Microscopic Characterization as a Tool for Separation of *Stemona* Groups

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ABSTRACT

Introduction: *Stemona* plants have been traditionally used as an insecticide, scabicide and pediculocide, and for the treatment of skin and respiratory diseases. *Stemona* can be separated into two groups according to their morphological characters and bioactive components i.e. stichoneurine and protostemonine groups. Protostemonine group contains alkaloids that possess potent insecticidal activity while stichoneurine group accumulates alkaloids with antitussive activity. In Thailand, a vernacular name "Non Tai Yak" refers to the roots of different species of *Stemona*, making it confusing to discern different species. The purposes of this study are to investigate the microscopic characteristics of the roots of seven species of *Stemona* growing in Thailand and to distinguish and identify these groups of *Stemona*. **Methods:** Cross-sectional histology of fresh root samples and powdered drug characteristics of 7 species of *Stemona* were studied under a microscope. **Results:** The roots of *Stemona* in the stichoneurine group (*S. tuberosa* and *S. phyllantha*) contained a non-lignified large pith while the roots of protostemonine group (*S. burkillii*, *S. cochinchinensis*, *S. collinsiae*, *S. curtisii* and *S. kerrii*) had a small lignified one. The powder of stichoneurine group contained numerous thin-walled parenchyma, but only few thick-walled parenchyma and lignified fibers and vessels were present. In contrast, thick-walled parenchyma and lignified fibers and vessels were frequently found in the powdered roots of protostemonine *Stemona*. These characteristics could be used to discern between *Stemona* in the stichoneurine and protostemonine groups. **Conclusions:** The microscopic characterizations can be used as a primary tool to categorize and separate 2 main *Stemona* groups.

Key words: Non Tai Yak, protostemonine, *Stemona*, Stemonaceae, stichoneurine

INTRODUCTION

Since ancient time, *Stemona* plants have been traditionally used as an insecticide, scabicide, pediculocide; used for treating skin and respiratory diseases, and also for killing head lice.[1-3] "Non Tai Yak" is a Thai vernacular name that refers to various species of *Stemona* in Thailand[4] and some other plants such as *Asparagus* sp. of the family Asparagaceae and *Clitoria* sp. of the family Leguminosae in some locations.[1] This plant has been used to protect plants against insect attack, the infection of fermented fish "Pla Raa" or fermented shrimp "Ka Pi" from housefly larvae. The inconsistency when providing and using the proper *Stemona* plant materials has led to the confusion in the scientific identification and in agricultural and pharmaceutical uses.[1] Recent taxonomic revision of the family Stemonaceae indicates that *Stemona* in Thailand comprises of 11 known species i.e. *S. aphylla* Craib, *S. burkillii* Prain, *S. cochinchinensis* Gagnep., *S. collinsiae* Craib, *S. curtisii* Hk. F., *S. involuta* Inthachub, *S. kerrii* Craib, *S. phyllantha* Gagnep., *S. pierrei* Gagnep., *S. rupestris* Inthachub and *S. tuberosa* Lour.[5] They can be separated into two main groups according to their morphological characters and bioactive component accumulation i.e. stichoneurine or tuberosa group and protostemonine or non-tuberosa group.[6] Stichoneurine group comprises of *S. tuberosa* and *S. phyllantha* and they are different from other *Stemona* plants because of their large and thick tuberous roots, large perianths and scented flowers.[5,6] However, the three dominant species of *Stemona* (*S. tuberosa*, *S. collinsiae* and *S. curtisii*) and some other species of *Stemona* in Thailand are still called "Non Tai Yak", making it confusing and causing misuses of these plants.

Phytochemical investigations of *Stemona* species revealed the presence of alkaloids, stilbenoids and chromenols. The

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alkaloids display a remarkable accumulation trend in *Stemona* species.[1,7] *Stemona* alkaloids constitute a unique chemical feature of the family Stemonaceae and cannot be detected in any other plant families thus far.[1,2] Classification of *Stemona* alkaloids based on biosynthetic considerations confirms three skeleton types i.e. stichoneurine- (tuberostemonine-), protostemonine-, and croomine-type alkaloids.[1] Stichoneurine group contains stichoneurine- and croomine-type alkaloids while protostemonine group contains protostemonine-type alkaloids.[1,7] Potent insect toxicity of *Stemona* plants is attributed to the derivatives of protostemonine-type alkaloids, especially the stemofoline derivatives,[1,8,9] whereas stichoneurine-type alkaloids possess only a remarkable insect repellance.[8] In contrast, stichoneurine- and croomine-type alkaloids were reported to be associated with antitussive activity.[10-14]

Herbal extracts from various *Stemona* plants have been used for over a century. *Stemona* has been developed into commercial products for bioinsecticide or antitussive drug. Since the variation of authentic *Stemona* raw materials affects their promised biological properties, the confusion stems out when *Stemona* roots as well as their powdered drug are used. Identification of these plants via morphological characteristics is limited by the presence of flowers and it cannot be determined in the form of powdered drug. Authentication of *Stemona* species using DNA-based techniques is effective but it is also expensive and time-consuming. This study presented a simpler method to identify and distinguish *Stemona* in stichoneurine or tuberosa group from protostemonine or non-tuberosa group by exploiting the dissimilarities between their microscopic characteristics.

MATERIALS AND METHODS

Plant materials

The samples of 7 *Stemona* species (*S. burkillii*, *S. cochinchinensis*, *S. collinsiae*, *S. curtisii*, *S. kerrii*, *S. phyllantha* and *S. tuberosa*) were collected from various locations in Thailand. Each sample was cultivated at National Corn and Sorghum Research Center, Nakorn-Ratchasima province in the North-East of Thailand. All root samples at the age of 4 year old were collected in August, 2009. The plant specimens were identified by Dr. Vichien Keeratinijakal, Kasetsart University, and the voucher specimens (VKS001-VKS007) were deposited at Pharmacognosy Department, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

Each fresh sample was dried at 60°C then powdered by an electronic mill. The powder was passed through a sieve no. 60 to yield fine powder. The powder of each sample was separately kept in a tightly-closed vial until used. Other portions of fresh root were used for histological inspection.

Microscopic methods

Cross-sectional histology of fresh root samples and powdered drug of the seven species of *Stemona* were examined under a microscope (Olympus, Japan) using mounting reagents. The characteristic tissues were photographed using a camera and drawn using a camera lucida (Olympus, Japan).

RESULTS

The tuberous roots of *Stemona* in stichoneurine group (*S. tuberosa* and *S. phyllantha*) were large and thick, 10-50 cm long, pale yellowish-brown in color, while the protostemonine group (*S. burkillii*, *S. cochinchinensis*, *S. collinsiae*, *S. curtisii* and *S. kerrii*) had slender pale yellowish-brown roots with varied length (4-50 cm). Cross-sectional histological characteristics of the fresh root samples of *Stemona* showed that the stichoneurine group had a larger pith compared to those in the protostemonine group. After applying aniline sulfate solution, the pith of the protostemonine *Stemona* turned yellow, making it a lignified pith, while the stichoneurine *Stemona* roots contained a non-lignified pith (Figure 1).

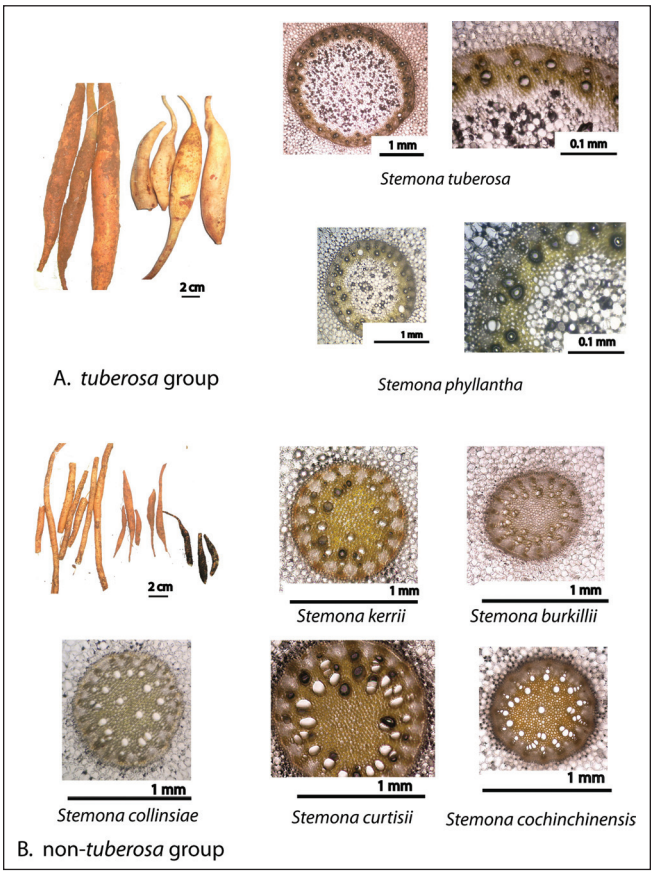

Figure 1: Cross-sectional histology of fresh root samples of various *Stemona* species applied with aniline sulfate showing characteristic features : (A) a large non-lignified pith of stichoneurine group, or tuberosa (B) a small lignified pith of protostemonine or non-tuberosa group.

