional ethical committee in resolution no. 1/18/2007 held on 23rd November 2007 at J N Medical college, Belgaum (Ethical committee IAEC reg. no. : 627/02/a/CPCSEA). The animals were fed on a standard pellet diet (Goldmohar rat feed, Mumbai) & water ad libitum. All the protocols were performed in accordance with institutional animal ethical committee as per the direction of the CPCSEA (Committee for the purpose of control and supervision of experiments on animals). Alloxan monohydrate was obtained from S. D. Fine chemicals limited, Mumbai. The other chemical reagents were of analytical grade or better.

METHODS

Standardization of crude & powdered drug(4)

Physical evaluation is the primary step adopted in the identification and standardization of crude drugs. It helps in the determination of adulterants and validates the authenticity of crude drug. Various parameters like organoleptic characters, powder microscopy, soluble extractives, loss on drying, ash values & total foreign organic matter were considered for the measurements.

Extraction(5)

The fresh air-dried, powered crude drug was extracted with petroleum ether (60–80°), benzene, chloroform, 95% ethanol & chloroform water I. P. by following simple maceration procedure at room temperature for seven days in a 2000–5000 ml conical flask with occasional shaking and stirring. The extract was filtered and concentrated to dryness at room temperature to avoid the decomposition of the natural metabolites. The dried extract was stored carefully for phytochemical investigation.

Phytochemical screening(4)

Preliminary phytochemical screening was carried out to study the presence of alkaloids, steroids, triterpenoids, essential oil, flavonoids, tannins, carbohydrates, and amino acids in the crude drug & their various extracts.

Thin Layer Chromatography of Major Chemical Constituents in Extracts(6)

TLC of extracts was carried out by using Silica gel GF 254 (activated) as adsorbent. Its slurry was prepared & poured on the clean, dried glass plate and spreaded on it as a uniform coating with thickness of 0.4 mm using glass rod. Plates were activated in hot air oven & maintained at 110°C for 30 minutes. The spots of 20µl of extracts were applied by using fine capillary tube of diameter less than 1 mm on the activated plates at the distance of 2 cm from one end of the plate. Chromatograms were developed by one way ascending TLC. Number and position of various constituents present in the drug was determined by spraying the plate with different spraying reagent. Sprayed plates were heated at 100°C for 10 minutes. Spots were marked and R_f value was calculated for spots.

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Test solution: Reflux 5 gm of powdered drug with 25 ml of hexane for 1 hour. Filter and evaporate to dryness. Dissolved the residue in 2 ml chloroform.

Solvent system[7]: n-hexane: Ethyl acetate (9:1), Visualization of spots: Sprayed the plate with Liebermann burchard reagent.

Acute oral toxicity studies (8)

The acute oral toxicity studies of extracts were carried out as per the guidelines of Organization for Economic Co-operation Development (OECD) guidelines, draft guidelines 423 adopted on 17th December 2001 received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of social justice and empowerment, Government of India.

Health young albino female rats of weighing between 150 to 250 gms (8 to 12 weeks old) were used for acute toxicity study to determine LD50 of various extracts. Three animals were used in each group and the starting dose lied in the range of 50 mg–5000mg/kg body weight. Totally 06 groups, each with three animals were used for assessing the toxicity study. Five groups received test dose and one group was selected as control group. Animals were observed at regular time intervals at least once during the first 30 minutes of initial dosing during the first 24 hrs. Additional observations like behavioural changes in skin, fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems and somato motor activity and behaviour pattern.

RESULTS

Macroscopic study of stem bark of *Crateva nurvala* revealed that it was 8–14 cm long and 4–7 cm wide with thickness

TABLE 1: PHYSICAL TEST OF CRUDE DRUGS

	Physical Test			
Crude drugs	**Nature**	**Colour**	**Odour**	**Taste**
Crateva nurvala	Coarse powder	Yellowish brown	Characteristic	Bitter

TABLE 2: EXTRACTIVE VALUES

Crude drugs	**Pet-ether% w/w**	**Benzene% w/w**	**Chloroform% w/w**	**Alcohol% w/w**	**Aqueous% w/w**
Crateva nurvala	1.46	3.90	3.62	10.65	20.45

of 6–10 mm. The thinner bark pieces were channelled but the thick bark pieces were flat and slightly curved. The outer surface was ash colored and rough due to presence of lenticels and transverse wrinkles. Inner surface was comparatively smooth and whitish brown to buff colored. Fracture was found to be short and splintery. The smoothened internal surfaces showed small yellowish dots indicating presence of stone cells.

Table 1 shows organoleptic characters of the crude drug. Powder microscopy of the drug showed the presence of collenchyma, starch grains with globular or elliptical in shape, lignified pericyclic fibres, brown coloured cork cells, stone cells with pitted thickening, prismatic calcium oxalate crystals and phloem fibres.

Other physical properties necessary in quality control of drug e.g. extractive values, loss on drying, total ash, acid insoluble ash, water soluble ash values and extract organoleptic features were also determined and results were tabulated (Table 2–5).

The preliminary phytochemical screening revealed the presence of triterpenoid, steroids chiefly in petroleum ether & benzene extracts; tannins, flavonoids, and carbohydrates in alcoholic and aqueous extracts; traces of volatile oil & glycosides (Table 6)

TABLE 3: LOSS ON DRYING

Crude drugs	**Loss on drying (% w/w)**
Crateva nurvala	4.37

TABLE 4: TOTAL ASH, ACID INSOLUBLE ASH AND WATER SOLUBLE ASH VALUES

Crude drugs	**Total ash value% w/w**	**Acid insoluble ash value% w/w**	**Water soluble ash% w/w**
Crateva nurvala	9.76	0.647	1.56

TLC of *Crateva nurvala* major Phytoconstituents of stem bark revealed a bright brownish red spot ($\mathbf{R_f = 0.30}$), which turns magenta on keeping. Other spots were $\mathbf{R_f = 0.71}$(Magenta color) $\mathbf{R_f = 0.15, 0.90}$ (light violet spots) also noted.

In acute toxicity study, all the extracts of *crateva nurvala* did not show significant toxicity signs when observed for

TABLE 5: PHYSICAL TEST OF EXTRACTS

Crude drugs	**Extracts**	**Nature**	**Colour**	**Odour**	**Taste**
Crateva nurvala	Pet-ether	Semisolid	Yellowish brown	Characteristic	Astringent
	Benzene	Solid	Yellowish brown	Characteristic	Astringent
	Chloroform	Semisolid	Reddish Brown	Characteristic	Astringent
	Alcohol	Semisolid	Reddish Brown	Characteristic	Astringent
	Aqueous	Semisolid	Reddish Brown	Characteristic	Astringent

TABLE 6: QUALITATIVE CHEMICAL INVESTIGATION OF CRUDE DRUG EXTRACTS

	TEST	*Crateva nurvala*				
	Extracts	Pet- ether	Benzene	Chloroform	Alcohol	Aqueous
1.	**Test for steroids**					
a)	Salkowski test	+	+	–	+	–
b)	Liebermann-burchard test	+	+	+	–	–
c)	Liebermann reaction	+	+	–	–	–
2.	**Test for steroidal glycosides**	–	–	–	–	–
3.	**Test for triterpenoids**					
a)	Salkowski test	+	+	–	–	–
b)	Liebermann-burchard test	+	+	+	–	–
4.	**Test for glycosides**					
a)	Legal test	–	–	–	–	–
b)	Keller killani test	–	–	–	–	–
c)	Modified Borntrager test	–	–	–	+	+
5.	**Test for saponins**					
a)	Foam test	–	–	–	–	–
b)	Haemolysis test	–	–	–	–	–
6.	**Test for carbohydrates**					
a)	Molisch's test	–	–	–	+	+
b)	Barfoed's test	–	–	–	–	–
c)	Benedicts test	–	–	–	+	+
d)	Fehling solution test	–	–	–	+	+
7.	**Test for alkaloids**					
a)	Mayer's reagent test	–	–	–	–	–
b)	Dragondroff's reagent test	–	–	–	–	–
c)	Hager's reagent test	–	–	–	–	–
d)	Wagner reagent test	–	–	–	–	–
8.	**Test for Flavonoids**					
a)	Shinoda test	–	–	–	+	+
b)	Zinc/Hcl reduction test	–	–	–	+	+
9.	**Test for tannins**					
a)	5% Ferric chloride test	–	–	–	+	+
b)	Lead acetate test	–	–	–	+	+
c)	Potassium dichromate test	–	–	–	+	+
10.	**Test for proteins**					
a)	Biuret test	–	–	–	–	–
b)	Million reagent test	–	–	–	–	–
11.	**Test for amino acids**					
a)	Ninhydrin test	–	–	–	–	–
12	**Test for mucilage**					
a)	Ruthenium red	–	–	–	–	–

TABLE 7: ACUTE ORAL TOXICITY STUDIES OF PLANT EXTRACTS

Sl.No	Extracts	LD50 Cut-Off	Vehicle
1	*Crateva nurvala* - Ethanolic extract	5000 mg/kg b.w	Tween 80
2	*Crateva nurvala* - Aqueous extract	5000 mg/kg b.w	Tween 80
3	*Crateva nurvala* - Chloroform extract	5000 mg/kg b.w	Tween 80
4	*Crateva nurvala* - Pet-ether extract	5000 mg/kg b.w	Tween 80
5	*Crateva nurvala* - Benzene extract	5000 mg/kg b.w	Tween 80

$1/10^{th}$ of this lethal dose will be used as effective dose (Therapeutic Dose) for pharmacological screening.

the parameters during the first four hours and followed by daily observations for 14 days and no mortality was also observed, the drug was found to be safe at the tested dose level of 5000 mg/kg b.wt. (Table 7).

DISCUSSION

The study of nature, colour, odour and taste of powdered & crude drug under investigation constitute an important feature of organoleptic evaluation. The determination of extractive values with range of solvents gives information about extractable non polar and polar as well as total extractable plant constituents. The determinations such as loss on drying and ash values indicate the status of air-dried drugs used for studies. The total ash values when comes in acceptable range it simply shows that no inorganic adulteration is present. Total ash value, acid insoluble ash and water-soluble ash were determined and results were in acceptable limits. The results of qualitative chemical investigations of *Crateva nurvala* bark extracts have revealed the presence of various chemical constituents mainly triterpenoids and flavonoids which may be responsible for its antilithic and antidiabetic property respectively. The results obtained were comparable and satisfied the standard literature.

CONCLUSION

In present study the pharmacognostic, phytochemical parameters & acute toxicity of *Crateva nurvala* stem bark were studied and results were in accordance with its pharmacopoeial standards and standard literature. Phytochemical screening reported the presence of triterpenoids, Flavonoids, tannins and steroidal compounds which support its action in traditional use in urolithiasis, skin disorders, anthelmintic, expectorant and antipyretic activity. Pharmacological screening of these extracts is under process and activity guided fractionation & formulation is the future aspect.

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Pharmacognostic studies on *Capparis sepiaria* (L.) R.Br.

Kalidass C.[1], Mohan V.R.[1]*, Amish Abragam D.[2]

[1]Ethnopharmacology unit, Research Department of Botany, V.O. Chidambaram College, Tutuicorin-628 008, Tamil Nadu, India.
[2]Research Department of Botany, St. John's College, Palayamkottai-2, Tamil Nadu, India.

Abstract

Capparis sepiaria (L.) R.Br. known to the *Palliyar* as Muruvilikodi is an important medicinal plant. The *Palliyar* tribe, inhabitants of the Saduragiri hills, Western Ghats, Tamil Nadu, India. use fresh bark, stem and leaves of this plant to treat eczema, dandruff and to reduce the body heat. The present investigation deals with the pharmacognostic studies of the root and stem of the said plant. Pharmacognostic studies include microscopic, physicochemical constant (ash and extractive valves), Fluorescence analysis and preliminary phytochemical evaluations.

Keywords: *Capparis sepiaria*, preliminary phytochemical and pharmacognosy.
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***Corresponding author e-mail:** vrmohan_2005@yahoo.com

INTRODUCTION

The use of plants as medicine is as old as human civilization. Men of all ages in both developing and undeveloped countries use plants in an attempt to cure various diseases and to get relief from physical sufferings. Herbal drugs, in India are also used as household remedy for common ailments since time immemorial. Our ancestrals had a profound knowledge of these medicinal plants and they knew innumerable remedies, a fact indicated in the writings of Siddhars of Tamil Nadu. Their expertise if documented properly would help the modern man find more effective prophylactic use of these herbs.
The relevance of pharmacognosy in standardization of herbal drugs has long been stressed. Many monographs on pharmacognosy have emerged as an aid in the pharmacognostic investigations (1). The process of standardization can be achieved by stepwise pharmacognostic studies (2). These studies help in identification and authentication of the plant material. Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy. Simple pharmacognostic techniques used in standardization of plant material include its morphological, anatomical and biochemical characteristics (3).

Capparis sepiaria is used in skin diseases (4). The decoction prepared from the stem bark along with powdered root of *Capparis sepiaria* is given in dropsy and gout (5). Fresh roots of *Capparis sepiaria* are crushed along with ginger and asafoetida and the paste is applied externally to cure mumps by the Bhil and Nayaka tribals of North Gujarat (6). Paste prepared from the leaves of *Capparis sepiaria* is applied as facial (7). Root paste with opium is applied on swelling of eyes (8). *Capparis sepiaria* is used as blood purifier, stomachic, tonic and appetizer. Its flowers, leaves and roots are used in cough and toxemia (9). Its root powder is used to treat skin diseases, tumours, inflammation and diseases of the muscles (10). *Capparis sepiaria* along with *Oxalis corniculata* and *Ricinus communis* are used for the treatment of aphthae (11). Two or three drops of fresh root juice of *Capparis sepiaria* is put in nasals to reduce the headache (12). Dried leaves of *Capparis sepiaria* are baked and pound to a fine powder. Ash of cotton cloth is mixed with it and applied on aphthae affected area (13). The objective of the present study is to evaluate various pharmacognostic standards like microscopy of leaf, stem and petiole, ash and extractive values, fluorescence analysis of powdered

whole plant and preliminary phytochemical analysis of *Capparis sepiaria*.

MATERIALS AND METHODS

The leaf, petiole and stem of *Capparis sepiaria* were collected from the well grown healthy plants inhabiting the natural forests of Saduragiri hills, Virudhunagar District, South-Eastern slopes of Western Ghats, Tamil Nadu, India. For anatomical investigations standard microtome techniques (14) were followed. Take T.S. of 10 to 12 µm thickness were prepared. These microtome sections were stained with 0.25% aqueous Toluidine blue (Metachromatic stain) adjusted to pH 4.7 (15). Photomicrographs were taken with NIKON trinocular photo micrographic unit. The most accepted descriptive terms were being used to describe the root and stem anatomy (16, 17).

Physico-chemical constant and fluorescence analyses

These studies were carried out as per the standard procedures (18). In the present study, the whole plant powder was treated with 1N aqueous sodium hydroxide and 1N alcoholic sodium hydroxide, acids like 1N hydrochloric acid, 50% sulphuric acid, nitric acid, picric acid, acetic acid and nitric acid with ammonia. These extracts were subjected to fluorescence analysis in visible/ daylight and UV light (254nm and 365nm). Various ash types and extractive values were determined by following standard method (19,20).

Preliminary phytochemical analysis

Shaded dried and powdered whole plant samples were successively extracted with petroleum ether, benzene, chloroform and alcohol. The extracts were filtered and concentrated using vaccum distillation. The different extracts were subjected to qualitative tests for the identification of various phytochemical constituents as per standard procedure (21,22).

RESULTS AND DISCUSSION

The anatomical features of the leaf, petiole and stem of *Capparis sepiaria* investigated are summarized in plate I and II. The surface of midrib is wavy and the epidermal layer in thin and less conspicuous. The ground tissue is parenchymatous and homogeneous; the cells are small and compact. The vascular strand is single, prominent and deeply urn shaped. It consists of dense and compact thick walled fibres and vessels arranged into thick radial segments. Phloem is wide and ensheaths the xylem strand (Plate-I. 1, 2). The vascular strand is 400 µm wide. The lateral vein is also prominent and planoconvox in sectional view. It has thick walled narrow epidermal layer, compact, thick walled ground parenchyma and wide arc of vascular strand comprising of long and thick radial segments of collateral bundles (Plate-I. 3, 4).

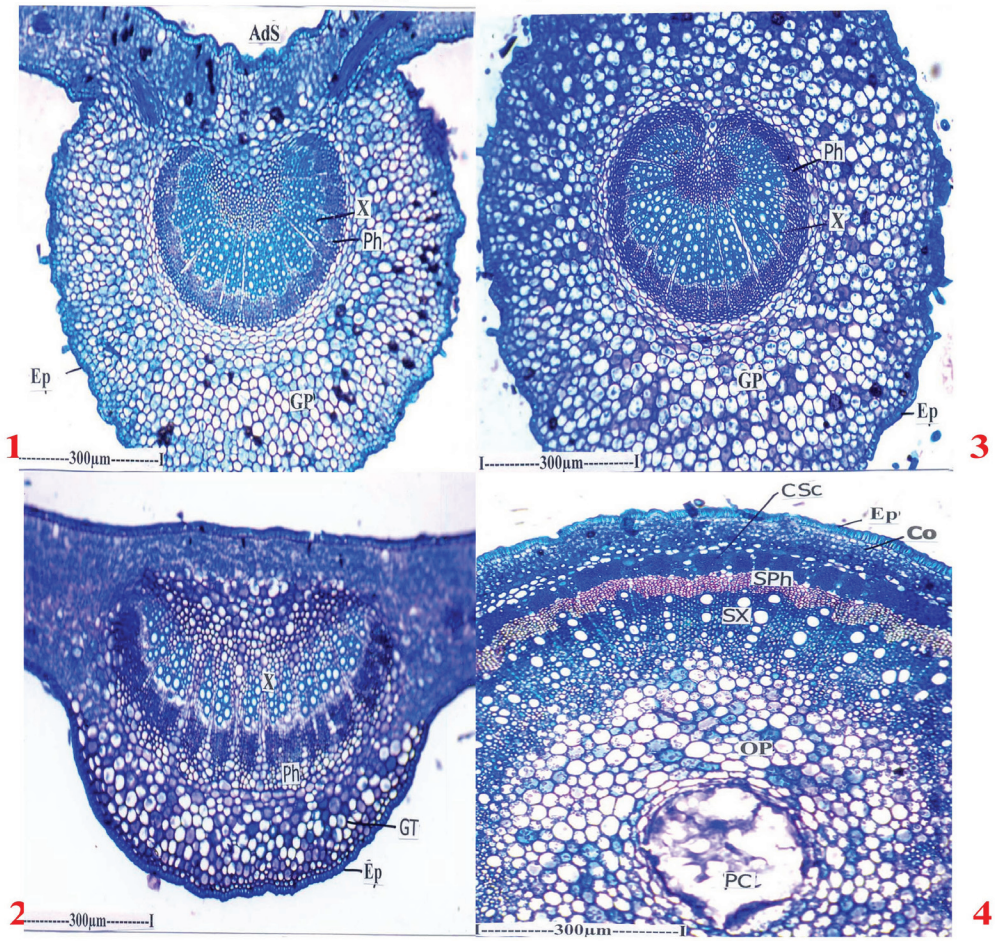
Lamina (Plate-I. 5) is smooth and even. It has narrow epidermal layers of small cells with thick cuticle. The mesophyll has undifferentiated compact layers of small cells. The vein lets have small circular vascular strands place in the median part of the mesophyll. The lateral veins are thick and straight. They form wide and distinct vein islets which polygonal in outline. The islets include distinct vein terminations which thick, profusely forked into dendroid pattern. The epidermal cells are small, polygonal and random in orientation. They have thick and straight anticlinal walls. The adaxial epidermis is apostomatic (Plate-I. 6). The abaxial epidermis is stomatiferous. The stomata are dense and are paracytic type (Plate-I. 7)

The petiole has a thin layer of thick walled, heavily cuticularised epidermal cells. The ground parenchyma has small, circular compact thick walled cells. The vascular strand is circular with narrow adaxial cleft. It consist of several, wedge shaped thick, compact segments, each segment having radial chains of xylem elements and thick walled fibres (Plate-II. 2). Phloem ensheaths the outer boundary of the xylem. A thick layer of sclerenchyma cells abust the phloem boundary.