Powders of all *Stemona* species appeared as creamish-white to creamish-yellow with a faint distinct odor and a sweet and bitter taste. The diagnostic characteristics of the powders of both groups of *Stemona* are shown in Figure 2 and are compared in Table 1. These characteristics are as follows.

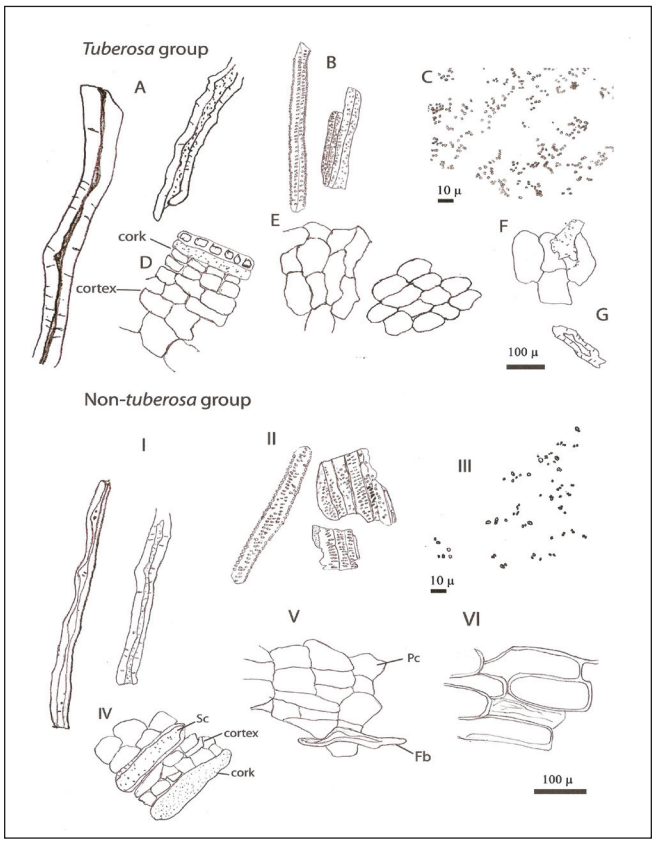

Figure 2: Powdered drug characteristics of *Stemona* roots. Tuberosa group comprised of: A, part of fibers; B, xylem vessels which arranged bordered pits; C, numerous small starch granules; D, part of cork and cortex in sectional view; E, parenchyma cells; F, parenchyma cells containing starch granules; G, sclereids. Non-tuberosa group was composed of : I, part of fibers; II, xylem vessels which arranged bordered pits and occasionally occurred in groups of interlocking cells; III, starch granules; IV, part of cork and cortex in sectional view with sclereids (Sc.); V, small part of fiber (Fb) connected to parenchyma cells (Pc); VI, thick walled parenchyma cells.

(1) Abundant starch granules that are simple, small, spherical to ovoid, or compound with two, three, four or occasionally up to six components.
(2) Abundant parenchyma from the cortex and stele. The cells are fairly large and vary from rounded to elongated rectangular in outline with thin wall. The cells are almost filled with starch granules.
(3) The lignified vessels of xylem occur in groups of interlocking cells. The vessels contain numerous bordered pits.
(4) Long fibers, fragmented.
(5) Thick-walled parenchyma of the xylem and medullary ray.

DISCUSSION

Stemona in stichoneurine group (*S. tuberosa* and *S. phyllantha*) had larger, thicker and longer tuberous roots than the protostemonine group (*S. burkillii*, *S. cochinchinensis*, *S. collinsiae*, *S. curtisii* and *S. kerrii*). The colors of the roots of both groups were the same pale yellowish-brown.

Cross-sectional histology of the roots of stichoneurine *Stemona* showed a non-lignified large pith containing numerous thin-walled parenchyma cells while the protostemonine *Stemona* roots had a small liginified pith with less abundant thin-walled parenchyma cells. The powdered drugs of the roots of stichoneurine group contained several thin-walled parenchyma but only few thick-walled parenchyma cells were found. The thick-walled parenchyma cells were frequently spotted in the powdered roots of the protostemonine group. The lignified fibers and vessels were frequently found in the powdered roots of protostemonine *Stemona* but rarely found in the root powders of the stichoneurine group. Numerous simple

TABLE 1 : Comparison of powdered drug characteristics of the roots of *Stemona* spp. in stichoneurine and protostemonine groups

***Stemona* species**	**Amount found**			
	Parenchyma	**Liginified cells**		**Starch granules**
		Vessels	**Fibers**	
Stichoneurine gr. *S. tuberosa* *S. phyllantha*	numerous thin-wall, rarely thick-wall	few	few	small, numerous
Protostemonine gr. *S. burkillii* *S. cochinchinensis* *S. collinsiae* *S. curtisii* *S. kerrii*	moderate thin-wall and thick-wall	moderate	moderate	small, numerous

and compound types of small starch granules were observed in both groups of *Stemona*.

CONCLUSION

Cross-sectional histology and powdered drug characteristics of the roots of various *Stemona* species growing in Thailand verified that the roots of the stichoneurine or tuberosa group had non-lignified larger pith containing numerous thin-walled parenchyma cells. The protostemonine *Stemona* roots had smaller liginified pith and were less abundant in thin-walled parenchyma. Thick-walled parenchyma cells were frequently found in the protostemonine *Stemona* roots, but rarely found in the roots of the stichoneurine group. The lignified fibers and vessels were frequently found in the root powders of protostemonine *Stemona* but rarely found in the stichoneurine group. These microscopic characterizations could be used as a primary tool to clearly identify groups of *Stemona*, and it could confirm their macroscopic characteristics. However, these characteristics could not distinguish each *Stemona* species. This is the first report on the utilization of microscopic characterizations of *Stemona* groups, particularly the ones growing in Thailand. The information will be of benefit to the correct identification of actual sources of *Stemona* for agricultural and pharmaceutical purposes.

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Pharmacognostic Evaluation of stem of *Berberis aristata* DC.

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ABSTRACT

Stem of *Berberis aristata* DC (Berberidaceae) is used in Indian traditional medicine for treating antibacterial, antiperiodic, antidiarrhoeal, ophthalmic, skin diseases and diabetes mellitus. It is an important commodity in folklore medicine of India for as laxative, ophthalmia and other eye diseases so far there no record on detailed pharmacognostic evaluation of this crude drug, hence this communication is useful for identification this plant material.

Key words: *Berberis aristata*, Pharmacognosy, macroscopy, microscopy

INTRODUCTION

Berberis aristata DC belonging to family Berberidaceae commonly called 'Indian barberry' in English and 'Daruhaldi' in Hindi is indigenous to India. It is an erect spinous shrub, often found in small patches on the hilly slopes. This shrub is found growing wild in the sub-Himalayan tract at altitude ranging from 1800-3000 meters. It also grows in the Nilgiri hills in South India and in Ceylon.[1] A large deciduous shrub usually 1.8-3.6 m high. The stem 4.5 m height and 20 cm diameter, nearly cylindrical, surface rough. Twing whitish or pale yellowish brown, Bark pale brown, closely and rather deeply furrowed, rough, blaze 5-7.5 mm bright yellow with coarse reticulate fibre. Leaves on long shoots, 3.8-10 cm long, 1.5-3.3 wide, obovate or elliptic, simple or either entire or with spinous toothed margin, base gradually narrowed, with prominent reticulate nerves, glossy dark green above, glossy pale green below but not glaucous beneath petiole o or distinct up 4 mm. Inflorescence a simple drooping racemes of 20 on single flower-head, they are yellow orange 2.5-7.5 mm long, pedicle stout, 4-6 mm long. The fruits are small berry 7-10 mm long, ovoid, blue-black thick pale bloom in colour.[2] The all parts of plant are economically important and when medicinal value is focused. Parts like wood, stem, root bark and fruit and its extract in traditional medice from long time. The *Berberis aristata* DC extract is called 'Rasaut', used as alternative and deobstruent and are used in skin diseases, menorrhagia, diarrhoea, jaundice and all affections of eyes. In bleeding piles it is administered with butter. Its ointment made with camphor and butter and applied pimples and boiles. The decoction of root bark is used as a wash for unhealthy ulcers, also in malarial fever. The stem is used for diaphoretic, laxative and useful in rheumatism. The of stem of *Berberis aristata* DC have been used in ethno medicine and in many Ayurvedic preparation for several medicinal properties alternative, antibacterial, antidiarrhoeal, ophthalmic, antidiabetic, eye, ear, oral cavity and in skin diseases.[3]

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PLANT MATERIAL

The Stem of *Berberis aristata* DC were purchased from Sanjivani Medicinal plant supplier, Nadiad, Gujarat, in month of September 2007, authenticated by Dr. A. S. Reddy, Taxonomist, Bioscience Department, Sardar Patel University. Vallabh Vidyanagar, Gujarat, India.

METHODS

Few fresh stem were used for studying the microscopic characters; histochemical test were performed as per the standard methods.[4] Dried stem coarsely powdered, used for physico-chemical analysis.[5] The coarsely powdered plant material was successively extracted with petroleum ether, benzene, chloroform, acetone, 90% ethanol and aqueous using Soxhlet apparatus and used for preliminary Phytochemical[6] and TLC[7] studies.

RESULT

Macroscopy

Stem pieces are nearly cylindrical, variable in length and thickness about 15 to 20 mm., bark about 0.4-0.8 cm thick, pale yellowish brown, soft, closely and deeply furrowed, surface rough, brittle, wood portion yellow, more or hard radiate with xylem rays. Pith present very small. Stems also branched; bark thin, fracture surface short and gets period off at places exposing the inner dark yellow wood.

Microscopy

Stem is circular in outline with outer well developed cork, narrow pericycle traversed by stone cells, central narrow pith surrounded by xylem and medullary rays pith surrounded by xylem and medullary rays are present occupying 60% area of the selection. T.S. of stem shows multilayered cork consisting of 3-45 rectangular to squarish radically arranged suberized cells, yellow coloured and thin walled arranged radially. Cortex narrow, composed of tangentially elongated parenchymatous tissue containing stone cell are isolated or in-group and starch grains. Pericycle characterized by discontinuous band of isolated or group of 2 to 5, lignified fibres. Sieve elements irregular in shape, thin walled a few cells containing yellowish-brown contents; Phloem fibre arranged in tangential rows, consisting of 2-4 cells, each fibre short, thick-walled and spindle shaped and lignified. Medullary ray in continuation with xylem and containing calcium oxalate crystal, cambium distinct. Xylem consists of vessels, tracheids, fibers and parenchyma. Xylem vessels numerous, small to medium sized, in single or in groups arranged radially. Centrally located parenchymatous pith. Simple starch grains and prismatic crystals of calcium oxalate are present throughout parenchymatous cell section. Occasionally dark brownish content found in ray cell.

Powder microscopy

Powder are yellow coloured shows fragments of cork cells, reticulate and spirally thickened xylem vessels, fibrous sclereids associated with stone cells, group of xylem fibres associated with parenchyma containing prismatic crystals of calcium oxalate, radically cut medullary ray crossing the fibres prismatic crystals of calcium oxalate and simple starch grains scattered as such and in the parenchymatous cells.

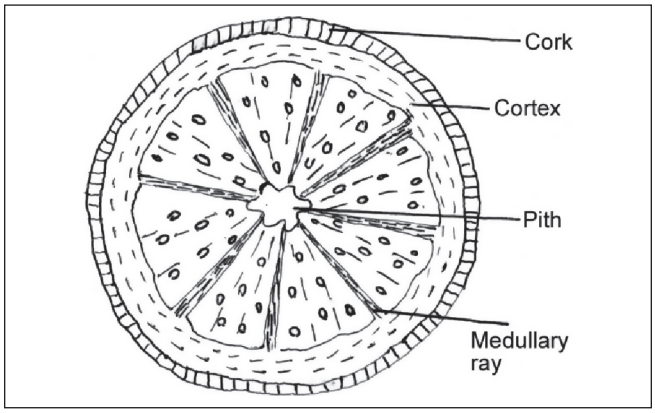


Figure 1: Diagrammatic T. S. of *Berberis aristata* DC stem.

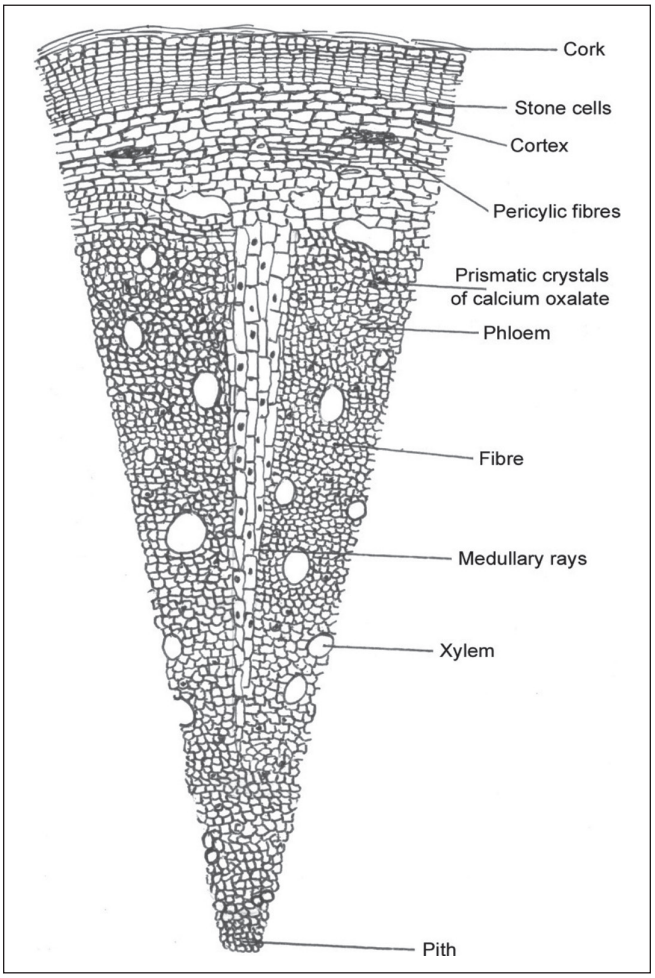
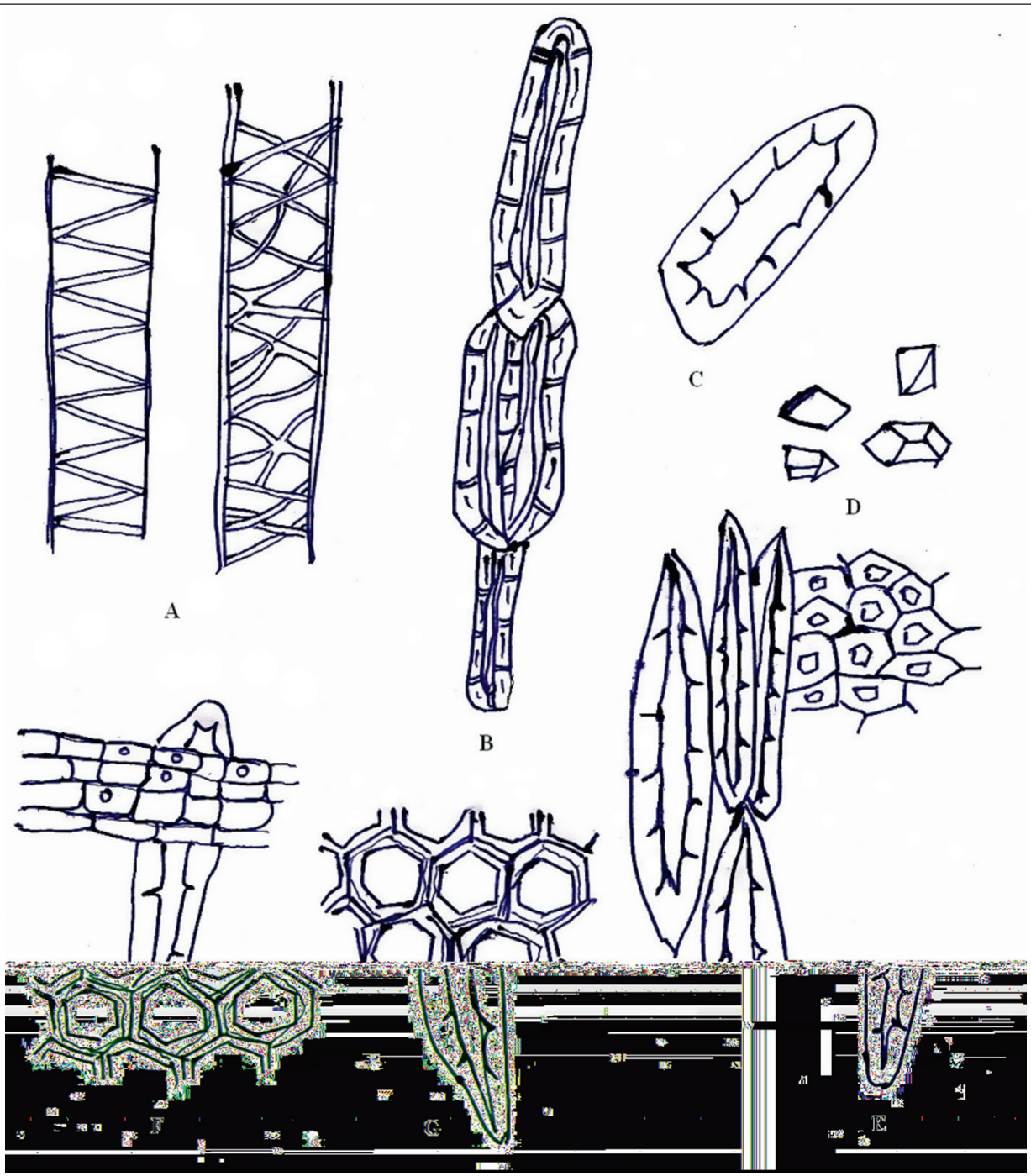