The stem is circular in sectional view. It has radially oblong, thick walled lignified cells. The cortex has outer zone of parenchyma and inner zone of continuous thick cylinder of sclerenchyma Secondary phloem has wide cylinder of diffusely distributed cells. Secondary xylem is thin and hollow cylinder; the xylem elements include wide, thick walled solitary vessels and thick walled fibres with wide lumen. The pith is wide and parenchymatous and has a central lysigenous canal.

The results of the ash and extractive values of *Capparis sepiaria* whole plant drug powder are depicted in Table-1. The total ash content of the powdered whole plant is 5.34% and extractive value in water is more than in ethanol. The results of fluorescence analysis of whole plant powder of *Capparis sepiaria* are shown in Table-2. The whole plant powder shows the characteristic fluorescent colour, when treated with IN aqueous NaOH and acetic acid under short UV light. The results of preliminary phytochemical screening of whole plant of *Capparis sepiaria* are presented in Table-3. The methanol extracts of the whole plant shows the presence of alkaloid, catachin, coumarin, phenol, saponin, tannin, terpenoid, sugar and xanthoprotein.

Plate I: *Capparis sepiaria* Linn.



Plate–I: *Capparis sepiaria* Linn.

Anatomy of the leaf
1. T.S of leaf midrib enlarged.

Anatomy of the lateral vein with lamina
2. T.S of leaf lateral vein – magnified.

Anatomy of the petiole
3. T.S of petiole ground plan.

Anatomy of the stem
4. T.S of stem a sector enlarged

AdS- Adaxial side; Ep-Epidermis; GP-Ground parenchyma; GT-Ground tissue; Ph-Phloem; X-Xylem; Co – Cortex; CSc – Corticosclereid; OP – Outer pith; PC – Inner pith cavity; Sph – Secondary phloem; SX – Secondary xylem.

The present study attempts a modest comprehensive investigation of the leaf, petiole and stem of *Capparis sepiaria*. Since the whole plant of *Capparis sepiaria* as the folklore claims has therapeutic qualities the present investigation has laid down a set of anatomical features of the leaf, petiole and stem which can be employed for its botanical diagnosis. The salient features of identification of the fragmentary sample are as follows.

Salient features of Capparis sepiaria

- It is a thorny straggling shrub commonly found in the semiarid forests.
- The leaf is characterized by wide circular abaxial midrib with deeply curved thick vascular strand and less distinct differentiation of the mesophyll tissue.
- Petiole is circular with undulate outline. Vascular strand is circular with adaxial cleft.

Table 1. Ash values and Extractive values of the powdered whole plant of *Capparis separia*

Type of Ash	% of Ash
Total ash value of powder	5.34±0.03
Water soluble ash	1.90±0.01
Alkalinity of water soluble ash	2.21±0.02
Acid insoluble ash	1.44±0.02
Nature of extract	**Extractive value (%)**
Alcohol (Ethonolic)	4.84±0.03
Water (Aqueous)	5.92±0.02

- The venation system consists of wide polygonal vein-islet and forked vein terminations.
- The epidermal cells are small polygonal, thick walled with straight anticlinal walls. The stomata are paracytic type.
- The stem has radially elongated thick walled epidermis, heterogeneous cortex and thin wide cylinder of xylem in which vessels in radial chains.
- A wide lysigenous canal is seen in the centre of the wide pith.

Table 2. Fluorescence analysis of the powdered whole plant of *Capparis sepiaria*

		UV Light	
Experiments	**Visible/Day light**	**254nm**	**365nm**
Drug powder as such	Pale green	Pale green	Dark green
Powder + 1N NaOH (aqueous)	Brownish yellow	Fluorescent green	Green
Powder + 1N NaOH (alcohol)	Pale brown	Pale yellow	Dark green
Powder + 1N HCL	Pale green	Pale yellow	Fluorescent green
Powder + 50% H_2So_4	Pink	Violet	Dark green
Drug powder + Nitric acid	Brown	Green	Dark green
Drug Powder + Picric acid	Pale yellow	Pale yellow	Dark brown
Drug Powder + Acetic acid	Pale green	Fluorescent green	Dark green
Drug Powder + HNO_3 + NH_3	Brown	Yellowish green	Pale brown

Table 3. Preliminary phytochemical screening of whole plant extract of *Capparis sepiaria*

Test	Petroleum ether	Benzene	Chloroform	Methanol
Alkaloid	–	–	–	+
Anthraquinone	+	–	–	–
Catachin	+	+	–	+
Coumarin	–	+	+	+
Flavonoid	–	+	–	–
Phenol	+	–	–	+
Quinone	–	–	–	–
Saponin	–	–	–	+
Steroid	+	–	–	–
Tannin	–	–	+	+
Terpenoid	+	–	–	+
Sugar	–	+	–	+
Glycosides	–	–	+	–
Xanthoprotein	+	–	–	+
Fixed oil	+	+	–	–

+: Present, –: Absent.

The physical constant evaluation of the drug is an important parameter in detecting adulteration or improper handling of drugs by African Pharmacopoeia (19) 1986). Equally important in the evaluation of crude drugs, is the ash value and acid insoluble ash value determination. The total ash is particularly important in the evaluation of purity of drugs, i.e., the presence or absence of foreign organic matter such as metallic salts and/or silica (23).

The ash value of *Capparis sepiaria* whole plant is 5.34%. This ash value is indicative of the impurities present in the drug. Since the ash value is constant for a given drug, this value is also one of the diagnostic parameters of the drug. In the present study, *Capparis sepiaria* whole plant has more water soluble ash than acid insoluble ash. The ash value is generally the index of the purity as well as identity of the drug.

Many phytocompounds fluoresce when suitably illuminated. The fluorescence colour is specific for each compound. A non-fluorescent compound may fluoresce if mixed with impurities that are fluorescent. The fluorescent method is adequately sensitive and enables the precise and accurate determination of the analyze over a satisfactory concentration range without several time consuming dilution steps prior to analysis of pharmaceutical samples (24). In the present study is the powdered whole plant of *Capparis sepiaria* emitted pale green under day light and short UV light and dark green in long UV light.

Presence or absence of certain important compounds in an extract is determined by colour reactions of the compounds with specific chemicals which act as dyes. This procedure is a simple preliminary prerequisite before going for detailed photochemical investigation. Various tests have been conducted qualitatively to find out the presence or absence of bioactive compounds. Different chemical compounds such as alkaloids, terpenoids, coumarin, tannin, saponin, flavonoids, quinines, anthraquinones, phenols and glycosides are detected in *Capparis sepiaria* whole plant extracts which could made the plant useful for treating different ailments as having a potential of providing useful drugs of human use.

In this dimension of pharmacognostic studies on *Capparis sepiaria* (L.) R.Br. is a substantial step and it further requires a long term study to evaluate pharmacological action as well as therapeutic efficacy and toxicity of said plant to establish as the drug. The pharmacognostic study of the *Capparis sepiaria* has been carried out for the first time. This could be useful in the preparation of the herbal section of proposed pharmacopoeia.

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Determination of Rutin Content and Antioxidant Activity of Extracts of *Butea monosperma* Flowers Extracted Using Various Extraction Methods

Edwin Jarald E*, Nalwaya Narendra, Meena Manish, Jain Anurekha, Edwin Sheeja[1]

*TIFAC-CORE In Green Pharmacy, B.R. Nahata College of Pharmacy, Mhow-Neemuch Road, Mandsaur – 458001, Madhya Pradesh, India.
[1]Unijules Life Sciences Ltd. & Associated Companies, Nagpur.

ABSTRACT

In our present study, we quantified rutin and also determined the *in vitro* antioxidant potential of aqueous extracts of *Butea monosperma* flowers prepared using soxhlet, decoction, ultrasonic and maceration methods. Presence of rutin was identified using chemical test and TLC method. Quantification of rutin in extracts was done by HPLC method. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitro blue tetrazolium (NBT) assay method was performed at different concentrations (100–400 µg/ml). All the extracts showed the presence of rutin. The percentage yield of crude extract and the quantity of rutin was found to be more in the extract prepared by soxhlet method than the other extracts. The antioxidant activity of extracts increased in a dose dependent manner. Concentration of soxhlet, decoction, ultrasonic and maceration extract needed for 50% inhibition of DPPH were found to be 52.80, 58.02, 60.86 and 52.62 µg/ml respectively. Similarly concentration needed for superoxide scavenging activity was found to be 543.92, 906.61, 752.44 and 680.04 µg/ml respectively. The results of our study showed that the *B. monosperma* possess a significant antioxidant activity and the maceration extract showed better antioxidant activity than the other extracts in DPPH model and in NBT model, the extract prepared using soxhlet showed better activity compared to other extracts.

Keywords: Antioxidant activity; *Butea monosperma*; Decoction; Maceration; Soxhlet; Ultrasonic.

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***Author for correspondence:** E-mail address: ejeru@rediffmail.com

INTRODUCTION

Butea monosperma Linn. (family-Leguminoceae) is a species of Butea commonly found all over India and Brahmadesh. The flowers are large 15 cm long, beautiful, bright orange red colored in rigid racemes, grow in spring (1). Rutin is the most widespread of all quercetin glycosides, which is also found in flowers of *B. monosperma*. Chemically it is quercetin-3-rutinoside and is sparingly soluble in water but freely soluble in methanol. After hydrolysis it affords an aglycone quercetin and two sugars i.e. rhamnose and glucose. Rutin as well as its aglycone quercetin both are used as vitamin P, in coronary thrombosis and purification of blood. Rutin is also used as antioxidant (2). Most of the diseases are mainly linked to oxidative stress due to free radicals. Antioxidant compounds play an important role as a health-protecting factor (3). Extraction is one of the most crucial points in the analytical chain in the effort of achieving a complete recovery of targeted compounds (4).

The present study deals with the comparative estimation rutin content and *in-vitro* antioxidant potential of aqueous extract of *Butea monosperma* flowers extracted using various extraction methods.

MATERIALS AND METHODS

Plant Material

Flowers of *Butea monosperma* were collected from local area of Mandsaur region. The collected material was dried and powdered.

Apparatus and Chemicals

HPLC System (Merck), UV Spectrophotometer (Thermospectronic Model of Elico India SL-159), Soxhlet assembly, Ultrasonicator (Scinentech), Water bath, TLC plates & chamber, Rutin Standard, HPLC grade methanol

and other chemicals & reagents of analytical grade were used.

Preparation of Test and Standard Solutions

The extracts were separately dissolved in ethanol and used for the *in-vitro* antioxidant study. The extracts and the pure rutin were dissolved in HPLC grade methanol separately and used for quantification of rutin. The stock solutions were serially diluted with HPLC grade methanol to obtain the lower dilutions.

Extraction Methods

Soxhlet extraction

Plant material (flowers) (25 g) was accurately weighed, packed in a soxhlet apparatus and extracted using water as solvent for 24 hrs at 50°C. After extraction the content of the flasks were filtered through filter paper and the filtrate was evaporated.

Maceration extraction

Plant material (flowers) (25 g) was taken in a 250 ml conical flask and 100 ml water was added. Then the flask was placed on a mechanical shaker for 24 hrs. After extraction the content of the flask was filtered through filter paper and the filtrate was evaporated.

Ultrasonic extraction

Ultrasound assisted extraction was performed in an ultrasonic bath. 25 g of plant material (flowers) was kept with water (100 ml) in a 250 ml conical flask and kept for sonication for 2 hrs at room temperature. After extraction, the contents were filtered and evaporated to dryness.

Decoction extraction

Plant material (25 g) was taken in a beaker and 100 ml of water was added. The plant material was allowed to boil for 15 mins. After extraction, the contents were filtered and evaporated to dryness.

Phytochemical screening

In order to determine the presence of rutin, a preliminary phytochemical study (colour reaction) with various extracts was performed.

Thin layer chromatography

To confirm the presence of rutin, TLC was performed using Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:27) as the solvent system. Presence of rutin could be detected by the presence of yellow coloured spot (5).

Determination of Rutin Content by HPLC

The extracts and pure rutin were dissolved in HPLC grade methanol separately and used for evaluation of rutin. The stock solutions were serially diluted with HPLC grade methanol to obtain a dilution of 10 µg/ml. Chromatographic separation was performed on a Lachrom liquid chromatographic system equipped with a RP-C18 column, reciprocating pump, UV detector and a rheodyne injector with 20 µl loop volume. Mobile phase of HPLC grade methanol was used and was delivered at a flow rate of 0.5 ml/min with detection at 257 nm. Then the peak area ratios of standard and sample solutions were calculated and the concentration of rutin was determined by using following formula.

$$\text{Concentration of Sample} = \frac{\text{AUC of Sample}}{\text{AUC of Standard}} \times \text{Concentration of Standard} \times \text{Dilution Factor}$$

In-vitro Antioxidant Activity
1, 1 –diphenyl-2-picryl hyadrazyl (DPPH) Method

Different concentrations of each herbal extracts were added at an equal volume, to methanolic solution of DPPH. After 15min at room temperature, the absorbance was recorded at 519 nm (6). It was repeated for 3 times. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

The percentage inhibition was calculated by following formula:

$$\% \text{ Inhibition} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

Nitro Blue Tetrazolium (NBT) Method

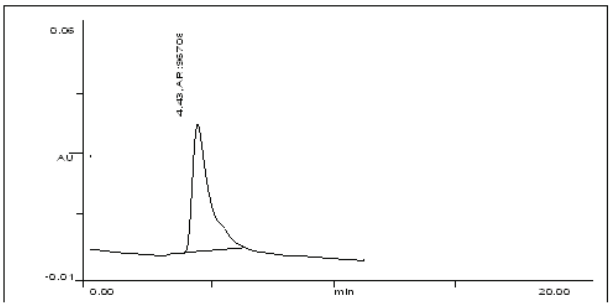
The method was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radical generated in riboflavin-light-NBT system. The reaction mixture contains EDTA, riboflavin, nitro blue tetrazolium (NBT), various concentrations of extracts and phosphate buffer (pH 7.6) in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp for 15 min and absorbance was measured at 560 nm (7).

The percentage inhibition was calculated by following formula:

$$\% \text{ Inhibition} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

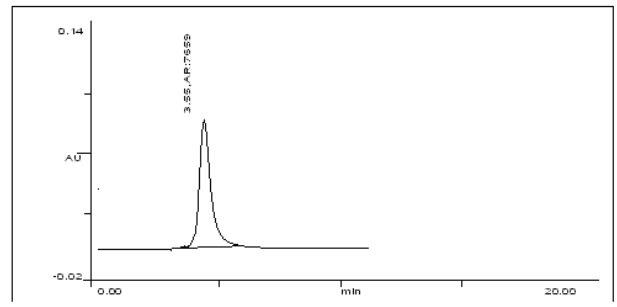
RESULTS AND DISCUSSION

In our present study, we quantified rutin and also determined the *in vitro* antioxidant potential of aqueous extract of *Butea monosperma* flower extracted using soxhlet, decoction, ultrasonic and maceration methods. Presence of rutin was identified using chemical test and TLC method (Table 1). Quantification of rutin in extracts was done by HPLC method (Fig. 1–5). All the extracts showed the presence of rutin. The percentage yield of crude extract and the quantity of rutin was found to be more in extract prepared by soxhlet method when compared to other extracts (Table 2). The results of our study showed that the *B. monosperma* possessed a significant antioxidant activity and the maceration extract showed better antioxidant activity than the other extracts in DPPH model and in NBT model, the extract prepared using soxhlet showed better activity than the other extracts. The antioxidant activity of aqueous extracts increased in a dose dependent manner. The IC_{50} values are presented in Table 3. Flavonoids are used for the prevention and cure of various diseases which is mainly associated with free radicals. Flavonoids acts as an antioxidant in biological systems and scavenge the free radicals thereby increasing the antioxidant defence in the body. The effect of Flavonoids and other putative antioxidants on biomarkers of oxidation have been studied in many pathological states that are thought to result from, or result in oxidative stress (3). In our study, better antioxidant activity was found in extracts prepared using soxhlet and maceration. The content of rutin is high in extract prepared using soxhlet and the content of rutin is less comparatively in the extract prepared using maceration. Even though the concentration of rutin is less in maceration extract the activity was equivalent to soxhlet extract. This may be due to the presence of other



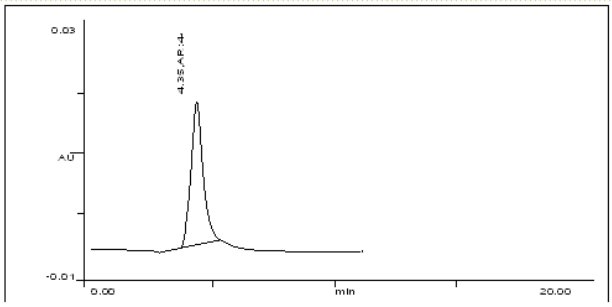
No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	4.43	8228	9670871	100.0000	100.0000	BB	0.802
		8e+03	9670871				