Figure 2: Detailed T. S. of *Berberis aristata* DC stem



A- Vessel with spiral and reticulate thickening
B- Fibre sclereids
C- Stone cells
D- Prismatic crystal of calcium oxalate.
E- Radially cut medullary rays containing starch grain associated with phloem fibres.
F- Cork cell in surface view
G- Fibre associated with parenchyma

Figure 3: Powder characters T. S. of *Berberis aristata* DC stem

DISCUSSION

The physico-chemical data obtained on analysis stem are shown in Table 1. Ash content was 13% indicating more amount of inorganic components are present. Most of mineral components present are water soluble in nature as indicated by 7 % of water soluble ash. The acid insoluble ash of 1 % was revealing the siliceous content is very less. Alcohol soluble extractive and water soluble extractive of 8 and 12% suggestive of presence of more polar secondary metabolites like alkaloids, glycosides, steroids, triterpenoides, coumarins, phenols and tannins. 1 % and 1.5 % of petroleum ether and chloroform (successive) extracts showed presence of fewer amounts of low polar compounds. Results of preliminary phytochemical studies and TLC are detailed in the Table 2 and 3 respectively.

Table 1: Physico chemical studies

Parameter	Mean value ± sd (n = 3)
Total Ash (% w/w)	13.05 ± 0.150
Water-soluble ash (% w/w)	6.55 ± 0.051
Acid-insoluble ash (% w/w)	1.32 ± 0.014
Alkalinity (CC of 0.1NHCl/g)	0.31 ± 0.025
Loss on drying atb105(% w/w)	7.89 ± 0.039
Alcohol soluble extractive (% w/w)	8.02 ± 0.450
Water soluble extractive (% w/w)	11.94 ± 0.086

Table 2: Preliminary Phytochemical study

Qualitative Test	Petroleum Ether (1.0 %w/w)	Benzene (1.2 %w/w)	Chloroform (1.5 %w/w)	Acetone (2.0 %w/w)	Ethanol (4.0 %w/w)	Aqueous (5.2 %w/w)
Alkaloid	–	–	+	–	+	+
Glycoside	–	–	–	–	+	+
Bitter Principle	–	+	–	+	+	–
Flavonoid	–	–	–	–	–	–
Tannin	–	–	–	+	+	+
Saponin	–	–	–	+	+	+
Coumarin	–	–	–	–	–	–

Table 3: Thin layer chromatography (TLC) study

Solvent Systems	Spray Reagent	R_f Of Spots after Derivatisation in Extract					
		Petroleum ether	Benzene	Chloroform	Acetone	Ethanol	Aqueous
Ethyl acetate : methanol, water (100 : 13.5:10)	Dragondroff reagent	–	–	0.26 (yellow brown) 0.32 (dark orange red) 0.40 (yellow brown) 0.58 (orange red) 0.67 (dark orange red)	–	0.23 (yellow brown) 0.54 (orange red) 0.67 (orange red) 0.81 (dark orange red)	0.28(yellow brown 0.43 (orange brown) 0.68 (orange red)
Ethyl acetate : methanol, water (100 :13.5:10)	10% ethanolic KOH	–	–	–	–	0.91, 0.50 (Yellow)	0.50, 0.53 (yellow)
Ethyl acetate : methanol, water (100:13.5:10)	Vanillin sulphuric acid reagent	–	0.90 (blue)	–	0.63 (blue green)	0.67 (blue green)	–
Ethyl acetate : methanol, water (100:13.5:10)	NP reagent	–	–	–	–	–	–
Chloroform glacial acetic acid : methanol water (64:32:12:8)	Vanillin sulphuric acid reagent	–	–	–	0.74 (blue violet)	0.94 (yellow brown)	0.95 (yellow brown)
Toluene ethyl acetate (93:7)	10 % ethanolic KOH reagent	–	–	–	–	–	–

CONCLUSION

Standards such as macroscopic, microscopic, physico-chemical, preliminary phytochemical studies and TLC were derived and described which are of diagnostic importance in authentication and quality control of stems of *Berberis aristata* DC.

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Pharmacognostical standardization of Ficus religiosa fruits

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ABSTRACT

Context: *Ficus religiosa* belonging to family Moraceae is a large glabrous tree, found throughout in India in the vicinity of temples. It is well known for curing a variety of ailments such as diarrhea, dysentery, vaginal and other urinogenital disorder, eczema, leprosy, rheumatism and used as anticonvulsant. **Aim:** The present study was undertaken to investigate the Pharmacognostical and Phytochemical parameters of fruits of *Ficus religiosa*. **Settings and Design:** Pharmacognostical investigations were carried out to study its macroscopical and microscopical characters. Various physiochemical parameters and histochemical color reactions were evaluated as per the IP method. **Results:** Macroscopical studies revealed that the fruit is purple colored, depress and globose shaped, 2-3 cm in diameter and sweet in taste. The results of microscopical studies showed the presence of epidermis, stone cells, pitted parenchymatous fibers, parenchymatous tissue, spiral vessels etc. The results of physiochemical parameters showed total ash- 6.74% w/w, water soluble ash- 5.40% w/w, acid insoluble ash-1.85%w/w, petroleum ether soluble extractive- 1.08% w/w, 90% methanol extractive-4.50% w/w, water soluble extractive-6.50% w/w. The qualitative evaluation of the extract indicated the presence of carbohydrates, steroids, free amino acids and phenolic compounds. Total phenolic content in the fruit was found to be 0.2% w/w.

Keywords: *Ficus religiosa* fruits, Moraceae, Phytochemical Evaluation, Total phenolic content

INTRODUCTION

After decades of serious obsession with the modern medicinal system, people have started looking at the ancient healing systems like Ayurveda, Siddha and Unani. This is because of the adverse effects associated with synthetic drugs. Herbal drugs play an important role in health care programs especially in developing countries. Ancient Indian literature incorporates a remarkably broad definition of medicinal plants and considers all plant parts to be potential sources of medicinal substances.[1] However, a key obstacle, which hindered the acceptance of the alternative medicines, is the lack of documentation and stringent control. Therefore, there is a need for documentation and stringent quality control.[2] With this backdrop it becomes extremely important to make an effort towards standardization of the herbal drugs. The process of standardization can be achieved by stepwise pharmacognostic studies.[3] The pharmacognostical studies are one of the major criteria for identification of herbal drugs.[4] Medicinal plants form a large group of economically important plants that provide the basic raw materials for indigenous pharmaceuticals.[5,6] One approach to the discovery of new drugs is the study of the bioactive constituents of higher plants. The investigation of plants used as remedies in the traditional folk medicine can be an interesting tool to identify several biologically active molecules from the 250,000 higher plant bioactive constituents with antiinflammatory, analgesic, antipyretic and anti ulcerogenic activity.[7] *Ficus religiosa (F. religiosa)* commonly known as peepal is a very big sacred tree and found throughout India in the vicinity of temples. *F. religiosa* leaf juice along with honey is used for treatment of asthma, cough, sexual disorders, diarrhoea, haematuria, earache and toothache, migraine, eye troubles, gastric problems and scabies.[8] Fruits are used for the treatment of asthma and respiratory disorders. Fruit paste is taken to cure scabies. Stem bark is used in the treatment of gonorrhoea, bleeding, cuts, wounds, paralysis, diabetes, diarrhea, bone fracture and used as antiseptic, astringent and antidote. Bark paste along with honey is used to treat

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cough and cold as well as accompanying mild fever. Aerial root juice is used for treatment of menustral problems.[9] In the present study an attempt has been made to highlight this medicinal fruit through pharmacognostic and phytochemical studies. As per the available literature no pharmacognostical study has been carried out on the fruits; hence the present investigation was undertaken to evaluate various pharmacognostical standards like macroscopy and microscopy of fruits; ash values, extractive values, microscopical characteristics of powdered fruits and preliminary phytochemical analysis of *F. religiosa* fruits.

MATERIAL AND METHODS

Collection of plant material

The fruits of *F. religiosa* were collected from Chandigarh in the month of Nov-Dec, 2008 depending upon its easy availability. Fruits were authenticated by Dr. Promila Pathak, Dept. Of Botany, Punjab University, Chandigarh. The fruits were shade dried, coarsely powdered and stored in an airtight container.

Chemicals and instruments

Compound microscope, glass slides, cover slips, watch glass and other common glass ware were the basic apparatus and instruments used for the study. Microphotographs were taken using a motic images microscope. Solvents used for extraction includes viz. petroleum ether, chloroform, ethyl acetate, ethanol (95%), water and reagents viz. phloroglucinol, glycerine, HCl, chloral hydrate and sodium hydroxide were procured from Central Drug House (P) Ltd., New Delhi, India.

Macroscopic and Microscopic analysis

The macroscopy and microscopy of the fruit and powder were studied according to the method of Brain and turner.[10] For the microscopical studies, cross sections were prepared and stained as per the procedure of Johansen.[11] The micropowder analysis was done according to the method of Brain and Turner[12] and Kokate.[13]

Physico-chemical analysis

Physico-chemical analysis i.e. percentage of ash values and extractive values were performed according to the official methods prescribed[14] and the WHO guidelines on the quality control methods for medicinal plant materials.[15]

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out by using standard procedures described by Kokate[16] and Harborne[17]. The shade dried and powdered fruits of *F. religiosa*, were subjected to maceration with different solvents like petroleum ether (60–80°C), 90% methanol and finally macerated with water so as to get respective extracts. All extracts were filtered individually, evaporated to dryness. After drying, the respective extracts were weighed and percentage yields were determined separately and stored in freeze condition for further use. The qualitative chemical tests, for identifying the presence of various phytoconstituents, were carried out on various extracts of *F. religiosa* fruits. The extracts were screened for the presence of tannins, saponins, sterols/triterpenes, alkaloids, glycosides, flavonoids, polyphenolic compounds, protein/amino acids and carbohydrates on Silica gel G (Merck) plates (0.25 mm thickness). Development was carried out with various solvent systems viz: ethyl acetate: formic acid: methanol (6: 0.6: 0.4 v/v/v), ethyl acetate: methanol: water (10: 1.3: 1.0 v/v/v), ethyl acetate: formic acid: acetic acid: water (10: 1.1: 1.1: 2.7 v/v/v), chloroform: methanol: water (6.4: 5.0: 1.0 v/v/v), benzene: ethyl acetate (8.6: 1.4 v/v) and ethyl acetate: methanol: water: acetic acid (6.5: 1.5: 1.5: 1.0 v/v/v/v). After development in the different solvents, the plates were sprayed with Dragendorrf's reagent, $AlCl_3$, hydroxylamine–ferric chloride, ninhydrine and antimony trichloride reagents for the discovery of alkaloids, flavonoids, proteins/amino acids and sterols/triterpenes respectively. Detection of glycosides, saponins, tannins, and carbohydrate are carried out using KOH, anisaldehyde–sulphuric acid, ferric chloride, and naphthoresorcinol reagents, respectively.[18]

Estimation of total phenolic compounds

Principle:

The total phenolic contents in the fruits of *F. religiosa* were determined by using Folin Ciocalteu's method. Folin Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. It works by measuring the amount of substance, being tested needed to inhibit the oxidation of the reagent. The sample extract dilution was oxidized with Folin Ciocalteu reagent and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue colour was measured at 765 nm after 30 min.

Preparation of standard solution:

Gallic acid was used to make the calibration curve. 10 mg of gallic acid was dissolved in 100 ml of 50% methanol (100 µg /ml) and then further diluted to 1, 2, 4, 6, 8 and 10 µg /ml. 1 ml aliquot of each dilution was taken in a test tube and diluted with 10 ml of distilled water. Then, 1.5 ml Folin Ciocalteu reagent was added and allowed to incubate at room temperature for 5 min. 4 ml of 20% (w/w) Na_2CO_3 were added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of the standard was measured at 765 nm and distilled water was taken as a blank.

Preparation of sample solution:

1 g of sample (fruit powder) was added to 15 ml of methanol (50%) and extracted for three times by maceration of 2 hours.

Then filtered and make up the volume with methanol (50%) in volumetric flask upto 50 ml. 1 ml aliquot of the sample was taken in a test tube and diluted with 10 ml of distilled water. Then, 1.5 ml Folin Ciocalteu reagent was added and allowed to incubate at room temperature for 5 min. 4 ml of 20% (w/v) Na_2CO_3 were added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature Absorbance of the sample was measured at 765 nm. Three parallel determinations were recorded. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as mg gallic acid equivalents (GAE) and percentage w/w.[19]

Calculation: Total phenolic contents (%) = GAE × V × D × 10^{-6} × 100/W

GAE - Gallic acid equivalent (µg/ml)

V - Total volume of sample (ml)

D - Dilution factor

W - Sample weight (g)

RESULTS AND DISCUSSION

Brief taxonomic description of the plant

The Sacred Figure (*F. religiosa*) or Bo-Tree (from the Sinhala bo) is a species of banyan figure native to Bangladesh, India, Nepal, Pakistan, Sri Lanka, southwest China and Indochina. In India, it occurs both wild and cultivated up to 5,000 ft (1,524 m).[20] *F. religiosa* is cultivated in various tropical areas of the world. In the United States, it is grown in southern California, Florida, and Hawaii. In Florida, seedlings were found in Homestead in 1975 and in Miami in 1988.[21] *F. religiosa is* a large, glabrous tree, with characteristic milky latex and the trunk often covered with epiphytes. It is a large dry season-deciduous or semi-evergreen tree with a pale stem up to 30 m tall and with a trunk diameter of up to 3 m, often appearing fluted on account of the numerous roots which have fused with the stem. The bark is light grey and peels off in patches. The leaves are leathery 4-8 inches long by 3-5 inches wide, somewhat egg-shaped or rounded, tailed at the tip and heart-shaped at the base, or sometimes rounded are large; alternate, with long petioles and a broadly ovate, subcoriacious lamina cordate in shape. The tip of leaf is long, lanceolate and cuspidate. The margin sinuate and the base truncate. The young leaves are frequently pink, change to copper and finally to green. Flowers minute within the receptacle. Receptacles-sessile, dark purple when ripe, basal bracts, broadly ovate-elliptic obtuse. Male flowers-Sessile K_{2-3}, ovate, lanceolate. A_1, anther single. Female flower $G_{(2)}$ and gall flowers sessile or pedicillate. K_{3-4}, lanceolate, gall flowers without perianth, style short, stigma round.[22,23] The fruit is a small figure 1-1.5 cm diameter, green ripening purple.