Figure 1. HPLC of Rutin



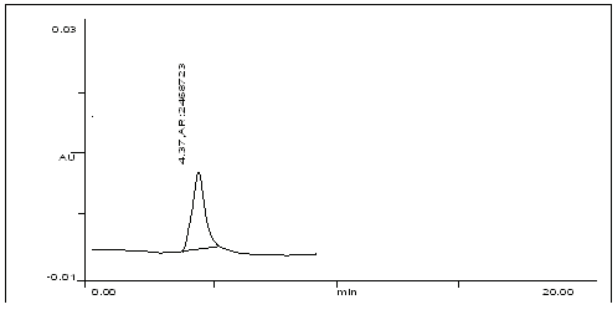
No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	3.55	175	76593	0.9188	0.4989	S	0.299
2	4.39	18872	15276451	99.0812	99.5011	BB	0.553
		2e+04	15353044				

Figure 2. HPLC of Aqueous Extract of *B. monosperma* Extracted by Soxhlet Method



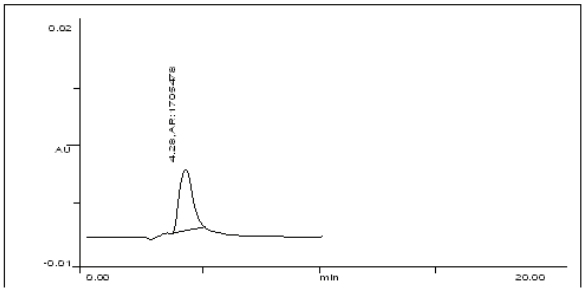
No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	4.35	5300	4436234	100.0000	100.0000	BB	0.571
		5e+03	4436234				

Figure 3. HPLC of Aqueous Extract of *B. monosperma* Extracted by Decoction Method



No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	4.37	2831	2468723	100.0000	100.0000	BB	0.595
		3e+03	2468723				

Figure 4. HPLC of Aqueous Extract of *B. monosperma* Extracted by Ultrasonication Method



No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	4.28	1778	1705478	100.0000	100.0000	BB	0.655
		2e+03	1705478				

Figure 5. HPLC of Aqueous Extract of *B. monosperma* Extracted by Maceration Method

Table 1. TLC of Rutin in the extracts of *B. monosperma*

Extracts	R_f Value and Colour
Standard	0.62 (Yellowish Green)
Soxhlet	0.63 (Yellowish Green)
Decoction	0.62 (Yellowish Green)
Ultrasonication	0.61 (Yellowish Green)
Maceration	0.62 (Yellowish Green)

Table 2. % Yield and Concentration of Rutin in *B. monosperma*

Extraction Methods	Yield* (% w/w)	Concentration of Rutin* (% w/w)
Soxhlet	47.33	82.32
Decoction	35.07	45.87
Ultrasonication	11.28	25.52
Maceration	10.12	17.63

* *Average of the three determination*

Table 3. IC_{50} Values* (µg/ml)

Extracts	Methods	
	DPPH	NBT
Soxhlet	52.80	543.92
Decoction	58.02	906.61
Ultrasonication	60.86	752.44
Maceration	52.62	680.04

* *Average of the three determination*

antioxidant chemicals in maceration extract. The results indicated that the developed procedure could be used for the estimation of rutin.

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Determination of Protocatechuic Acid by HPTLC Method in *Amomum subulatum* Roxb. Fruit Extracts

Manek Ravi*[1] , Patel N. M.[2], Bhargava Anurag[2], Vaghasiya Jitendra[1], Jivani N. P.[1], Koradia Shailesh[1]

[1]RBPMPC, Kailashnagar, Atkot-360040, Dist: Rajkot, Gujarat, India
[2]BMCPER, Modasa, Dist: Sabarkantha, Gujarat, India

ABSTRACT

The aim of present study is development of simple, rapid and accurate HPTLC method for estimation of Protocatechuic acid in various extracts of *Amumum subulatum* Roxb. fruit constituents(Family Zingiberacea), commonly known as 'Badi Elaichi'. The powdered drug was subjected to extraction by soxhlet apparatus using methanol and acetone separately as well as Petroleum ether (40–60), chloroform, methanol and water successively. The extracts were screened for presence of phytoconstituents using preliminary chemical tests. Protocatechuic acid was estimated in methanol and acetone extract by HPTLC method. Detection and quantification was performed at wavelength 254nm. The Acetone and methanol extracts were found to contain 1.04846 and 0.8634 %w/w Protocatechuic acid respectively by using validated method. Since this method resolves and quantifies protocatechuic acid accurately and precisely, it can be useful for quantification of the compound in herbal formulation.

Keywords: Protocatechuic Acid, HPTLC, *Amomum subulatum* Roxb.
Editor: Mueen Ahmed KK, Phcog.Net

***E-mail address:** ravi_manek@yahoo.com

INTRODUCTION

The quality of herbal medicine is implication of safety and efficacy, which is profile of constituents present in it. It is difficult to establish quality control parameters of plant based drug due to complex nature and inheritant variability of chemical constituents. So, modern analytical techniques should be implicated to over come this problem (1).

Since ancient time, seeds of *Amomum subulatum* Roxb. have been valued for its aroma, as spice, flavor and condiment. The seeds are reported in Ayurvedic system of medicine and are an official drug in Ayurvedic Pharmacopoeia and are marketed under the name of '*Elcho*' or '*Badi Elaichi*' (2). Traditionally it has been used for digestive problems treating flatulence, loss of appetite, gastric complaints, congestion of liver and also recommended in cases of inflammatory condition of eyes (3). Orally administered *A. subulatum* could be useful in prevention of hyperlipidaemia and provide antioxidant protection (4). An anti-wrinkle cream containing *A. subulatum* was evaluated in the treatment of facial skin wrinkles by prospective, open, phase III clinical trial and showed that the active constituents of *A. subulatum* (protocatechualdehyde and protocatechuic acid) have potent antioxidant activity (5). Greater cardamom (*A. subulatum* have significant ability to inhibit lipid peroxidation in rat liver homogenate due to their polyphenol content, strong reducing power and superoxide radical scavenging activity (6). Protocatechualdehyde, Protocatechuic acid, 1,7-bis (3,4-dihydroxyphenyl) hepta-4E,6E-dien-3-one and 2,3,7-trihydroxy-5-(3,4-dihydroxy-E-styryl)-6,7,8,9-tetrahydro-5H-benzocycloheptene was isolated from greater cardamom and evaluated for its antioxidant acivity (7).

It was found to possess antioxidant activity, attributed to presence of protocatechuic acid. Since protocatechuic acid is marker compound responsible for its antioxidant activity. So, *A. subulatum* fruit constituent extracts are subjected to HPTLC analysis by developing a method for estimation of protocatechuic acid in methanol and acetone extract. The proposed method has been validated as per ICH guidelines (8–9)

MATERIAL AND METHODS

Present study was conducted at BMCPER, Modasa, Gujarat and RBPMPC, Atkot, Dist Rajkot, Gujarat, India during January 2007 to November 2008.

Collection and authentication of the fruits and seeds

The fruits of *Amomum subulatum* Roxb were collected from local market of Modasa and authenticated by Dr. H. B. Singh Scientist and Head of Raw Materials Herbarium & Museum Dept of National Institute of Science and Communication and Information Resources, New Delhi (NISCAIR) and preserved at the herbarium in Dept. of Pharmacognosy, B. M. Shah College of Pharmaceutical Education and Research, Modasa.

Extraction and Phytochemical Investigations

100g powder of fruit constituents of *A. subulatum* were extracted with Methanol and Acetone separately. Successive extraction was performed by using Petroleum ether (40–60), chloroform and Methanol successively by soxhlet apparatus and lastly remaining marc was refluxed with water. The extracts were concentrated and air dried, weighed and percentage yield was determined. Qualitative chemical tests for identifying various phytoconstituents present were carried out on all extracts of *A. subulatum* Roxb fruit constituents (10).

Estimation of Protocatechuic acid by HPTLC in Methanol and Acetone extract of A. subulatum Roxb *fruit constiuents*

Materials: Standard Protocatechuic acid was a purchased from LGC Promochem Pvt. Ltd. Bangalore All the chemicals used in the experiments are of analytical grade.

Experimental condition

Sample applicator: Camag Linomat V Automatic Sample Spotter
Stationary phase: precoated silica gel plates 60 F254 (10 cm × 10 cm, with 0.2 mm thickness, E. Merck, Darmstadt, Germany) The plates were prewashed by methanol and activated at 60 °C for 5 min prior to chromatography.
Solvent system: Chloroform: Acetic acid (9:1) (11)
Development chamber: CAMAG glass twin-through chamber (10 × 10 cm) previously saturated with the solvent for 60 min (temperature 25.2 °C, relative humidity 40%). The development distance was 8 cm. Subsequent to the scanning.
Scanner: Camag TLC scanner III in absorbance mode at 254 nm and operated by Win- Cats software 4.03 version.

Evaluation was *via* peak areas with linear regression

Calibration Curve of Standard Protocatechuic acid

A stock solution of Protocatechuic acid was prepared by dissolving 10mg of compound in ethanol and volume was made up to 10 ml in volumetric flask. From this solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8µl spots were applied on plate as shown in figure 1.

Estimation of Protocatechuic acid in Alcoholic and Acetone Extract

To determine content of Protocatechuic acid in Methanolic and acetone extract, an accurately weighed 50 mg of extracts were transferred to 10ml volumetric

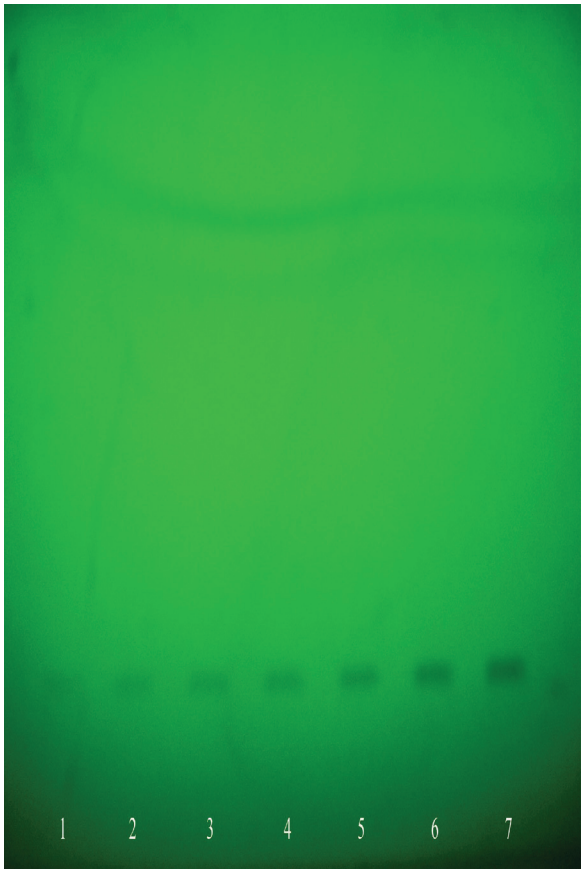

Figure 1. Image of HPTLC plate (254nm) for calibration curve
1 - 100 ng of Protocatechuic acid Standard, 2 - 200 ng of Protocatechuic acid Standard, 3 - 300 ng of Protocatechuic acid Standard, 4 - 400 ng of Protocatechuic acid Standard, 5 - 500 ng of Protocatechuic acid Standard, 6 - 600 ng of Protocatechuic acid Standard, 7 - 800 ng of Protocatechuic acid Standard

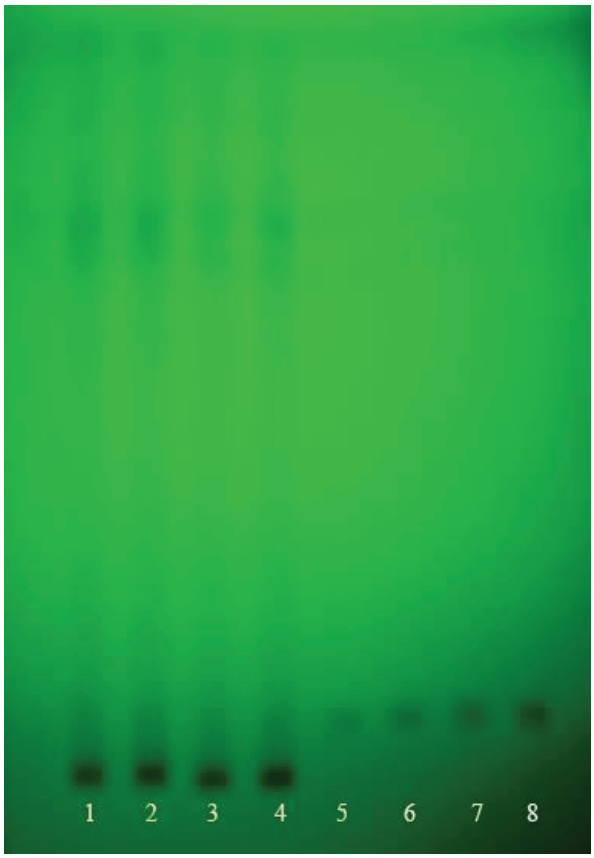

Figure 2. Image of HPTLC plate (254nm)
1. 5µl Acetone extract (5mg/ml), **2.** 10µl Acetone extract (5mg/ml), **3.** 5µl Methanol extract (5mg/ml), **4.** 10µl Methanol extract (5mg/ml), **5.** 0.2µl Protocatechuic acid Standard (1mg/ml), **6.** 0.4µl Protocatechuic acid Standard (1mg/ml), **7.** 0.6µl Protocatechuic acid Standard (1mg/ml), **8.** 0.8µl Protocatechuic acid Standard (1mg/ml), Solvent system Chloroform: Acetic acid (9:1), Detection at 254nm.

flask separately. Then dissolved in ethanol and diluted up to 10ml with ethanol. The solutions were filtered with what man no. 1 filter paper. Spots of 5 and 10 µl of both the solutions were applied to TLC plate along with 0.2, 0.4, 0.6 and 0.8 µl of Protocatechuic acid Standard (1mg/ml) spots on same plate as shown in figure 2. Peak of Protocatechuic acid in extract solution was identified by matching the Rf with peak obtained in Protocatechuic acid Standard solution.

The method was validated in terms of linearity, precision, repeatability, specificity, Limit of detection (LOD), Limit of quantification (LOQ) (8–9).

RESULTS

Result in Table 1 shows that 15.06% w/w methanol extract having dark brownish black color with characteristic odour and semisolid consistency, 14.56% w/w Acetone extract having dark brownish black color with characteristic odour and semisolid consistency were obtained. While successive extraction was performed with Petroleum ether, chloroform, methanol and water successively and it % yield, colour, odour and consistency are shown in Table 1

Qualitative chemical examinations of various extracts revealed the presence of carbohydrates, flavonoids, amino acids, steroids, triterpenoids, glycosides, and tannins. Methanol and Acetone extracts showed presence of carbohydrates, flavonoids, amino acids, steroids, triterpenoids, glycosides, and tannins and phenolics. Petroleum ether showed presence of steroids and terpenoids, while successive chloroform extract showed presence of steroidal compounds. (Table 2)

Estimation of Protocatechuic acid by HPTLC Methanol and Acetone extract of A. subulatum Roxb.

HPTLC Finger Printing of both extract

Figure 3 shows that in Acetone extract 8 peaks were observed its Rf and area is shown in table 3, out of which Peak no 2 at Rf 0.16 was assigned as Protocatechuic acid by matching Rf with standard Protocatechuic acid which is shown in image of HPTLC plate. (Figure 2)

Table 1. Physical characters of various extracts of *A. subulatum* Roxb. Fruit constituents

Extract	% Dry wt in gms.	Colour	Odour	Consistency
Methanol	15.06	Dark Brownish black	Characteristic	Semisolid
Acetone	14.56	Dark Brownish black	Characteristic	Semisolid
Successive extraction				
Petroleum Ether (40–60°C)	08.11	Brownish	Characteristic	Waxy
Chloroform	03.05	Brownish	Characteristic	Semisolid
Methanol	14.16	Dark Brownish black	Characteristic	Semisolid
Aqueous	02.09	Brown	Characteristic	Semisolid

Table 2. Phytochemical screening of various extracts of *A. subulatum* Roxb fruit constituents

Nature	Methanol	Acetone	SPE	SCH	SMET	SAQ
Alkaloids	–	–	–	–	–	–
Carbohydrates	+	+	–	–	+	+
Flavonoids	+	+	–	–	+	+
Amino acids	+	+	–	–	+	+
Steroids	+	+	+	+	+	–
Triterpenoids	+	+	+	–	+	–
Saponins	–	–	–	–	–	–
Glycosides	+	+	–	–	+	+
Tanins & Phenolics	+	+	–	–	+	+

]S.P.E=Petroleum Ether, SCH. = Chloroform, SMET=Methanol, SAQ= Aqueous

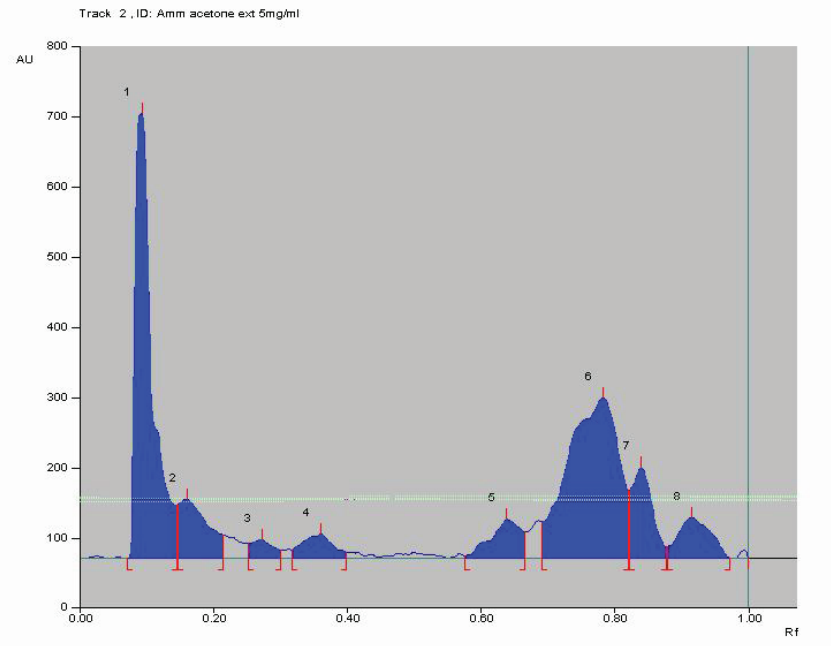

Figure 3. HPTLC finger print chromatogram of 10µl Acetone extract (5mg/ml) solution of *Amomum subulatum* Roxb.

Table 3. Rf and area of peaks observed in HPTLC chromatogram of 10µl Acetone extract (5mg/ml) solution of *Amomum subulatum* Roxb.