Description of Fruit

Macromorphology:

F. religiosa fruits are syconus inflorescence containing drupe fruits, having depressed and globose shape with 2-3 cm in diameter. The colors of the fruit are green when unripe and purple when ripe. The outer surface of the fruit is smooth and the position of fruit is sessile in axillary pairs (Figure. 1). Fruits of this plant are odorless and ripe fruits are sweet in taste. The fruits (figures) are small, axillary, paired, sessile, obovoid or globose, purplish when ripe. The fruit, is developed from an entire inflorescence, the fleshy part being hollow receptacle, the entire inflorescence axis, to the interior of which very numerous small flowers are attached. The fruits of these flowers are drupes, the stones of which are minute seeds present in figures. These stones are about 1.5 to 2.0 mm long each contains an endospermic seed with a curved embryo. At one point of the surface may be seen the orifice of the receptacle surrounded by small bracts and at another part the short remains of the stalk is usually present. When young, the receptacle contains laticiferous vessels filled with milky latex; as it ripens the latex disappears, the fleshy walls fills with sugar and becomes edible.

Micromorphology:

Transverse section of F. religiosa fruit showed that the internal structure was divided into four compartments viz. Epidermis, Hypodermis, Pericyclic fibres and Ground tissue (Figure. 2) Epidermis was single layered covered with thin cuticle followed by 4-5 layered hypodermis which consists of compactly arranged collenchymatous cell surrounded by 12-15 layered sclerenchymatous pericyclic fibres. The innermost layer was ground tissue which was made up of parenchymatous cells (Figure. 3) in which stone cells (Figure. 4) and spiral vessels (Figure. 5) were scattered. Furthermore transverse section of single drupe fruit showed the presence of innermost curved embryo surrounded by

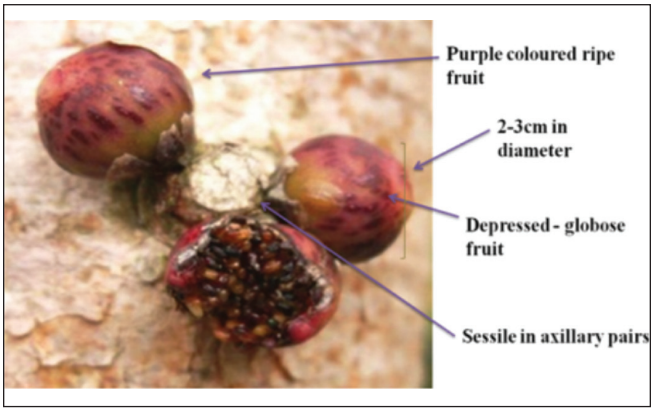

Figure 1: T.S. of whole inflorescence X 10X

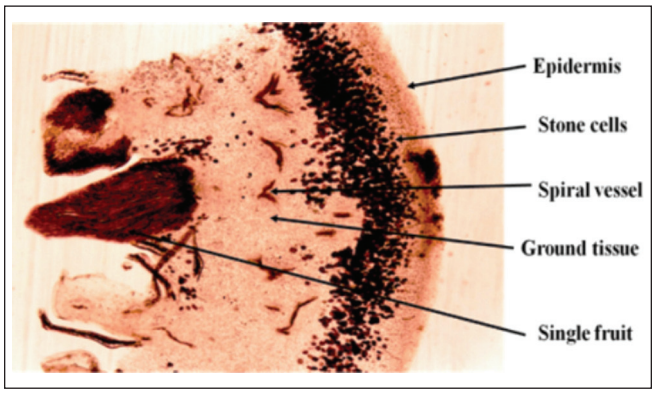

Figure 2: T.S. of single drupe fruit X 40X

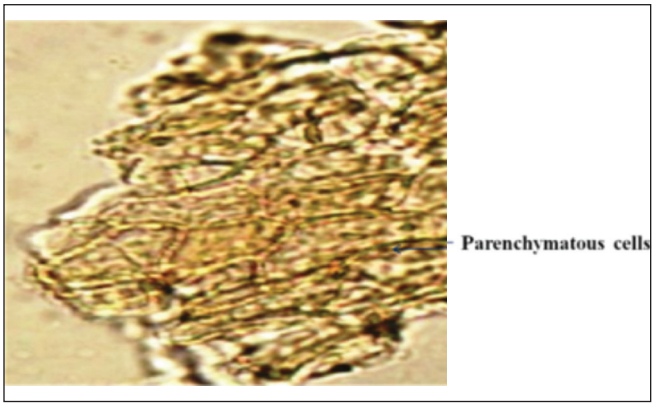

Figure 3: Pitted parenchymatous fibres X 100X

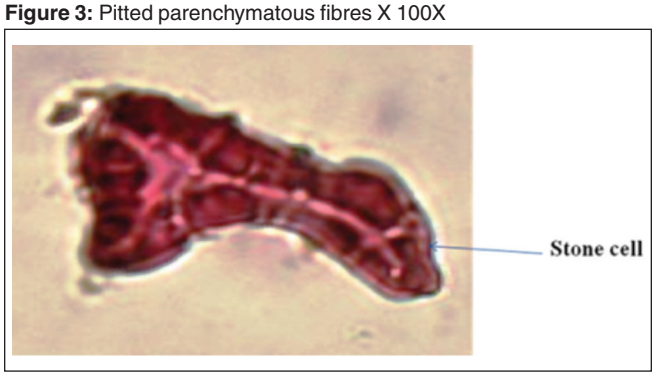

Figure 4: Stone cells X 40X

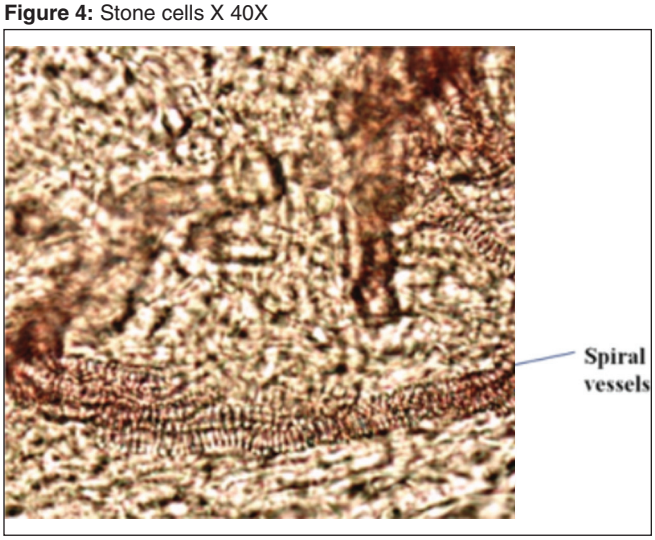

Figure 5: Spiral Vessels X 40X

thick walled, polygonal sclerenchymatous cells (Also revealed in the L.S. Figure. 6).

Physico-chemical studies

Ash values of a drug give an idea of the earthy matter or the inorganic composition and other impurities present along with the drug. The percentage of total ash, acid insoluble ash and water soluble ash were carried out (Table 1). The percentage extractive values were calculated and shown in (Table 2). Also the three extracts viz. petroleum ether, 90% methanolic and aqueous extracts were weighed and percentage yields were determined separately. The color, consistency and appearance of the extracts were reported in (Table 3).