Peak No.	Rf	Area
1	0.09	12149.8
2	0.16	2734.5
3	0.27	657.5
4	0.35	1190.8
5	0.64	1891.7
6	0.78	13094.5
7	0.84	2981.1
8	0.91	2119.3

Figure 4 shows that in Methanol extract 11 peaks were observed its Rf and area is shown in table 4. Here, Peak no 2 at Rf 0.16 was assigned as Protocatechuic acid by matching Rf with standard Protocatechuic acid which is shown in image of HPTLC plate. (Figure 2)

Estimation of Protocatechuic acid in Acetone and Methanol extracts

Standard Protocatechuic acid showed single peak in HPTLC Chromatogram and single spots were observed on HPTLC plate as shown in figure 1, 5 and 6. Concentration of Protocatechuic acid in acetone extract and methanol extract were found to be 1.048 and 0.863 %w/w

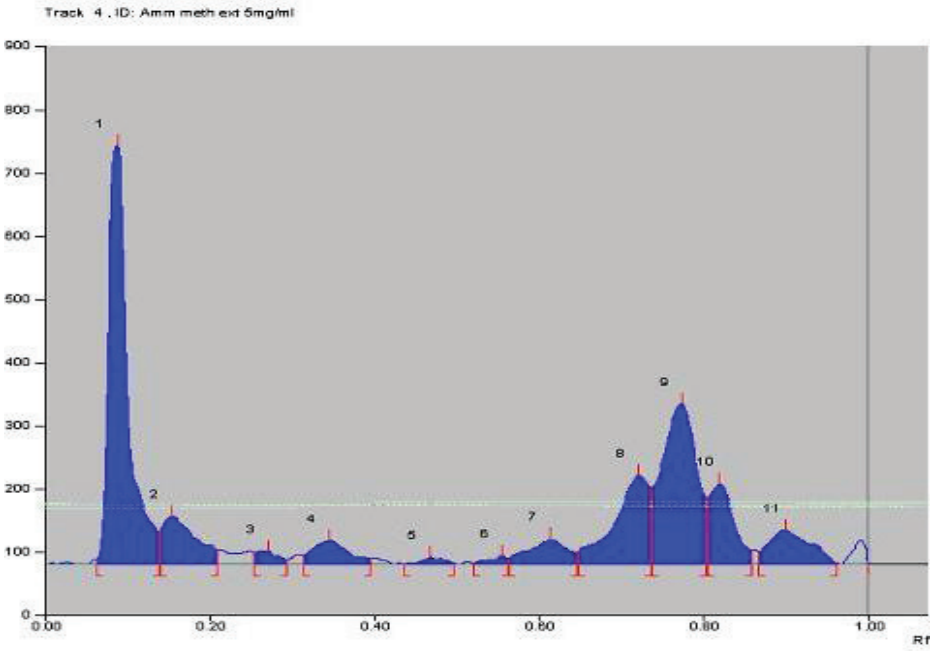

Figure 4. HPTLC finger print of chromatogram 10µl Methanolic extract (5mg/ml) Solution of *Amomum subulatum* Roxb.

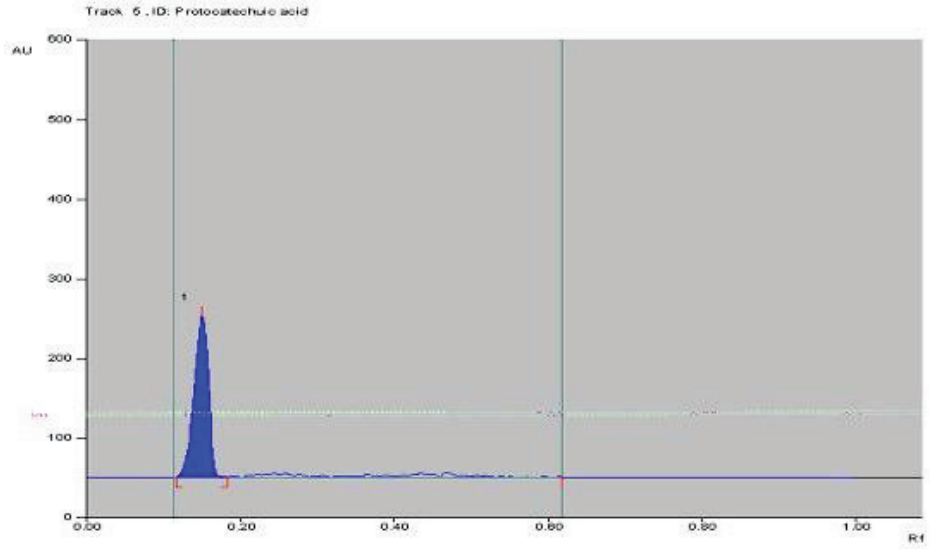

Figure 5. Chromatogram of standard Protocatechuic acid (Rf 0.16); Mobile phase: Chloroform: Acetic acid (9:1)

respectively calculated by regression equation y=4.6142x + 315.61, obtained from calibration curve of standard Protocatechuic acid. (Figure 7)

Validation of HPTLC method

Linearity

As shown in table 5, the correlation coefficient of calibration curve of Protocatechuic acid was found to be 0.9994, thus exhibits good linearity between concentration and area.

Accuracy (% Recovery)

The percentage recovery of Protocatechuic acid in methanol and acetone extract was found to be 99.83% and 98.84%, respectively (Table 6 and 7). Thus accuracy of the method has shown satisfactory results.

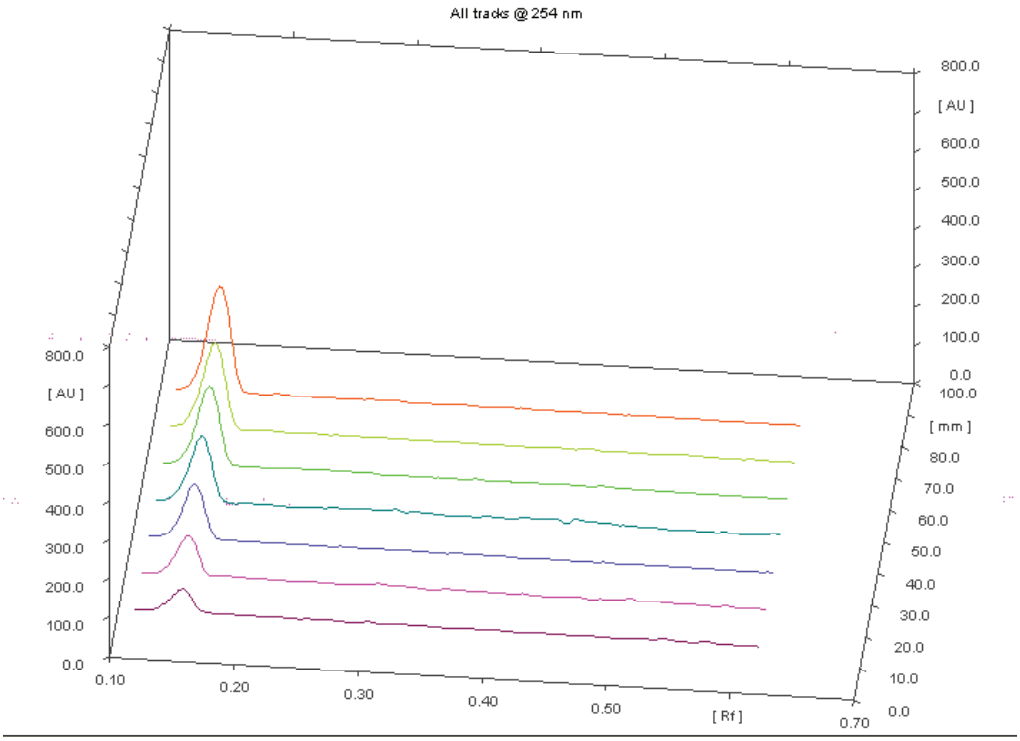

Figure 6. Three dimensional image of calibration spots of Protocatechuic acid (all tracks at 254nm)

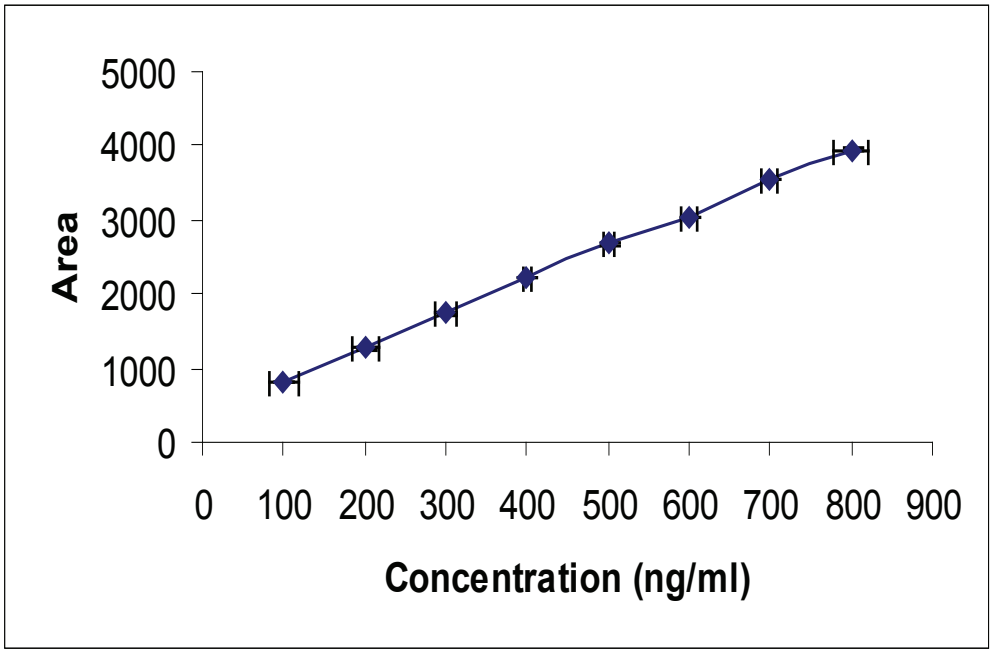

Figure 7. Calibration curve of Protocatechuic acid Standard. (r^2 0.9994), Data expressed as mean ± SEM, n=5

Table 5. Validation parameters for estimation of Protocatechuic acid by HPTLC Method

Parameters	Results
Linearity range	100 - 800 ng/spot
Precision (%CV)	
Repeatability of measurements	0.24 - 0.36 %
Repeatability of application	0.29 - 0.68 %
Interday	1.95 - 2.06 %
Intraday	1.12 - 1.56 %
Correlation coefficient (r2)	0.9994
limit of Detection	33 ng/spot
Limit of Quantification	100 ng/spot
Accuracy (% recovery)	
Methanol extract	97.57–102.01%
Acetone extract	96.65 - 100.79%
Specificity	Specific

Specificity

As Peak of standard Protocatechuic acid as well as Protocatechuic acid peak in sample was matching, therefore the method was found to be specific.

Limit of Detection and Limit of Quantitation

The minimum detectable limit and quantitation limit of Protocatechuic acid was found to be 33 ng/spot and 100 ng/spot, respectively.

DISCUSSION

Methanol and acetone extracts showed presence of carbohydrates, flavonoids, amino acids, steroids, triterpenoids, glycosides, tannins and phenolics which confirms the previous findings (3). HPTLC finger printing of acetone and methanol extract separated 8 components and 11 components, respectively. It confirms presence of protocatechuic acid in both extract (11). Here, acetone extract showed higher concentration of protocatechuic acid as compared to methanol extract. In the past studies only Acetone was used for the extraction of protocatechuic acid (7), but present study revealed that methanol can also extract protocatechuic acid.

Validation of HPTLC method of estimation of protocatechuic acid exhibits good linearity between concentration and area. Precision, accuracy and specificity of the method has shown satisfactory results.

Table 6. Results of recovery study of the method for Protocatechuic acid in Methanolic extract of *Amomum subulatum* Roxb. Fruit constituents

Amount of sample taken	Amount of Protocatechuic acid found (mg)	Amount of Protocatechuic acid added (mg)	Amount of Protocatechuic acid taken (mg)	Amount of Protocatechuic acid found (Mean±SD, n=5) (mg)	% Recovery
25 mg	0.226	2.000	2.226	2.172 ± 0.1181	97.57
50 mg	0.432	2.000	2.432	2.481 ± 0.2477	102.01
75 mg	0.657	2.000	2.657	2.655 ± 0.4182	99.92

Average recovery : 99.83%

Table 7. Results of recovery study of the method for Protocatechuic acid in Acetone extract of *Amomum subulatum* Roxb. Fruit constituents

Amount of sample taken	Amount of Protocatechuic acid found (mg)	Amount of Protocatechuic acid added (mg)	Amount of Protocatechuic acid taken (mg)	Amount of Protocatechuic acid found (Mean±SD, n=5) (mg)	% Recovery
25 mg	0.267	2.000	2.267	2.191 ± 0.8170	96.65
50 mg	0.524	2.000	2.524	2.501 ± 0.4345	99.09
75 mg	0.790	2.000	2.790	2.812 ± 0.5324	100.79

Average recovery : 98.84%

CONCLUSION

The HPTLC method was found to be rapid, simple and accurate for quantitative estimation of Protocatechuic acid in different extracts. The Protocatechuic acid is main bio-marker compound of *Amomum subulatum* Roxb. fruit constituents. Hence the assay results of this compound can be useful for evaluation of marketed product.

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A Rapid High-Performance Liquid Chromatographic Method for Quantitative Analysis of Anti-cancerous Active Components in *Gloriosa superba* Tubers

Rajagopal Chitra*, Kandhasamy Rajamani

Medicinal Plants Unit, Horticulture College and Research Institute, Tamil Nadu Agricultural University ,Coimbatore - 641003, Tamil Nadu.

ABSTRACT

Extracts of *Gloriosa superba* tubers with promising anti-cancerous capacity, used in traditional medicine, have been analyzed by high-performance liquid chromatography (HPLC) coupled with diode-array detection (DAD). Two kinds of amino alkaloids were successfully separated and quantified in tubers from eighteen accessions. The colchicine and colchicoside content of tubers from different accessions varied greatly. The colchicine content was found to be at a higher level (0.70 per cent) in the accession 'Viralimalai cultivated', whereas the accession 'Sathyamangalam wild' recorded the highest colchicoside (6.62 per cent) content.

Keywords: HPLC-UV, *Gloriosa superba* tubers, colchicine and colchicoside, anti-cancerous compounds, Gloriosa accessions.

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Corresponding author: Medicinal Plants Unit, Horticulture College and Research Institute, TNAU, Coimbatore
E-Mail id: chitra.varadharaj@gmail.com; Phone no: 09942766922

INTRODUCTION

Medicinal plants are now recognized, throughout the world, as an important component of natural resources of the respective countries. Seeds and tubers contain valuable alkaloids *viz*., colchicine and colchicoside as the major constituents, which are used to treat gout and rheumatism. Due to the action of colchicoside on spindle fibre formation during cell division, the plant has been identified as a potential anti-cancerous drug. In the Indian Systems of Medicine, the tubers are used as tonic, antiperiodic, antihelmenthic and also against snake bite (1). It is traditionally used for the treatment of bruises, colic, chronic ulcers, haemorrhoids and cancer, and is also employed as a tonic and purgative (2). Duke (1985) has also reported the abortifacient action of the plant rhizome (3).

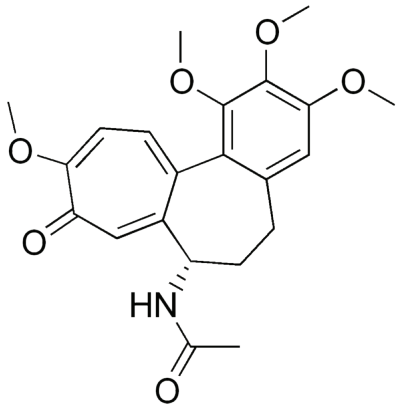
Colchicine, an important alkaloid used in the treatment of gout and in plant breeding work for including polyploidy, is at present, extracted from the corms of *Colchicum autumnale*, occurring wild in some parts of Europe. Recently, there had been an increase in the demand of this alkaloid but the supplies from the conventional sources had not been sufficient to cope with it. A worldwide search for an alternative plant source is being carried out but no suitable raw material has yet been found. Among the Indian plants the corms of *Colchicum luteum* and the seeds of *Iphigenia stellata* containing 0.25% and 0.9% of colchicine respectively are not available in sufficient quantities to warrant any commercial utilization (4). *G. superba* is another plant containing colchicine.

Several analytical methods for the determination of colchicine in pharmaceutical preparations, in biological fluids and in plant extracts have been described (5, 6, 7 & 8). The aim of the present study is to quantify the colchicine and colchicoside contents and to identify the accessions of *Gloriosa* with high alkaloids content.

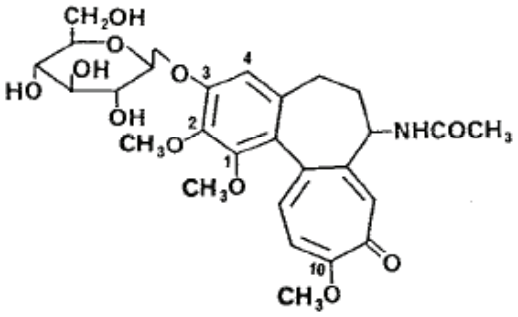
MATERIALS AND METHODS

Plant material and preparation of sample

Tubers of eighteen accessions of *Gloriosa*, collected from different places of Tamil Nadu (15 accessions) and Andhra Pradesh (3 accessions) were planted at the Medicinal Plants Unit, Botanical Garden, Tamil Nadu Agricultural University, Coimbatore. After harvest, the tubers of 18



Colchicine



Colchicoside

Figure 1. *Gloriosa superba* Chemical constituents

accessions were dug out, cleaned and used as experimental materials.

The tubers were sliced into small pieces for freeze drying at –20°C. After 7 days, the freeze dried plant material was ground to fine powder and then used for extraction of alkaloids. 0.5 g of powdered plant material was extracted twice with 25 ml of petroleum ether with frequent shaking for 1 hour, followed each time by filtration. The solid residues were air dried and then extracted with 10 ml of dichloromethane at room temperature for 30 min with frequent shaking. Then 10% solution of ammonia (0.5 ml) was added to the mixture with vigorous shaking for 10 min; the mixture was left undisturbed for 30 min and then filtered. The residue was washed twice with 10 ml of dichloromethane and then combined with the filtrate. The organic phase was evaporated to dryness and then dissolved in 1 ml of HPLC grade methanol to yield the test sample (9).