Preliminary phytochemical screening

Preliminary phytochemical screening revealed the presence of steroids, carbohydrates, amino acids and phenolic compounds. Results showed the presence of carbohydrates in aqueous extract, steroids in pet ether (60-80°C), amino acids and phenolic compounds in both aqueous and 90%

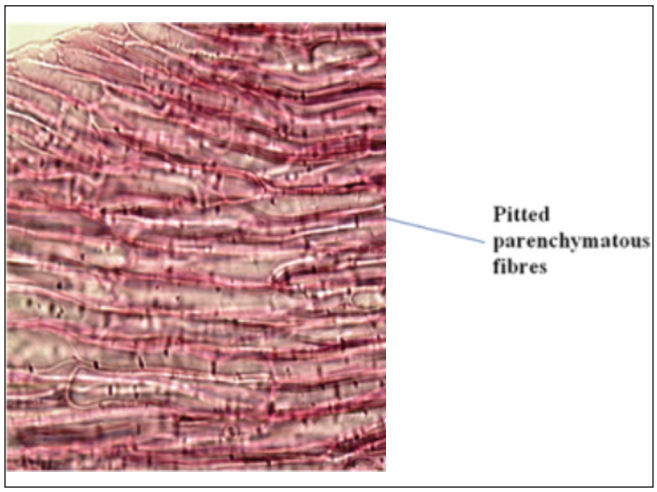

Figure 6: L.S. of single drupe fruit X 40X

Table 1: The percentage ash values of fruits of *F. religiosa*

Type	Yield % w/w
Total ash	6.74
Water soluble ash	5.40
Acid insoluble ash	1.85

Table 2: The percentage extractive values of fruits of *F. religiosa*

Type	Yield % w/w
Methanol(hot)	4.50
Methanol(cold)	3.85
Aqueous(hot)	6.50
Aqueous(cold)	5.50

Table 3: Color, consistency and percentage yield of extracts of fruits of *F. religiosa*

Type of extract	Method of extraction	Amount of extract (g)	%yield w/w	Appearance
Pet. Ether	Soxhlet extraction	5.40	1.08	Green-brown coloured waxy material
90% Methanol	Soxhlet extraction	26.50	5.40	Brown coloured semisolid mass
Aqueous	Cold maceration	13.40	2.70	Brown coloured powder

Table 4: Qualitative chemical tests of the extracts of *Ficus religiosa* fruits

Class of compound	Petroleum ether extract (60-80 oC)	90% methanol extract	Aqueous extract
Carbohydrates			
• Molish's test	–	–	+
• Fehling test	–	–	+
• Benedict test	–	–	+
Proteins			
• Biuret test	–	–	–
• Million's test	–	–	–
Steroids			
• Salkowiski test	+	–	–
• Liebermann-Burchard test	+	–	–
Phenolic compounds			
• 5% $FeCl_3$ solution	–	+	+
• Lead acetate solution	–	+	+
• $KMnO_4$	–	+	+
• $K_2Cr_2O_7$	–	+	+
• Gelatin solution	–	+	+
Flavonoids			
• Shinoda test	–	–	–
• Lead acetate test	–	–	–
Amino acids			
• Ninhydrin test	–	+	+
Alkaloids			
• Dragendorff 's test	–	–	–
• Mayer 's test	–	–	–
• Hager 's test	–	–	–
• Wagner 's test	–	–	–
Saponins			
• Foam test	–	–	–
Glycosides			
• Borntrager 's test	–	–	–
• Legal test	–	–	–

methanol extract (Table 4). The tested plant showed positive results for variable amounts of unsaturated sterols and polyphenols. In the methanolic extract amino acids and phenolic compounds are present in considerable amounts. Alkaloids, flavonoids, tannins, saponins, proteins and glycosides were not found in any of the tested extracts. The presence of these constituents was further confirmed by TLC studies on various extracts. TLC showed the presence of steroids mainly in petroleum ether extract, two prominent spots appears after derivatization with anisaldehyde-sulphuric acid reagent. Presence of polyphenols was confirmed in methanolic after spraying of KOH and anisaldehyde-sulphuric acid reagent; five spots appeared on the plate. Amino acids and polyphenols were also found in aqueous extract, while all other phytoconstituents were absent from all extracts.

Total phenolic content of *F. religiosa fruits*

The total phenolic content estimated in fruits of *F. religiosa* was 0.2% w/w of dry fruit powder. The calibration curve of standard gallic acid is shown in Figure. 7.

CONCLUSION

The Pharmacognostical study of *F. religiosa* fruit was done for the purpose of standardization. Standardization of natural products is a complex task due to their heterogenous

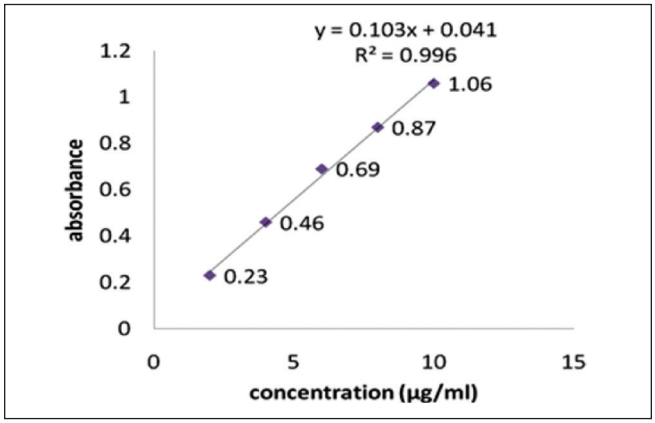

Figure 7: Calibration curve of Gallic acid

composition, which is in the form of whole plant, plant part/extracts obtained thereof. To ensure reproducible quality of herbal products, proper identification of starting material is essential. The parameters studied were macroscopy, microscopy, physico-chemical properties and phytochemical screening. The results of macroscopical study revealed that the fruit, known as syconus, is developed from an entire inflorescence, the fleshy part being hollow receptacle (the entire inflorescence axis) to the interior of which very numerous small flowers are attached. Fruit is green colored when unripe and purple upon ripening. Ripe fruit is sweet in taste and found odorless. The outer surface is smooth; shape is depressed and globose and 2-3cm in diameter. Thus our findings confirm the characters of fruits of moraceae family.[24]

Microscopical studies revealed the presence of single layered epidermis covered with thin cuticle followed by 4-5 layers of hypodermis, sclerenchymatous pericyclic fibers 12-15 layers and stone cells and spiral vessels are scattered in ground tissue. Area of ground tissue is 3/4th of total fruit and each single drupe is embedded in ground tissue.

The fruit was also characterized for it physico-chemical properties. Water soluble and alcohol soluble extractive values increased gradually with ripening of fruit.

The results of phytochemical screening of petroleum ether (60-80°C), methanolic and aqueous extracts have shown the presence of carbohydrates, proteins, amino acids, phenolic compounds and steroids by positive reaction with the respective test reagent.

The result of Folin Ciocalteu method revealed the presence of total phenolic compound content 0.2% w/w which was not reported earlier. These are the antioxidant compounds which act as free radical terminators.[25] This plant can also play a role in plant defensive mechanism by counteracting reactive oxygen species (ROS), thus minimizing molecular damage due to microorganisms, insects and herbivores.[26]

The fruits of *F. religiosa* were selected for the pharmacognostical standardization on the basis of literature review, as no such study has been reported earlier.

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Pharmacognostical evaluation on roots of *Cissus repanda* vahl. a folk medicine

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ABSTRACT

Cissus repanda Vahl. (Syn. C.rosea, Vitis repanda and V. rosea). Family Vitaceae, commonly known as 'Panivel' in Hindi, is a folklore medicinal herb, reputed for the healing properties of its roots and stem. The trible people and Traditional practitioners of Orissa, Gujarat, Andhra Pradesh and some parts of Karnataka Dist. are found to be prescribing the root powder of this plant in case of bone fractures, cuts, boils and wounds. As yet the roots of plant has not found to be reported and hence the Roots of this plant was investigated thoroughly as per the pharmacopoeial parameters. Physicochemical parameters shows high value of acid insoluble ash indicating high polarity, preliminary phytochemical investigations shows the presence of alkaloid, tannin, mucilage and calcium salts. The microscopic characters of root shows Mucilage, rosette and acicular crystals of calcium oxalate, starch grains, tannin Stealar region shows fibres, scalariform vessels and pitted parenchyma and multiseriate medullary rays.

Key words: *Cissus repanda, Vitis rosea,* Vitaceae, Evaluation, Physicochemical analysis.