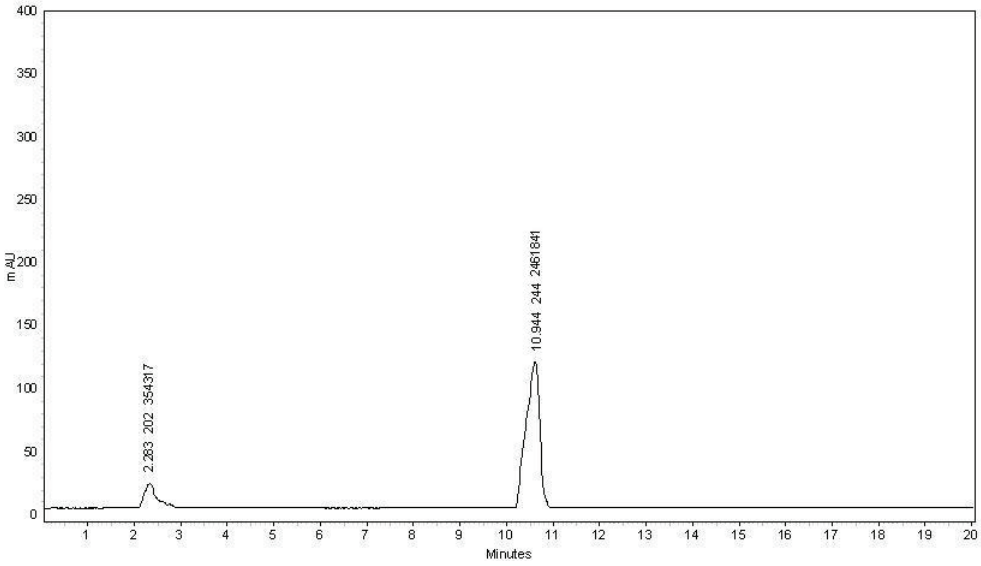
HPLC-UV Analysis

Identification of colchicine and colchicoside were done by comparing the retention time of the sample with that of the standard obtained from Sigma, USA and Alchem International Ltd, New Delhi respectively. A stock solution containing 100 mg of colchicine and colchicoside standard in 10 ml HPLC-grade methanol was prepared separately for calibration and for the control standards. Quantitative determination of the alkaloids viz., colchicine and colchicoside was carried out by RP-HPLC using a C18 column and a C18 pre-column packed with Kromasil. The mobile phase consisted of water and acetonitrile (70:30). A Hewlett-Packard series 1050 liquid chromatograph equipped with a quaternary pump system, a diode array detector operating at 350 nm, and data processing were used for the analyses. The colchicine and colchicoside contents were calculated using the following formulae in terms of ppm after which it was converted into per cent from: Sample quantify (ppm) = (A_s / A_{std}) X (M / M_1) X (V / V_1) X F, where A_s - Sample area, A_{std} - Standard area, M - µl of standard injected, M_1 - Weight of the sample taken, V - Volume of final extract, V_1 - µl of sample injected and F - Recovery factor.

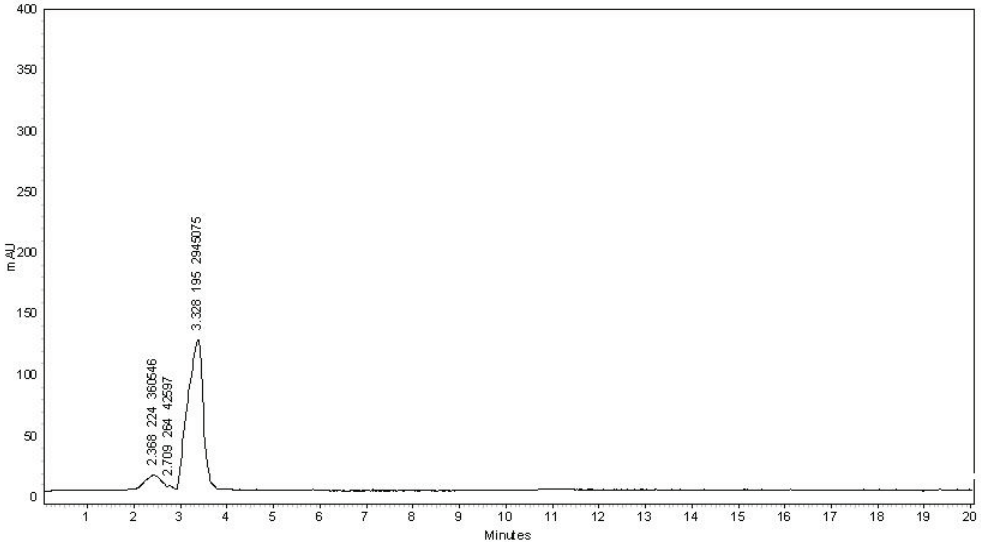
RESULTS AND DISCUSSION

A simple reversed-phase HPLC-UV (PAD) method with gradient elution was developed in order to determine colchicines and colchicoside in tubers. A typical HPLC chromatogram of the tuber extract from *Gloriosa superba* is shown in Fig. 2A: colchicine eluted at ca. 11.7 min. and Fig. 2B: colchicoside eluted at ca. 3.5 min. By measuring peak areas at 245 nm for colchicines and 210 nm for colchicoside, a calibration curve was constructed which was linear ($r^2 > 0.99$) in the range 1.0–250 ppm. Two control standards containing 70 and 200 ppm of colchicine and colchicoside were used to ensure accuracy and precision; their RSD (%) values were within 2% of the actual concentrations. The limit of detection of the method was determined to be 50 ng/mL. The method described has the advantage of using a simple gradient elution in the reversed-phase mode without adding buffers. Addition of acetic acid to the mobile phase renders the method compatible with HPLC-MS requirements.

Results show that all the accessions studied exhibited significant variation in colchicine and colchicoside contents. The concentration levels of colchicine and colchicoside determined in 18 accessions of *Gloriosa*



A. Colchicine



B. Colchicoside

Figure 2. Typical Chromatogram of colchicine and colchicoside standard

are represented in Table 1, and the chromatogram of standards and anti-cancerous compounds are depicted in Figures 2, 3 and 4 respectively. The colchicine content was found to be at a higher level (0.70 per cent) in the accession 'Viralimalai cultivated', whereas the accession 'Sathyamangalam wild' recorded the highest colchicoside (6.62 per cent) content. Four accessions recorded significantly superior values than the grand mean of 0.12 per cent of colchicine and seven accessions showed higher values than the grand mean of 1.29 per cent of colchicoside. Previous scientific reports cite colchicine concentration ranging from 0.37 per cent to 0.61 per cent (10) and colchicoside concentration ranging from 0.10 per cent to 0.82 per cent (11) in *G. superba*. Thus corms and seeds of *C. autumnale* can contain up to 1.2% colchicine (12), whilst seeds and bulbs of the plant growing in Albania contained 0.33 and 0.02% of colchicine, respectively (13). In a cultivation study using different NPK fertilizer

Table 1: Colchicine and colchicoside content in tubers of different accessions of *Gloriosa superba*

Accessions	Colchicine (%)	Colchicoside (%)
Nallampalayam cultivated	0.03	0.27
Kallimanthayam cultivated	0.01	0.07
Sathyamangalam wild	0.01	6.22
Aruppukotai wild	0.01	0.49
Aruppukotai cultivated	0.01	2.35
Kankayam cultivated	0.08	1.99
Kallimanthayam wild	0.01	0.35
Ottanchadram cultivated	0.24	1.40
Moolanur cultivated	0.06	2.68
Jeyankondam cultivated	0.13	0.33
Udangudi cultivated	0.20	1.88
Viralimalai cultivated	0.70	0.15
Pudukottai cultivated	0.06	0.36
Andhra cultivated – I	0.55	1.04
Andhra wild	0.02	0.95
Z-Melur cultivated	0.01	1.68
Poondurai wild	0.01	0.52
Andhra cultivated -II	0.02	0.39
Mean	0.12	1.29
SE(d)	0.004	0.018
CD (0.05%)	0.008	0.037

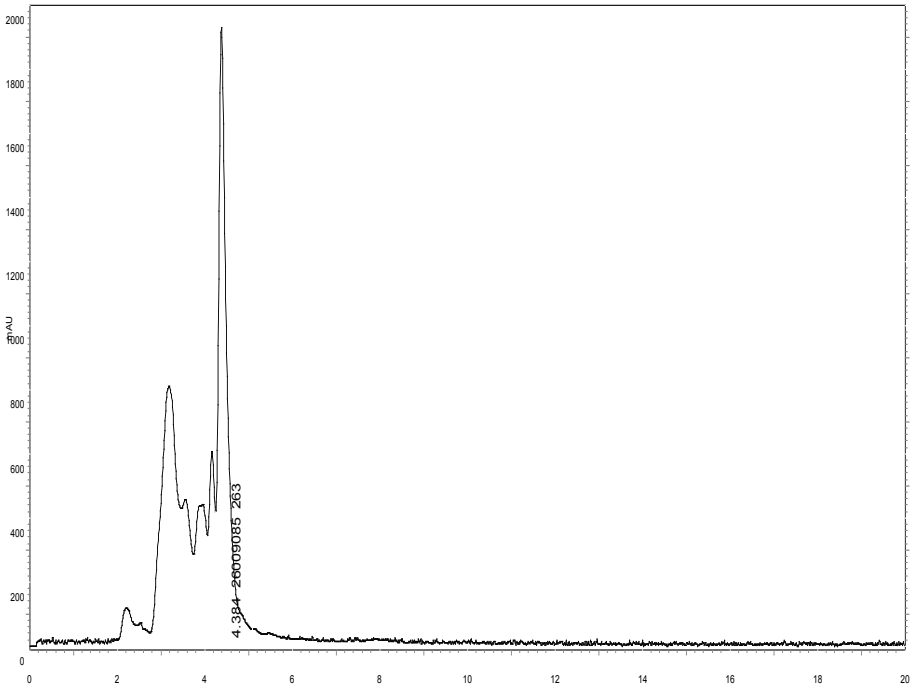


Figure 3. Typical HPLC chromatogram of colchicine of the tuber extract from *Gloriosa superba* accession Viralimalai cultivated measured at 239 nm. (For extraction and analytical protocols see Experimental section.)

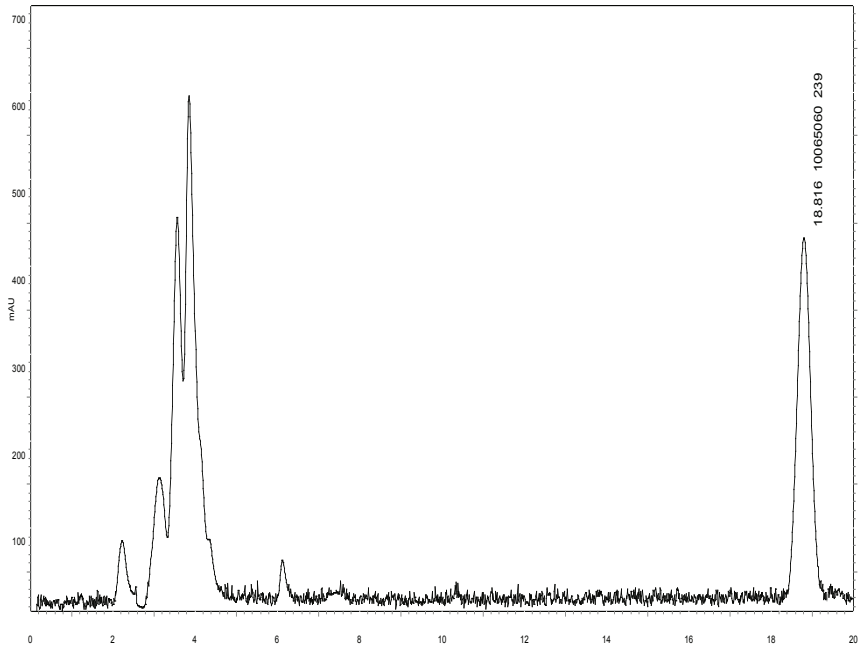


Figure 4. Typical HPLC chromatogram of colchicoside of the tuber extract from *Gloriosa superba* accession Sathyamangalam wild measured at 203 nm. (For extraction and analytical protocols see Experimental section.)

levels, Al-Fayyad *et al*. (2002) reported the presence of colchicine in corms (0.052%), flowers (0.025%), and leaves (0.013%) of *Colchicum hierosolymitanum* using a different analytical method (8). The colchicine and colchicoside being secondary metabolites are often influenced by the environmental and seasonal factors. The relatively high colchicine and colchicoside content of the two accessions examined in this study encourages the cultivation of these accessions in Tamil Nadu particularly in consideration of their adaptability to grow under drought condition. From the results, it is evident that there is wide variation in the biochemical compounds of *Gloriosa* accessions, which can be further exploited to popularize the useful accessions for extraction of drugs.

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Preparation and Evaluation of Cordia Fruit Gum as Tablet Binder

Patil D.N.[1*], Kulkarni A.R.[2], Hatapakki B.C.[1] and Patil B.S.[3]

[1]Department of Pharmacognosy & Phytochemistry, K.L.E.S's, College of Pharmacy, Gadag - 582101., Karnataka (India) Ph.No. (08372) 230042, 221667, Fax No. (08372) 221443.
E-mail: dnpatilgadag@rediffmail.com
[2]Department of Pharmacology, K.L.E.S's, College of Pharmacy, Belgaum-590041. Karnataka (India)
[3]Department of Pharmacognosy, SET's, College of Pharmacy, Dharwad. Karnataka (India)

Abstract

In the present work, we have formulated the oral tablets of paracetamol by using cordial fruit gum as a binder. The four different tablet formulations were prepared by wet granulation method. The binder concentrations used in the formulation were 2, 4, 6 & 8 % w/w. The evaluation of granules showed 0.643 to 0.746 mm granule size, 26.65 to 32.10 ° angles of repose and 21.8 to 13.4 % fines. Tablets were compressed to hardness at about 7.5 to 8.2 kg/cm^2. The evaluation of tablet showed 1.58 to 1.10 % friability, 12 to 22 min disintegration time and more than 90 % dissolution in 75 min. Tablets at 6 % w/w binder concentration showed more optimum results as tablet binder. The cordial fruit gum was found to be useful for the preparation of uncoated tablet dosage form.

Keywords: Cordia fruit gum, Binder, Paracetamol and Dissolution
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INTRODUCTION

The cordia fruit gums obtained from trees of *Cordia dichotoma* (Boraginaceae), it is a medium sized tree with a short, usually crooked trunk 3–4 ft. in girth (1). The fruits are globose, yellowish-brown, pink or black and pulpy. The plant grows in India, Sri Lanka and other warmer regions. The medicinal attributes of *C. dichotoma* have been known since a long time. The fruits of the plant are used as cooling, astringent, emollient, expectorant, anthelmintic, purgative and diuretic (2). A number of pharmacological properties such as analgesic, anti-inflammatory and hepatoprotective have been reported (3–5). The ripe fruits are traditionally eaten by Indian as pickle. The fruit mucilage is used as a gum for pasting sheets of paper board and as a emulsifier in the pharmaceutical excipients. The various gums were evaluated as tablet binder (6). In this present investigation cordia fruit gum has been evaluated as a binder in pharmaceutical dosage form.

MATERIALS AND METHODS

Paracetamol IP and microcrystalline cellulose were obtained as a gift sample from Kem Well House, Bangalore. All other materials used in this study were purchased from s.d fine chemicals Mumbai. Fresh white gum of *Cordia dichotoma* was collected from authenticated plant fruits in local area of Gadag district (India).

Purification of gum

The well dried cordia fruit gum was powdered in mortar and passed through sieve no. 80. Gum was solublised in distilled water. The concentrated solution was precipitated by acetone. The precipitate was separated and dried at 60°C. The dried gum was powdered and stored in tightly closed container.

Standardization of gum

The cordia fruit gum was standardized for following properties. Loss on drying: 5 gm of gum was dried at 100 ± 5 °C till the constant weight is obtained. The loss on drying was found to be less than 10 % w/w. Ash value: 1gm of gum was accurately weighed and evenly distributed it in the crucible. It was dried at 105 °C for one hour and ignited in muffle furnace at 600 ± 25 °C. Percentage ash content was found to be less than 8.5% w/w. 2 to 8 % w/w gum solutions have pH 6.8 to 6.2.

Preparation and evaluation of granules

Wet granulation method was used to prepare granules of drug. The formulation was developed by using Paracetamol IP as model drug. Binder solution of gum was prepared by dissolving it in distilled water. The binder concentrations used were 2, 4, 6, 8 % w/w in solution. Binder level was adjusted by lowering the level of MCC in the formula. All ingredients were dry mixed manually in mortar. Binder solution was slowly added into mixture. The wet mass was granulated by passing them manually through a number 12 mesh sieve. Granules were dried at 50 °C in oven and again received through number 16 mesh sieve. The granules were evaluated for percentage of fines and particle size. Granules were mixed with 6 % talc and evaluated for flow property (7–8). The tablet formulation was developed for 600 mg tablet weight as shown in Table 1.

Preparation and evaluation of tablets

The tablets were compressed by using Cadmach single punch tablet machine fitted with flat faced punches. The batch size prepared was of 100 tablets. The prepared tablets were stored in closed container for 30 days. No evidence of chemical change was observed. The tablets were evaluated for content uniformity, hardness, friability, disintegration time and dissolution study (9–12). Dissolution study was carried out in 900ml 0.1 N HCL medium using paddle type Dissolution Test Apparatus. The dissolution was carried out at 37 ± 10 ° C and 50 rpm paddle speed. The 10 ml samples were withdrawn at 10 min intervals. 10 ml dissolution medium was added into dissolution chamber as a replacement for sampling after each interval. Absorbance was measured at 243 nm using UV spectrometer (Simadzu).

RESULTS AND DISCUSSION

The binder gum obtained from natural origin and has pH between 6.8–6.2. The prepared granules were evaluated for percentage of fines, particle size and flow properties. The results are shown in Table 2. It was observed that the percentage of fines was reduced as the concentration of binder was increased. The flow property of granules was determined by angle of repose and it was found that values were between 26–32°. The increased percentage of fines reduces particle interlocking and friction, thus decreasing angle of repose. All batches showed good flow property. Granule size distributed between 0.64–0.74 mm. Three batches of tablets of each binder concentration were prepared. The prepared tablets were evaluated for content uniformity, hardness, friability and Disintegration time. The results are indicated in Table 3. All batches of tablets exhibited a good uniformity in content. The hardness of tablet increased with increase in percentage of binding agent. The friability values decreased with increase in binder concentration. The disintegration time also increased with increase in binder concentration. All the evaluation parameters were found to be within the pharmacopoeial limits at binder concentrations 6–8 % w/w. Increase in binder concentration therefore resulted in a corresponding decrease in friability and increase in disintegration time. Dissolution study showed that the drug release from the tablets containing 2–8 % w/w binder was more than 90 % in 75 min. Tablets at 6% w/w concentration shows more optimum results as tablet binder. The drug release from tablets decreased with increase in binder concentration.

Table 2: Evaluation of granules prepared from cordia fruit gum as binder

	Binder concentration (%w/w)			
Characteristic	***2***	***4***	***6***	***8***
Percentage of fines	21.8	19.5	15.8	13.4
Particle size (mm)	0.643	0.688	0.742	0.746
Angle of repose (°)	26.65	27.54	30.68	32.10

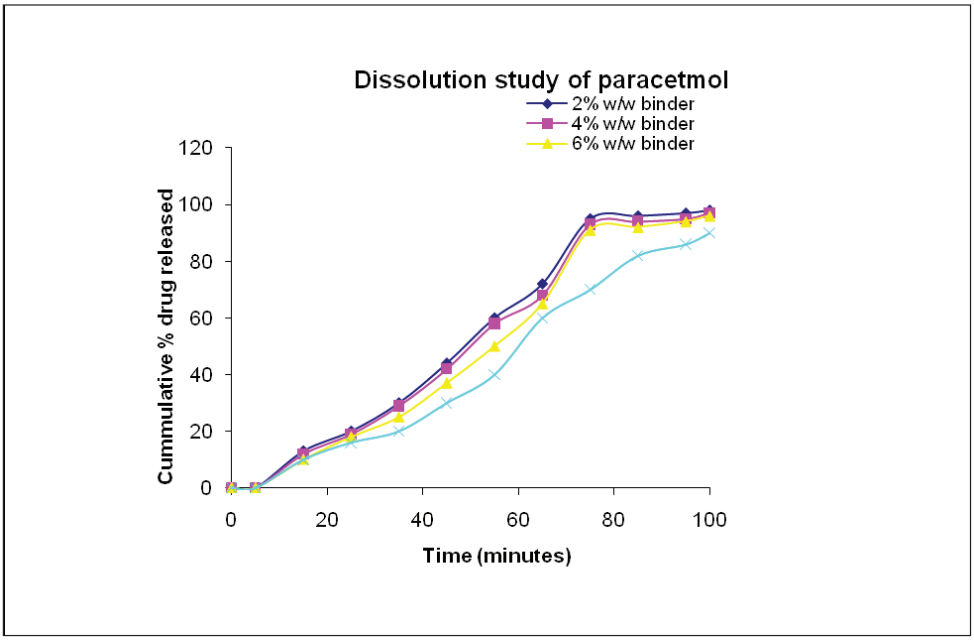
Table 1: Composition of Tablets Containing Cordia fruit Gum as Binder

	Variation of Binder Concentration by lowering the level of MCC			
Ingredients	**Batch I**	**Batch II**	**Batch III**	**Batch IV**
Paracetamol	400 mg	400 mg	400 mg	400 mg
Microcrystalline Cellulose(*MCC*)	164 mg	152 mg	140 mg	128 mg
Binder in Dist. water(*Cordia fruit gum*)	2 % w/v	4 % w/v	*6 % w/v	8 % w/v
Talc	24 mg	24 mg	24 mg	24 mg

*Indicates good concentration of binding agent.
In the formula weight of one tablet (600 mg) is mentioned, but each batch was calculated and taken for 100 tablets.

Table 3: Evaluation of tablets prepared with cordia fruit binder

	Binder concentration (%w/w)			
Characteristic	***2***	***4***	***6***	***8***
Content Uniformity (%)	95.20	98.56	98.98	98.90
Hardness kg/cm^2	7.5	7.8	8.0	8.2
Friability (%)	1.58	1.47	1.25	1.10
Disintegration time	12 min	13 min20 sec	16 min 45 sec	22 min



CONCLUSION

The cordia fruit gum exhibited good binding properties for uncoated tablets. The increased concentration of gum showed small retardation in drug release from tablet.

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A survey on knowledge and attitude of pharmacy, health science and medical students towards traditional medicine as well as willingness of students and doctors towards the integration of traditional and modern medicine in Gondar university.

Muthuswamy Ragunathan[1]*, Solomon MeQuanente Abay[2]

[1]Department of Pharmacognosy, Cherraan's College of Pharmacy, 521-Siruvani Main Road, Coimbatore-39, Tamilnadu state, India

[2]Department of Pharmacology, Addis Ababa University, Medical Faculty, PO Box 9086, Addis Ababa, Ethiopia

ABSTRACT

A cross-sectional study was carried out to assess the knowledge, attitude, and willingness of modern health professionals (students and doctors) to the integration of traditional and modern medicines. Stratified random sampling technique for students as well as quota sampling for doctors wer used. In addition, a self-administered questionnaire was employed in the study. The findings of the study showed that most students (respondents) have an encouraging general knowledge on traditional medicine. However, 54.9% respondents said that they do not have in-depth awareness about it. Most students and doctors believe that health service coverage in the nation can be increased through bringing together of traditional and modern practitioners and they themselves are willing to actively participate in such co-operations. About 69% of students and 80% of doctors in the study are willing to integrate the traditional and modern medicines in their profession. The impact of traditional medicine course is found to be very significant in bringing attitudinal and willingness developments on the students. Hence, 81.93% respondents (students) are willing to take the course. On the other hand, most of the students and doctors said that, they do not know the National Policy of Traditional Medicine. Thus, despite the above promising findings, the endeavors of the government and the university are found to be unsatisfying. This shows the exclusive effort should be taken by the government among the health professionals towards integration of traditional medicine system to modern medicine.

Keywords: Traditional medicine; Knowledge; Integration; Attitude; Complementary and Alternative medicine; Ethiopia

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***Author of Correspondence:** Prof. Ragunathan Muthuswamy; mobile; 00919865656279 E-mail: ragunathranilmonica@gmail.com; ragunathan@fastmail.fm

INTRODUCTION

The 1978 Alma-ata conference pointed the essence of incorporating traditional medicine in the general health care system in order to make health care service available to all the population. Besides the OAU (Organization of African Union), heads of state and government declared the period 2000–2010 G.C. as the African decade on African Traditional Medicine. Thus, every country in the African region is expected to adopt a method of traditional medicine incorporation that would be suitable: integrative, inclusive, or tolerant as the case may be (1). According to WHO, the promotion of traditional medicine in health care services particularly at PHC (primary health care) level should be intensified using a selection of essential remedies particularly herbs in PHC and policy support for integration of traditional and modern medicines (2).

Many countries such as China, Vietnam, and Republic of Korea have an integrated system of traditional and modern medicine (3). In China, traditional Chinese medicine, class is opened to Western Medical students, and mass media is extensively used to promote the integration of the two medical systems throughout the nation (4).

In South Africa, an integrative training pilot study on traditional medical system has come up with a favorable clinical efficacy, cost-effectiveness, and provision of improved quality of life (5). Health professionals

particularly pharmacy, nursing and medical students around South Africa were recommended to take CAM (complementary and alternative medicine) course. Because health professionals have a responsibility to understand CAM given that, more than half of the population already uses herbal remedies (6). However, in Ethiopia, despite the support and encouragement to traditional medicine in policies, the government is criticized to have a limited effort to practically implement such policies (7).

Any government health program is believed to be ineffective with out a good awareness, positive attitude, and higher commitment of all the participants in the implementation of such programs. Therefore, this study is conducted to assess knowledge, attitude and willingness of modern health professionals (Students and Doctors) to the integration of the two medical systems namely; traditional and modern medicines

Study area

This study has been made among the students of College of Medicine and Health Sciences (CMHS) Campus in University of Gondar and on the population of Medical Doctors in the University Hospital. Both areas are located side by side in Gondar town, 748 km North West of Addis Ababa, Ethiopia. The college has three schools namely: school of health science, school of medicine and school of pharmacy. The college provides diploma, degree, masters, and contains 2200 regular degree students. In addition, the University Hospital has about 36 active medical doctors. It has been offering in-patient and out patient health care services to the local and surrounding communities.

MATERIALS AND METHODS

Study Design

A cross-sectional study was carried out using self-administered questionnaire. The questionnaire addressed issues related to demographic characteristics, knowledge on and attitude towards traditional medicine (only to students) as well as willingness to integrate the two medical systems . In addition, questions to assess endeavors of government, university as well as students & doctors themselves were involved and related factors affecting the awareness of students on traditional medicine were also included in the questionnaire.

The main objective of the study is assessment of knowledge and attitude of students as well as willingness of students and doctors to the integration of traditional and modern medicines. A stratified random sampling technique was used to maintain samples from a population of regular health science, medical and pharmacy students. Besides, doctors were selected using a quota sampling technique.

Data collectors introduce participants in the study before the actual distribution of the self-administered questionnaire. Data was collected on 327 students and 30 medical doctors who had given their full consent and were willing to be involved in the study. The collected data was cleared and analyzed using SPSS version 15. A sample size of 327 students was used and it will allow for a 50% prevalence estimate, with 95% confidence interval ranging from 43%–57% of the target population 2200. Discussion and conclusion are made based on the findings.

Source of Population

The source population was all the 2200 population of CMHS students distributed in to 11 departments and the 36 medical doctors in the university hospital. This include health science departments from year I to year III, medical students from year I to year VI and pharmacy students from year I to year IV.

Study Population

Students as well as doctors selected from the source population using the appropriate sampling procedures.

Exclusion Criteria

The study did not involve extension students no matter how they are in the same department with the same or different batch. In addition, those who are attending their masters program were not included. Most importantly, the study also excluded non-medical doctors as well as respondents who are not willing to participate in the study.

Sampling Technique

The population of students is stratified in to schools and departments so that a stratified random sampling technique was used to maintain the study population. In addition, quota-sampling technique was used for the population of doctors.

Variables of the Study

Independent variables

These are socio demographic like age, sex, marital status, religion, economic income.

Dependent variables

Knowledge, attitude, intention to take the course, perception to integrate traditional and modern medicines.

Data Collection Procedure

Students who have previous experience of data collection were recruited and lecture was given for 45 minutes about the research purpose, how to fill the questionnaire. Next pretest was conducted on eight students within the college of medical health sciences. Then difficult questions were modified, and the corrected questionnaires were distributed. Data was collected using self-administered English version questionnaire in the classroom before the class began and after the class ends. For doctors, the questionnaires were given during tea break by the principal investigators. Finally, data was reviewed, checked for completeness, consistency and relevance by the principal investigator of the study analyzed by using SPSS - 15 biostatical software package.

Ethical Consideration

Ethical clearance was taken from the department of pharmacognosy. The subjects of this research had already informed about the objective and purpose of the study and why they were selected to be study participants. Respondents also were informed that they could discontinue or refuse to participate in the study at any time they want. At last, emphasis was given to respondents that all information given through the survey time should be recorded and kept anonymously so that their confidentiality should be ensured.

RESULTS

The knowledge, attitude, and willingness of students to the integration of traditional and modern medicine

Socio demographic characteristics

From 327 participants in the study, males (75.8%) accounted the majority. About 71.6% of respondents were found in the age group between 18 to 22 years. Orthodox and Muslim respondents accounted to 64.8% and 20.8% respectively. Considering the proportion of students from the three schools, 63.9% were from school of health science, 22.6% were from school of medicine and the rest 13.5% were from the school of pharmacy (Table 1).

Knowledge of students about traditional medicine

About 89% respondents said that peoples in developing countries mostly use traditional medicine. Thus 49.2%, 42.4% and 43.1 % participants respectively said cultural influence, unavailability of modern medicine and poor economic status are factors for reliance of peoples on traditional medicine for their health care needs.

Asked on how traditional healing skill is acquired, 65.8% respondents said from family practice, 35.8%

Table 1: Socio demographic characteristics of respondents (students) who were participated in the study (University of Gondar – May 2008)

Variables		Frequency	% (total = 327)
Sex	Male	n = 248	75.8%
	Female	n = 79	24.2%
Age	<18 year	n = 42	12.8%
	18year–22 year	n = 234	71.6%
	22 year–24 year	n = 35	10.7%
	>24 year	n = 16	4.9%
Religion	Orthodox	n = 212	64.8%)
	Protestant	n = 68	20.8%)
	Muslim	n = 36	11%
	Catholic	n = 2	0.6%
	Others	n = 7	2.8%
School	Health science	n = 209	63.9%
	School of Medicine	n = 74	22.6%
	School of Pharmacy	n = 44	13.5%

Note: The total 327 is adjusted according to non-responses

Table 2: General knowledge about traditional medicine by respondents (students) who were participated in the study (University Of Gondar – May 2008)

Variable	Frequency	% (total = 327)
Peoples in developing countries mostly use TM.	n = 290	(89%)
Why people generally use TM		
– In availability to MM	n = 155	47.4%
– Cultural influence	n = 161	49.2%
– Poor economic status	n = 141	43.1%
– Lack of education	n = 133	40.7%
– Presence of incurable diseases in do MM.	n = 85	26%
How healing skill is acquired		
• family experience	n = 215	65.8%
• Training	n = 117	35.8%
• Holy spirit gift	n = 91	27.8%
• Any interested person can do it	n = 35	10.7%
Risk of TM		
• Some harmful practice	n = 260	79.5%
• unhygienic practice	n = 213	65.1%
• Too costly	n = 50	15.3%
TM has some advantage over MM	n = 240	73.6%
Tm can serve as source of knowledge of MM	n = 299	96.1%
Have not good (in-depth) awareness on TM	n = 195	54.1%

Note: The total 327 is adjusted according to non-responses
TM = Traditional Medicine
MM = Modern Medicine

through training (apprenticeship) and 27.8% said from Holy-spirit gift. Besides 10.7% respondents said any interested person can also practice regardless of acquisition of the healing skill.

Harmful practices are said to be present a long with the practice of traditional medicine by 79.5% participants. In addition, almost all (96.1%) said traditional medicine could serve as source of knowledge to the modern medicine, and 73.6% said it has some advantages over modern medicine. However, as high as 54.9% participants said, they do not have developed in-depth awareness on traditional medicine (Table 2).

Attitude and willingness of respondents (students) to integration of traditional and modern medicine

About 94.2% and 70.2% respondents respectively said that Ethiopian traditional medicine needs an improvement and government shall take a better control and support to the sector. Where as 60.2% participants said they have suspicion on healing skill of traditional healers so that they do not mostly accept their skills.

Conversely, 81.3% of respondents believe that health service coverage in Ethiopia can be increased by the coordination of traditional and modern practitioners and 92.3% are wanted to actively participated in such co-operations and would like to share their professional knowledge to the healers.

Asked about integration of traditional and modern systems of medicines, 69% of the total participants are willing to integrate the two medical systems in their profession. About 81.93% respondents showed, their interest to take traditional medicine course and the need to aware students about national traditional medicine policy is mentioned by 63.7% respondents (Table 3).

Factors affecting awareness of students (respondents) on traditional medicine

The students (participants) were asked about influencing factors of their awareness on traditional medicine and the findings are given as follows.

The absence of traditional medicine course and shortage of related literatures (Books, Journals etc.) were respectively mentioned by 63.3% and 51.7% respondents. In addition, 44.3% participants said poor student initiation is another cause for lower awareness

Table 3: Attitude towards traditional medicine and willingness to the integration of traditional and modern medicines by respondents (students) who were participated in the study (University of Gondar – May 2008)

variables	Frequency	% (n total = 327)
– TM in Ethiopia need Improvement	n = 308	94.25%
– Government shall take better control and support to TM sector	n = 229	70.2%
– Do not mostly accept skill of healer	n = 197	60.2%
– Health service coverage in Ethiopia can be increased by coordination of traditional and modern practitioners.	n = 260	81.3%
– willing to cooperate with traditional practitioners	n = 298	92.3%
– Willing to integrate traditional and modern medicines	n = 223	69%
– want to take traditional medicine course	n = 263	81.9%
– Government shall publicize the national policy on traditional medicine	n = 216	69.7%

Note: The total 327 is adjusted according to non-responses
TM = Traditional medicine

of students on traditional medicine. Where as absence of seminar about traditional medicine is mentioned by 43.5% respondents. On the other hand, 62.3% participants said they have come across about traditional medicine in a media but it is only about an advertisement (Table 4).

Comparison of respondents (students) who have taken traditional medicine course and those who have not taken

Twenty-one students of pharmacy 3th and 4th years have taken the course and all (100%) are willing to cooperate with traditional practitioners. Moreover, 90.2% of them believe that health service coverage in the nation can be increased through such cooperation. Willingness to integrate traditional and modern medicines is mentioned by 95.2 % of these respondents.

Conversely, 306 (83.6%) respondents have not taken the course and 90.5% of participants expressed their willingness to cooperate with traditional practitioners. Whereas 78.6% respondents believe that health service coverage of the nation can be increased by coordination of traditional and modern practitioners. Willingness to integrate traditional and modern medicines is expressed by only 66.3% of respondents who have not taken the course.

The Students, university, and government endeavors towards obtaining better awareness on traditional medicine by the students.

Students are appreciably doing well to gain better awareness on traditional medicine. They discussed with people and listened TV and radio about traditional medicine where as 32.7% participants said they read book journal etc regarding traditional medicine.

On the other hand, 270 (83.1%) respondents said that the university is not doing enough to promote their awareness on traditional medicine nor does the government as 93.6% respondents did not take a traditional medicines course which significantly concerns it.

Willingness of doctors (respondents) to the integration of traditional and modern medicines as well as related opinions

Socio demographic characteristics

A total of 30 doctors of which 76.7% are males were participated in this study. The majority are in the age groups 20–30years. Only 20% respondents have specialized where as the rest 80% are general practitioners (Table 5).

Table 4: Factors affecting awareness of students on traditional medicine data given by respondents who were participated in the study (University of Gondar – May 2008)

Variable	Frequency	% (n total = 327)
• Absence of Traditional medicine course	n = 207	63.35%
• lack of writing (Book, Journal etc)	n = 169	51.7%
• Poor student initiation to know	n = 145	44.3%
• Absence of seminar	n = 141	43.1%

Note: The total 327 is adjusted according to non-responses

Table 5: Socio demographic characteristics of respondents (doctors) who were participated in the study (University Of Gondar May 2008)

Variables		Frequency	% (n total = 30)
Sex	Male	n = 23	76.7%
	Female	n = 7	23.3%
Age	• 20 Yr – 29yr	n = 24	80%
	• 30 – 40Yr	n = 6	20%
	• > 40yr	n = 0	0%
Religion	• Orthodox	n = 24	80%
	• Protestant	n = 4	13.3%
	• Muslim	n = 2	6.7%
Educational level	• Specialist	n = 24	80%
	• General practitioner	n = 6	20%
Ward of practice	– pediatrics	n = 11	36.7%
	– Internal medicine	n = 8	26.7%
	– gynecology and austerities	n = 7	23.3%
	– Public health	n = 3	10%
	– Surgery	n = 1	3.3%

Willingness to integrate traditional and modern medicines by doctors (respondents) and other related opinions

About 80% of the respondents expressed their willingness to integrate traditional and modern medicines in their professional practice. However, 67% participants said that they do not know about the national policy of traditional medicine. Asked on their awareness on traditional medicines, 90% respondents said they did not develop good awareness at a student level and 70% said they have not good awareness once after they are at work. Only 6.7 % of respondents are found to some times read about traditional medicine in general.

86.7% respondents mention the fact that Ethiopia has lots of potential to be used in traditional medicine. For instance, a similar proportion of participants (86.7%) said minor training of Traditional Birth Attendants (TBA) could significantly decrease maternal mortality associated with delivery. Relatively, 80% of the participants believe that health service coverage of the nation can be increased through coordination of traditional and modern practitioners and 80% said they themselves are willing to co operate with traditional healers and would like to share their professional knowledge.

Half (50%) of the respondents said that traditional medicine is some times better than modern medicine in the management of certain illnesses and 6.7% of them have ever advised their clients to utilize traditional medicine. In addition, about 80% respondents said they take history of traditional medicine usage while diagnosing patients (Table 6).

Table 6: Willingness of respondents (doctors) for the integration of traditional and modern medicines as well as related opinions (University of Gondar – May 2008)

Variables	Frequency	% (Total = 30)
– Willing to integrate traditional and modern medicines	n = 24	80%
– know government policy on traditional medicines	n = 10	33%
– Developed good awareness on traditional medicine at a student level	n = 3	10%
– Got a better opportunity to know well about traditional medicine at work	n = 9	30%
– Usually read about traditional medicine	n = 2	6.7%
– Ethiopia has a lot potential to be used in traditional medicine secretor	n = 26	86.7%
– Training of traditional birth attendants can decrease maternal mortality due to delivery	n = 26	86.7%
– Willing to cooperate with traditional practitioners	n = 24	80%
– Health service coverage of the nation can be increased by cooperation of traditional and modern practitioners	n = 24	24%
– Traditional medicine is sometimes better than modern medicine to manage certain illnesses	n = 15	50%
– have ever advised patients to use traditional medicines	n = 2	6.7%
– Take history of traditional medicine usage from patients.	n = 24	80%

DISCUSSION

This study has shown that most respondents expressed for the possible increase of the nation's health service coverage through coordination of traditional and modern practitioners. This is congruent to what Tsegaye reported in his study (8). The respondents in his study said, coordination could increase health status of the nation at some level. This is clearly supported by the point that about 80,000 traditional healers, drug collectors and vendors are expected to be found throughout the Ethiopia (9). Beside most participants also said traditional medicine of Ethiopia, need a better government control and support. One of such a support could be establishing cooperation with modern health professional as most participants in this study (students and doctors) are willing to cooperate with traditional healers.

The 69% students and 80% doctors showed willingness to integration of traditional and modern medicines. Such finding will alert decision makers to give a more than usual concern to establish practical cooperation among the practitioners of the two medical systems. It is congruent to what Bishaw (1999) pointed in his finding that most doctors in his study were willing to integrate ,cooperate with traditional practitioners which also said that healers shall be brought in to the existing health care institutions . Similarly, 96.3 % respondents in a study (medical students above 4th year and doctors in Jimma Institute of Health Sciences and health institutions in Jimma zone) said that they are willing to work in coordination, integration with traditional practitioners(8).

But, for such cooperation to exist, a strong legal recognition to traditional medicine is essential as the Chinese constitution stated the equal value of traditional Chinese medicine as to the modern medicine (4). Most respondents (students and doctors) said that they do not have acquired in-depth awareness traditional medicine and relatively said that they do not know the government traditional medicine policy. Conversely, the impact of the course is found to be very significant on the attitude and willingness of students to the integration of the two medical systems . Thus, changes in the provision of health professional training are vital and occupy an essential role in realizing integration of traditional and modern medical systems.

In a survey on more than 100 pharmacy students in South Africa, 90% of them said that CAM should be a core part of their education since it enables them to look for evidence and then make informed decisions in the best of their patients. (6). Thus, government of Ethiopia shall

incorporate traditional medicine course into the training program of modern health professionals. From the above finding it is also clear to understand that the government has not still made better concern towards promotion of awareness of modern health professional.

The finding that most students in the study (62.3%) have come across advertisement on traditional medicine shows that professionals do have a good exposure to the media through which the government can effectively publicize programs and strategies on traditional medicine of the nation and hence can bring consensus.

The factors affecting awareness of students on traditional medicine are found to be absence of related course about it, shortage of writings (books, journals, etc), absence of seminars as well as poor student initiation to know about traditional medicine. All these issues, however; are not insurmountable by the joint effort between the university and the government. In general, half of the doctors in the study said that traditional medicine is sometimes better than modern medicine in the management of certain illnesses and hence two of them have ever advised patients to use traditional medicine. This shows the need to study document and publicize the effective healing wisdom of traditional healers which could possibly lead to a referral system among the two medical systems.

The fact that most (80%) doctors in the study said they take history of traditional medicine usage from their clients, is very appreciable as its use is popularly accepted and could have a significant effect on the patient outcome. Despite a more promising finding, the endeavor of the government and the university are found to be unsatisfying. At least the health professionals (mainly doctors) are not made to be aware of national traditional medicine policy which really shows the exclusive nature of government effort to realize the integration of the two medical systems.

CONCLUSION

The willingness of students and doctors to the integration of traditional and modern medicines shows that an absolute focus on modern medicine alone cannot improve health status of the country better than an integrated health care system. Thus change in the training curricula of health professional is essential and holds a pivotal role to realize integration of traditional and modern medical systems.

The fact that most respondents(students)have come across an advertisement on traditional medicine through media shows the government can effectively publicize its health program in general and about traditional medicine in particular using the media. So that national consensus could be maintained and unbiased recommendations are given by the health professional to the best interest of their patients.

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Changes in Antioxidant Status of *Chlorophytum borivilianum* Under Stress Conditions

Nikam Vijaya K.* and Chavan Prakash D.

Department of Botany, Shivaji University, Kolhapur – 416 004.

ABSTRACT

Safed musli (*Chlorophytum borivilianum* Sant. and Fernad.) plants were raised in fertile soil in earthen pots and subjected to various durations of water stress and waterlogging (5 and 10 days). Lipid peroxidation in the leaf tissue was found to be greatly promoted by water stress and waterlogging treatment. In tuber tissue, the level of lipid peroxidation was increased due to water stress conditions. The SH content (which is an indicator of antioxidant capacity of cell) in leaf and tuber tissue was found to be reduced by both kinds of soil constraints and 10 days waterlogging was most effective in this respect. The level of an important antioxidant ascorbic acid in the leaf tissue was markedly increased by both stress factors while in case of tuber tissue, such trend was noticeable only in water stressed plants. The activity of antioxidant enzymes catalase and peroxidase in the leaf tissue were stimulated due to water stress and waterlogging condition. In case of tuber tissue, activity of catalase was stimulated by water stress; while, waterlogging stress caused reduction in activity of this antioxidant enzyme.

Keywords: Medicinal plant, *Chlorophytum*, water stress, waterlogging, antioxidants.

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*** Author for Correspondance Email:** vkn_vijaya@yahoo.co.in; prakash777_2000@yahoo.com

INTRODUCTION

In plant cells, oxidative stress reactions involve generation of toxic free radicals from the reaction of the molecular oxygen to superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and hydroxyl radicals (OH). The toxic radicals can be removed through the mobilization of antioxidant reserves, which react both enzymatically and chemically with the toxic molecular species and their products. To counter the hazardous effects of Reactive Oxygen Species (ROS) which are generated under stress, plants have evolved a complex antioxidative defense system composed of both antioxidant enzymes and metabolites such as ascorbate peroxidase, catalase, superoxide dismutase, glutathione reductase, ascorbic acid, reduced glutathione, oxidized glutathione, and vitamin E (1). The efficiency of development of such mechanisms in the plant species is positively corrected with the stress tolerance potential of that species. Safed musli is one of the promising medicinal plants having a great commercial potential (2) and attempt have been made to cultivate this species in different agroclimatic conditions. The influence of water stress and waterlogging on the medicinal plant *C. borivilianum* (Safed musli) and its associated oxidative metabolism has not been studied. Hence, in the present investigation, an attempt is made for to study some enzymatic and non-enzymatic antioxidants in leaf and tuber tissue from both water stressed and waterlogged Safed musli plant.

MATERIAL AND METHODS

The plant materials of Safed musli (tubers with crown) were collected from musli growers of village Pattankodoli Dist.Kolhapur. The healthy tubers with crown were cultivated in earthenware pots (35 cm diameter with hole at the bottom) in garden soil containing farmyard manure in proportion of 3:1 in the month of June. The pots were equally watered, twice a week and every care was taken to raise healthy and vigorously growing plants in each pot. Three plants were grown in each pot. Three month old healthy plants in these pots were subjected to water stress (5days and 10days) and waterlogging treatment (5day and 10days). The plants which received a regular water supply served as control. The extent of lipid peroxidation, the level of antioxidants and the activities

of antioxidant enzymes were estimated from the control and stressed plants. The thiobarbituric acid assay for lipid peroxidation was carried out by the method of Heath and Packer (3). Ascorbic acid content was estimated as per the method described by Sadasivam and Manikam (4). Sulphydryl content was determined following the method of Ellman (5) with slight modifications. To study peroxidases activity the method of Maehly (6) was followed. Catalase activity was assayed by following the method of Luck (7) as described by Sadasivam and Manikam (4). The soluble proteins in the enzyme extract were determined according to the method of Lowry *et al*., (8). The values depicted in the figures represent mean of three determinations.

RESULTS AND DISCUSSION

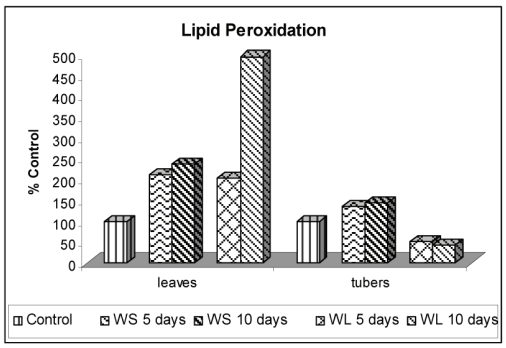
The influence of water stress and waterlogging on extent of lipid peroxidation (as indicated by MDA content) in leaf and tuber tissue of *C. borivilianum* is shown in Fig. 1. It is evident from figure that lipid peroxidation in the leaf tissue is greatly promoted by both water stress and waterlogging. In case of tuber tissue, lipid peroxidation is higher under water stress condition but it is lowered due to waterlogging condition. Many studies have shown greater accumulation of MDA and H_2O_2 in response to drought (9). Valentovic *et al*., (10) reported increase in lipid peroxidation in roots and second leaf of water stressed maize cultivars. Dong *et al*., (11) in wheat and Lin *et al*., (12) in rice reported that waterlogging induced plant senescence was associated with degradation of chlorophyll and the accumulation of the MDA. The increase in lipid peroxidation clearly indicates the possibility of membrane damage in the leaf tissue of water stressed and waterlogged Safed musli plants.

The sulfhydryl (-SH) content in the leaves and tubers of water stressed and waterlogged *C.borivilianum* is shown in Fig. 1. It is clear from figure that the SH content in leaf and tuber tissue is reduced by both water stress and waterlogging treatment. Levitt (13) proposed SH hypothesis or sulfhydryl-disulphide (SH = SS) hypothesis of freezing injury and resistance. The protection of –SH groups in proteins from the formation of disulfide bridges is considered to of great importance for providing cellular resistance to dehydration caused by drought and heat (13). Loggini *et al*., (14) compared two wheat cultivars with different drought tolerance capacity. They found that both cultivars responded with a decline in total glutathione concentrations and showed a higher reduced glutathione to oxidized glutathione ratio (GSH/GSSG) after a month of drought . Hurng and Kao (15) reported level of total glutathione, reduced form glutathione or oxidized glutathione levels in flooded tobacco leaves were lower than these in control leaves during first two days of waterlogging, afterwards the level increased by increasing flooding. The lowering of SH level in *C.borivilianum* indicates its sensitivity to both kinds of stresses.

The effect of water stress and waterlogging on the ascorbic acid content in leaves and tubers of *C. borivilianum* is shown in Fig. 1. It is clear from figure that the level of this important antioxidant in the leaf tissue is markedly increased in response to water stress and waterlogging. While, in case of tubers such a trend is noticeable only in plants subjected to 10 days water stress. Zhang *et al*., (16) reported elevation in the content of ascorbic acid in leaves of *Brassica parachinesis* due to drought stress. Nayyar and Gupta, (17) compared responses of C_3 (wheat) and C_4 (maize) plants to water deficit. They observed that, maize had significantly higher ascorbic acid content than wheat. The experiments of Hurng and Kao, (15) revealed that flooding increased the levels of total ascorbate in senescing tobacco leaves. Lin *et al*., (18) reported slight increase in total ascorbic acid and reduced ascorbic acid in roots of tomato and egg plants under waterlogged conditions. Under both water stress and waterlogging stress level of ascorbic acid is increased in leaf tissue of *C. borivilianum*. Such an increase in ascorbic acid is certainly of great value in protection against toxic accumulation of H_2O_2.

The effect of water stress and waterlogging on the activity of enzyme peroxidases in leaf and tuber tissue of *C. borivilianum* is recorded in Fig. 1. It is evident from the figure that the activity of enzyme peroxidases in the leaf tissue is stimulated by water stress and waterlogging treatments. In case of tuber, the activity of peroxidases is elevated in 5 days water stress and 10 days waterlogging treatments. According to Sen *et al*., (19), peroxidase activity increased faster in the water stressed rice leaves during senescence. El-Tayab (20) recently recorded increase in peroxidase activity in *Vicia faba* cultivars due to water stress. Hwang *et al*., (21), also noticed an increase in activity of peroxidase in waterlogged sweet potato plants. In case of *C. borivilianum* leaves a significant increase in peroxidase activity by both kinds of stresses is noticed. Since this enzyme system plays multiple roles in plants besides free radical scavenging some other effects of such elevation in enzyme activity are possible.

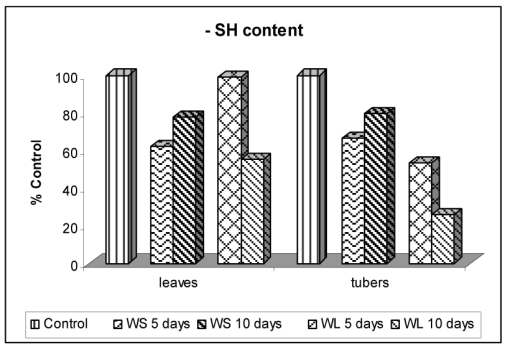
Influence of water stress and waterlogging on the activity of enzyme catalase is shown in Fig. 1. It is clear from figure that an enhancement in leaf catalase activity is brought about by to water stress and waterlogging treatments. This increase is more marked in water stressed leaves. In case of tuber, activity of enzyme catalase is also stimulated by water stress. However, waterlogging



Average values:

a) Control leaf = 84.23 µ moles MDA g^{-1} Fresh weight.

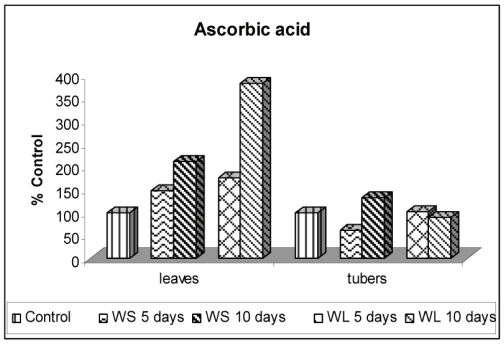
b) Control tuber = 1.76 X 10^{-5} Co M



Average values:

a) Control leaf = 1.08 X 10^{-5} Co M (Co-Original concentration)

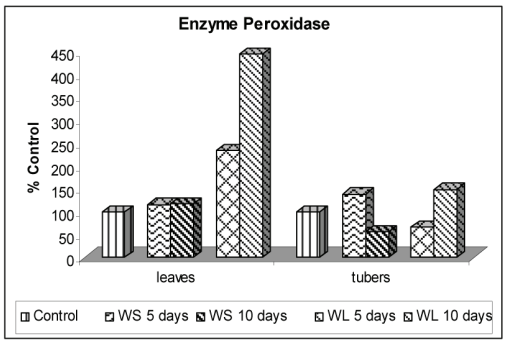
b) Control tuber = 113.20 µ moles MDA g^{-1} Fresh weight.



Average values:

a) Control leaf = 2.07 mg g^{-1} Fresh weight

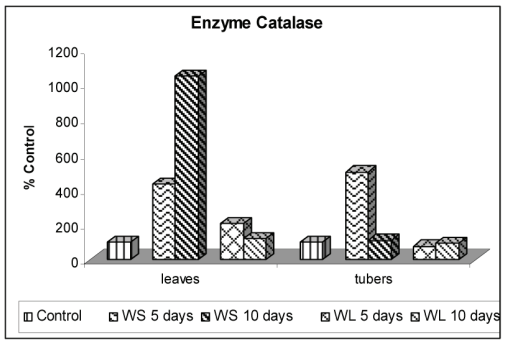
b) Control tuber = 1.26 mg g^{-1} Fresh weight



Average values:

a) Control leaf = 0.43 unit min^{-1} mg^{-1} protein.

b) Control tuber = 0.35 unit min^{-1} mg^{-1} protein.



Average values:

a) Control leaf = 3.76 unit min^{-1} mg^{-1} protein.

b) Control tuber = 7.55 unit min^{-1} mg^{-1} protein.

Figure 1: Effect of water stress (WS) and waterlogging (WL) on Ascorbic acid content in *C. borivilianum*

stress has caused a reduction in catalase activity in the tuber tissue. Water stress is reported to cause an increase in catalase activity in leaves of rice, wheat and cowpea (17, 19, 22). In case of *Chlorophytum* due to water stress catalase is markedly increased in the leaf tissue. While, in tuber tissue such increase is evident only under moderate stress conditions. The combined action of catalase and superoxide dismutase converts the toxic O_2^-, H_2O_2 to water and molecular oxygen, preventing the cellular damage under unfavorable conditions like water stress (23, 24).

Keles and Unyayar, (25) reported increase in catalase activity in seedlings of *Helianthus annuus* (cvs. Nantio F1 and Ozdemirdey) under waterlogging stress. Li *et al*., (26) noticed increase in catalase activity in *Phellodendron amurens* seedlings due to waterlogging conditions. In the present investigation, the study of catalase activity shows that the leaf enzyme activity is almost doubled due to 5 days waterlogging stress. However, in the tuber tissue which is a major target of waterlogging stress the enzyme activity is reduced due to the stress. An increase in catalase can indirectly cause an ameliorating effect in the waterlogged root tissue suffering from anaerobic conditions, by provision of O_2 produced through H_2O_2breakdown. In case of waterlogged *Chlorophytum* tubers such possibility is obscure since catalase activity is reduced due to stress.

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Development and Evaluation of Polyherbal Formulations for Hair Growth Activity

Sabarwal Neetu*, Varghese Dhanila, Barik Rakesh, Khandelwal Anjali, Jain Ashish, Jain Sanjay

Smriti College of Pharmaceutical Education, 4/1 Pipliya Kumar Kakad, Maya Kheri Road, Indore M. P., India-452010

ABSTRACT

Tridax procumbens (Linn.), *Hibiscus rosasinensis* (Linn.), *Trigonella foenum graecum* (Linn.), and *Embilica officinalis* (Linn.) are traditionally acclaimed herbs and widely used in indigenous system of medicine for their hair growth promoting potential. In the present study it was envisaged to formulate in-house polyherbal formulations of the four herbs in varying ratios and evaluating the formulations for their hair growth initiating and hair growth promoting activity. Experimental animal models for evaluation were qualitative hair growth analysis undertaken by visual observation of two parameters, hair growth initiation time and hair growth completion time. The HO4 formulation which showed significant reduction in hair growth initiation time was also evaluated using other experimental models including primary skin irritation test, vascular permeability effect, protection against cyclophosphamide induced alopecia and hair growth activity test. Hair growth initiation time was markedly reduced by the formulation. No signs of erythema and /or edema were recorded and formulation was thus free from any observable irritant effect. Formulation showed increased vascular permeability and was significantly more effective in increasing cutaneous capillary permeability over a 24 h period after administration as compared with standard. Topical application of formulation protected cyclophosphamide induced alopecia significantly as compared to control. The formulation showed significant increase in hair length and hair count as compared to standard and control. The results thus corroborate with the traditionally acclaimed hair growth-promoting capabilities of the plants. The prepared polyherbal formulation also holds potential for the treatment of alopecia.

Keywords: alopecia, hair growth, hair oil, minoxidil, polyherbal

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***Author For Correspondence:** E-Mail- neetusabarwal19@gmail.com Phone No. - +919425376066 Fax No. - 0731-2802262

INTRODUCTION

Hair loss is a dermatologic disorder, and the surge for discovering natural products with hair growth promoting potential is continuous (1). Hair loss is a universal problem having affected both sexes of all races to different extents for as long as mankind has existed (2). The main problems associated with hairs are pigmentation (fading), dandruff and falling of hairs (shedding) (3). Various factors contributing to hair loss includes genetic predisposition, hormonal factors, disease states such as typhoid, malaria, jaundice and use of chemotherapeutic agents. The telogen/ anagen effluvium is also considered to be the reason for hair loss. Hair loss causes a very stressful state of mind for hair fall sufferers (4). Topical application of biological response modifiers and antiandrogens are currently available therapies for the management of alopecia. However, the low success rate and associated adverse effects limits their clinical use (5). Natural products are fancy in cosmetics and about thousand kinds of plant extracts have been examined with respect to hair growth activity (6). In traditional Indian system of medicine many plants and herbal formulations are reported for hair growth promotion as well as improvement of quality of hairs, but lack of sound scientific backing and information limits their use (7). The herb *Tridax procumbens* is found as weed throughout India and used in the indigenous system of medicine for the treatment of variety of human ailments and as hair growth promoters (8–9). *Hibiscus rosasinensis* is a glabrous shrub widely cultivated in the tropics. It is well accepted that the leaves and flowers of the plant have hair growth promoting and antigreying properties (10–11). The plant *Emblica officinalis* is a rich source of vitamin C and contains appreciable amount of pectin rich in mineral matters like phosphorus, iron and calcium (12). *Trigonella foenum graecum* is used as high protein fodder and for its cleansing and softening activity. It promotes scalp health and prevents hair falling (13). In

the present study efforts have been made to formulate and evaluate hair growth promoting activity of polyherbal formulations, which includes in various concentrations the herbs mentioned above in an oil base.

MATERIALS AND METHODS

Collection and identification of plants

The aerial parts of *Tridax procumbens* (Compositae) and flowers of *Hibiscus rosasinensis* (Malvaceae) were collected locally from Indore in the month of August 2008. The dried fruits of *Emblica officinalis* (Euphorbiaceae) and seeds of *Trigonella foenum graecum* (Leguminosae) were purchased from the local market. The plant parts were identified with standard herbarium specimens available in AICRP on Medicinal & Aromatic plants, J.N. Krishi Vishwa Vidyalaya, College of Agriculture, Indore, Madhya Pradesh. A voucher specimen has been maintained in Institute museum for further reference. The plants were dried under sunlight, crushed to moderately coarse powder and stored in air tight container. Powdered drugs were subjected to pharmacognostic studies for confirmation. Coconut oil, sesame oil, almond oil, aloe oil and castor oil, the constituents of oil base were purchased from the local market.

Drugs and chemicals

Cyclophosphamide (50 mg/kg intraperitoneally) as alopecia inducer, Rootz-M5 (Minoxidil topical solution USP 5%) as reference standard for promoting hair growth, Evan's blue dye (20 mg/kg), Histamine dihydrochloride (30µg/ml) as reference standard for enhancing vascular permeability and normal saline as control.

Animals

Swiss albino mice of either sex weighing 20–25 gm and Swiss albino rabbits of either sex weighing 2.5–4 kg from Veterinary College, Mhow (Madhya Pradesh) were used for hair growth studies. The animals were housed at room temperature (24±2° C), 12h/12h light dark cycles and fed with standard pellets diet *ad libitum* and allowed free access to drinking water. The animals were allowed to acclimatize for one week before the experiments.

Ethical approval

All experimental protocols were approved by Institutional Animal Care and Ethics Committee headed by CPCSEA (Committee for Purpose of Control and Supervision of Experiments on Animals).

Preliminary screening of herbs for hair growth potential

The oil extracts of *Tridax procumbens, Hibiscus rosasinensis, Emblica officinalis* and *Trigonella foenum graecum* were individually prepared by cloth pouch decoction (14) method in the concentration range of 2, 4, 6, 8 and 10% w/w in the oil base containing coconut oil, sesame oil, castor oil, almond oil and aloe oil in a ratio of 4:2:2:1:1.

Hair growth initiation test

Quantitative modified model for the study of hair growth initiation studies was carried out on mice that were divided into six groups of five animals each. Hair clippers were used to remove the hair from dorsal portion of all test animals. Hair remover (Anne French) was used to ensure complete removal of hairs from denuded area which was nearly 4 cm^2. Finally the test sites were cleaned with surgical spirit. Group I served as control, group II was treated with Minoxidil topical solution USP 5% and served as positive control, Group III, IV, V and VI were given topical application of oil extracts of *Tridax procumbens*, *Hibiscus rosasinensis, Emblica officinalis* and *Trigonella foenum graecum* in the concentration range of 2,4,6,8 and 10% w/w oil respectively. This treatment was continued for 24 days, during the course the hair growth initiation pattern was observed and reported (15).

Table 1. Selection of concentrations of herbs for maximum hair growth activity

Formulation code % w/w	Amount of drugs/100ml of oil			
	T.procumbens	*H.rosasinensis*	*E.officinalis*	*T.foenum graecum*
2.5 (HO1)	2.5	2.5	2.5	2.5
5 (HO2)	5	5	5	5
7.5 (HO3)	7.5	7.5	7.5	7.5
10 (HO4)	10	10	10	10

Preparation of herbal hair formulations

After confirming the preliminary hair growth initiation potential of individual herb oil extracts all the herbs were mixed in four concentrations viz. 2.5, 5, 7.5 and 10% w/w for determining synergistic effect of all the herbs for maximum hair growth activity (Table 1).

Qualitative hair growth study

Qualitative hair growth analysis was undertaken for the formulations HO1, HO2, HO3, and HO4 by visual observation of two parameters: hair growth initiation time (i.e. minimum time to initiate hair growth on denuded skin region) and hair growth completion time (i.e. minimum time to completely cover the denuded skin region with new hair). The formulation showing significant reduction in hair growth initiation time and minimal time for hair growth completion was selected among other developed formulations and was further evaluated using experimental animal models (15). Physicochemical and microbiological assessment of the formulation was also carried out according to British Pharmacopoeia.

Primary skin irritation test

The HO4 formulation was tested for primary skin irritation test following the standard test method of ASTM F 719/81 with slight modification using albino rabbits. Six healthy rabbits of either sex weighing 2.5–4 kg were selected for the study. Each rabbit was caged individually and food and water given *ad libitum* during the test period. 24 hrs prior to the test, the hair from the back of each rabbit was shaved to expose sufficiently large test areas. The test site was cleaned with surgical spirit. Measured quantity of the hair oil was applied over the test site. The test site was observed for erythema and edema for 48 hrs after application. This test was conducted to evaluate the irritancy of the prepared polyherbal hair oil formulations on intact skin of rabbit (16).

Vascular permeability test

Swiss adult albino rabbits were used for the study. Skin sites were prepared 24 hrs prior to the administration of the test sample by clipping the hairs on the flanks, extending towards the dorsal region of the animals. All the sites were depilated. Three skin sites of approximately 4 cm^2 each (on either side) were demarcated on each rabbit, for the application of polyherbal hair oil formulation HO4, control (normal saline) and reference agent (Histamine dihydrochloride 30 µg /ml). Evan's blue dye (20 mg/kg body weight) in normal saline was administered i.v. through the marginal ear vein of the rabbit. One hour after Evan's blue dye administration 0.1 ml of formulation, histamine dihydrochloride (30 µg/ml) and normal saline were randomly administered by intradermal injection at the prepared site in each rabbit. Increased vascular permeability was assessed by dye diffusion and observations were recorded at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 18 and 24 hrs after intradermal injection of control and test agents. The intensity of the reaction was graded as 1=negligible, 2=mild, 3= moderate, 4=marked and expressed as average increase in vascular permeability (17).

Protection against cyclophosphamide induced alopecia

The effect of formulation HO4 was evaluated in chemotherapy induced alopecia in mice. Swiss albino mice were used for this test. The skin site of 4 cm^2 was depilated and demarcated on each mice. The mice were divided into three groups for the formulation, negative control and positive control. Each group was containing six mice. Test oil was applied topically to the depilated area daily for 10 days. On the 11^{th} day of Cyclophosphamide (50mg/kg intraperitoneally) was injected to induce hair loss. For the next few days mice were observed for the hair loss if any (18).

Hair growth activity test

Two parameters studied for hair growth activity were hair count and hair length. Swiss albino mice of either sex were used. Twenty four hours prior to the test, the skin sites 4 cm^2 was depilated and demarcated on each mice. The mice were divided into three groups each having six mice for the formulation HO4, negative control and positive control. Test drugs were applied topically to the depilated area daily for 28 days. The numbers of hairs were counted on 7^{th}, 14^{th} and 28^{th} day using magnifying lens and hair length was measured on day 7 and 21 during the experiment (19).

Statistical analysis

Statistical analysis was carried out employing analysis of variance (ANOVA) and Tukey Kramer multiple comparison tests. P values <0.05 were considered significant.

RESULTS AND DISCUSSION

Preliminary screening of herbs for hair growth potential

In hair growth initiation test for preliminary screening of herbs the activity of various concentration of oil extracts were recorded and significant results were obtained.

Qualitative hair growth study

The four formulations (HO1, HO2, HO3, and HO4) were subjected to hair growth initiation and completion test for determining the minimum number of days in which hair growth pattern can be observed (Table 2).

Initiation of hair growth in denuded area was considerably reduced by all the oil formulations. In control group animals, data recording was done in the second week, whereas data were recorded in first week in groups treated with developed formulations and Minoxidil. As against 10 days in control animals, hair growth initiation time was 7 days in HO1 and HO2, 6 days in HO3 and 5 days in HO4. In Minoxidil treated standard group, hair growth initiated after 5 days. Data clearly exhibit comparable performance of HO4 against Minoxidil in this parameter. Out of these four combinations the maximum activity was observed in formulation HO4 having 10% of each ingredient which was selected for further physicochemical, microbiological and biological evaluation.

Table 2. Qualitative hair growth study of combined formulations

Treatment	No. of Mice	Hair growth initiation time (days)	Hair growth completion time (days)
Control	5	10	27
Standard	5	5	19
HO1	5	7	19
HO2	5	7	18
HO3	5	6	18
HO4	5	5	17

Table 3. Results of various physicochemical parameters

Parameters	Result
Appearance	Greenish black
Odour	Characteristic
Density at 30° C (gm/ml)	0.8500–0.8600
Kinematic viscosity (mm^2/s)	15–18
Refractive index at 30° C	1.473
Acid value	2.24
Saponification value	280.5
Specific Gravity	0.934
pH	7.5

Physicochemical evaluation and microbiological assessment

Various physicochemical parameters were estimated and found within the specified limits according to BP (Table 3).

In the microbiological assessment no viable aerobic count (cfu) was found.

Primary skin irritation test

This test was conducted to evaluate the irritation by the prepared formulations on the intact skin of rabbits. None of the prepared formulations showed any erythema and/or edema, indicating that prepared formulations was not irritant on the skin of rabbits. Thus it can be concluded that the prepared formulations are free from observable irritant effect.

Vascular Permeability test

The formulation was found to increase cutaneous capillary permeability significantly and showed marked increase after 3 h. Vascular permeability enhancing effect of polyherbal hair oil formulation is given in Figure 1. The vascular permeability enhancing (VPE) effects of formulation were significant to positive control (Histamine dihydrochloride) and negative control (normal saline). After topical application of the formulation, VPE activity reached moderate levels within 1 h. VPE activity of the formulation declined slowly, mild values were recorded grossly between 10 h and 18 h. Vasodilation is a characteristic feature of hair growth. Evan's blue dye, injected intravenously forms colored complex with serum albumin which leaks out into the extra cellular space only when vascular capillary permeability is sufficiently increased to allow the passage of albumin molecules leading to blue discoloration of the skin. The area and intensity of the blue discoloration is proportional to increase in vascular permeability within limits. The result of the study showed that the

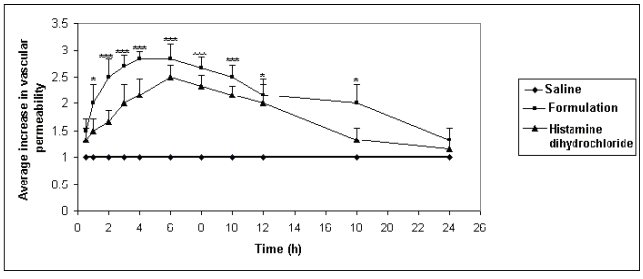

Figure 1. Vascular permeability enhancing effect of polyherbal hair oil formulation.
Mean ± S.E.M., n=6/group. Significant levels *** p< 0.001, *p< 0.01 vs. standard

formulation were significantly more effective in increasing cutaneous capillary permeability over a 24 h period after administration (20).

Protection against cyclophosphamide induced alopecia

In the experimental study the effects of polyherbal hair oil formulations were evaluated in cyclophosphamide induced alopecia in mice. Mice with a synchronized hair cycle were treated with cyclophosphamide. The drug treatment usually induces dystrophic changes in growing hair follicles, resulting in premature regression as a result of massive apoptosis in the hair bulb, leading to subsequent hair loss. Topical application of formulation protected cyclophosphamide induced alopecia significantly as compared to control (Table 4). However the exact mechanism of protection is not known and further studies are a pre requisite in order to evaluate the exact mechanism behind protection from cyclophosphamide induced alopecia (21).

Table 4. Effect of hair oil formulation on cyclophosphamide induced alopecia

Group	Hair follicle per mm of skin after 11th day of cyclophosphamide treatment
Negative Control	21±0.365
Positive Control	50.83±0.307
Formulation	42.16±0.307*

Mean ± S.E.M., n=6/group. Significant levels * p< 0.05 vs. positive control

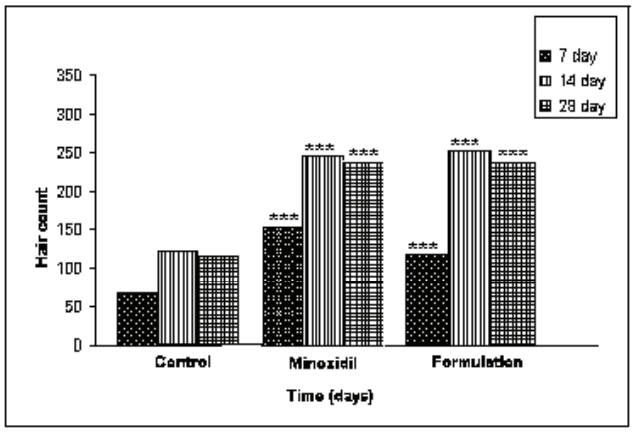

Figure 2. Effect of hair oil formulation on hair growth activity (hair count) of mice.
Mean ± S.E.M., n=6/group. Significant levels *** p< 0.001 vs. control

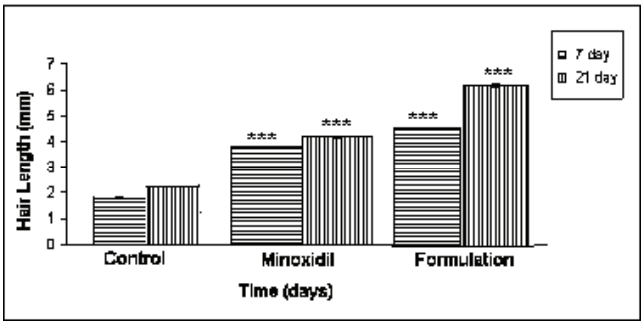

Figure 3. Effect of hair oil formulation on hair growth activity (hair length) of mice.
Mean ± S.E.M., n=6/group. Significant levels *** p< 0.001 vs. control

Hair growth activity test

The hair growth (i.e. the length and number of hair) on each mice was recorded. The formulation showed significant increase in hair length and hair count as compared to control (Figure 2 & 3).

CONCLUSION

The hair growth studies finally proved that formulation containing different herbs showed synergistic effects by significant increase in hair growth activity. Excellent results of hair growth were seen in formulation prepared by cloth pouch decoction method used for oil preparation. Among the prepared formulations, HO4 was the best as evidenced by least hair growth initiation and completion time and it was found to be superior to Minoxidil treatment. It may be rewarding if studies to unfold the mechanism of action of this herbal formulation are undertaken using this bed.

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