INTRODUCTION

Cissus repanda Vahl. (Vitaceae) commonly known as 'Panivel' in Hindi, is an important medicinal plant distributed from Kumaun to Arunachal Pradesh, Tripura, Assam, Bihar, Orissa, Madhya Pradesh, and Western Ghats region up to 1350 m.[1]

It is a large climber, with soft, very porous wood with corky bark. The stem yields potable water on cutting thus the name "Panivel" (Pani-Water Vel-creeper). Leaves: simple, broadly ovate, 12-20 cm in diameter, repeatedly toothed, and tomatoes beneath less above, base deeply cordate, petiole 15-20 cm long, stipules oblong and tendrils dichotomous opposite to leaf. Inflorescence lax, umbellate branched. Flower: bracteate, bracteolate, actinomorphic, bisexual, tetramerous, hypogynous flowers reddish brown in colour. Calyx: sepals 4, fused and valvate. Corolla: petals 4, free and valvate. Androecium: stamens 4, opposite to the petals arise from the base of the disc, basified. Gynaecium: ovary 2 celled, with many ovules in each cell, ovary superior, style subulate, and stigma small. Fruit one seeded fleshy berry.[2] (Plate 1-1.1, 1.2)

The medicinal potential of *C.repanda* has been known to traditional system and widely used in folklore medicine. *C.repanda* is a well known plant and its roots and powder has been traditionally used in the form paste for cuts, wounds and bone fractures.[3,4] In spite of its reputation in these ailments it has not yet been investigated scientifically and hence it was thought worth to study it in detail. The present paper highlights macroscopic, microscopic, physicochemical and Thin Layer Chromatographic analysis of methanolic extract of roots.

MATERIALS AND METHODS

Collection

Fresh plants of *C.repanda* Vahl. were uprooted from the natural habitat from Orissa, Karnataka and Deharadun. The collected samples were identified, authenticated by using various floras and texts. The verified specimen was preserved in the departmental herbarium museum vide no. 6001/2009 for future reference.

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The matured roots were separated from aerial parts, cut in to small pieces and shade dried, coarsely powdered (40 mesh) drug was used for Phytochemical and for study of the diagnostic characters of the powder. The rest of the sample was preserved in the solution of F.A.A. (70% Ethyl alcohol: Glacial acetic acid: Formalin in the ratio of 90:5:5) for the histological profile.

PHARMACOGNOSTIC EVALUATION[5,6,7]

Organoleptic evaluation

The colour, odour, and taste of the root and the powder were recorded separately.

Microscopic evaluation

Free hand sections were taken, cleared with chloral hydrate and then with phloroglucinol and hydrochloric acid. Histochemical tests for few constituents like tannin, mucilage etc. were also carried out. Sections and powder diagnostic characters were drawn with camera lucida and also took microphotographs by using Carl Zeiss binocular microscope.

Physical evaluation[6,7,8]

In physical evaluation, moisture content, ash values viz., total ash, acid insoluble ash, and extractive values viz., alcohol soluble extractive value, water soluble extractive values were determined. The ash value represents the inorganic salts present in the drug. Extracts obtained by exhausting crude drugs are indicative of approximate measures of certain chemical compounds they contain, the diversity in chemical nature and properties of contents of drug. The determinations were performed in triplicate and results are expressed as mean ± SD. The percentage w/w values were calculated with reference to the air-dried drug.

Preliminary Phytochemical Screening[9]

Ten gram of dried root powder was subjected to continuous soxhlet extraction with petroleum ether (60-80°C), chloroform, ethyl acetate, methanol and water for 8 hrs and the extract was evaporated to dryness. The dried extract was weighed, and percentage yields were calculated. The extract was further subjected for the presence of various constituents like alkaloids, tannins, phenolics and for saponin glycosides.

RESULTS AND DISCUSSION

The root is long, tuberous with smooth surface elongated 15 to 20 cm in diameter, fracter fibrous, colour externally dark brown and internally yellowish orange, odour slightly aromatic, taste at the beginning mucilaginous and later on causing itching sensation in the throat. The thick transverse section of root is somewhat spherical in outline, shows outermost narrow cork, cortex and central stellar region. (Plate.1-1.3, 1.4)

Detailed transverse section tangentially running 20 to 25 rows of suberised cork cells, phellogen is narrow one or two rows followed by 2 to 3 rows of parenchymatous cells of phelloderm, cortical parenchymatous zone laying under this being 4 to 5 layers embedded with tannin, starch grains. A few cortical cells contain rosette and acicular crystals of calcium oxalate, number of Mucilage cells also present in cortex. The central xylem is very wide composed of radially arranged groups of 2 to 5 vessels of pitted and scaleriform, few thick walled fibres and parenchyma, alternating with the wide multiseriate medullary rays embedded with starch and occasional acicular crystals of calcium oxalate. Phloem encircling the xylem is narrow and wedge shaped consisting of parenchyma, sieve elements with medullary rays and are getting wider to the perifery from the centre and reach up to the inner border of the cortex with few non lignified fibres. The ray cells consist starch grains some acicular crystals of calcium oxalate. (Plate. 2 - 2.1, 2.2, 2.3, 2.4)

Powder microscopy

The powder of *C.repanda* root is light brown in colour, and slightly aromatic in odour, sharp mucilaginous in taste and producing itching in throat.

The diagnostic microscopical characters of the powder are cork in surface and transversly cut view, rosette and

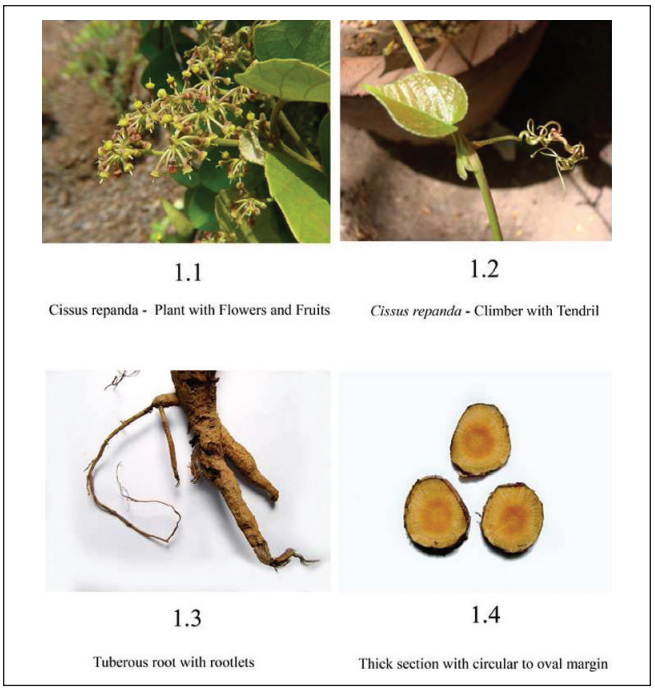


Plate 1: Cissus repanda Vahl.

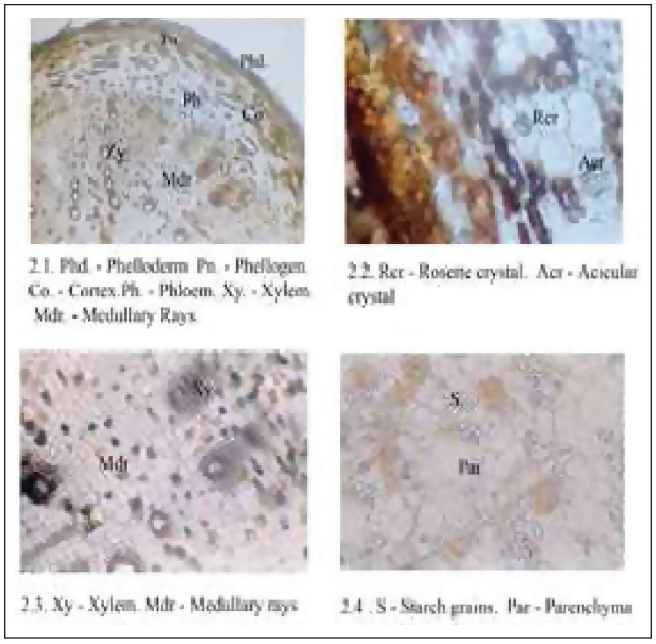
acicular crystals of calcium oxalate scattered as such throughout or embeded in the parenchyma cells of cortex, radially cut medullary rays, simple oval to pear shaped starch grains from cortex and medullary rays, tannin content cells of cortical region, simple fibres of phloem, scalariform and pitted vessels of stealar region. (Plate. 3-A to H)

Preliminary Phytochemical Evaluation

The various physical parameters of root and root powder viz., moisture content, ash values viz., total ash, acid insoluble ash, water soluble ash, and extractive values viz., alcohol soluble extractive value, water soluble extractive values were determined. The results of this study were shown in table. (Table-1)

The methanol extracts of the powdered root of *C.repanda* showed the presence of alkaloids, glycosides. Aqueous Showed that presence of alkaloids, saponin, tannin and phenolics, calcium, mucilage. These secondary plant metabolites are known to possess various pharmacological effects might be responsible for the various actions exerted by *C.repanda*. (Table-2)

The Thin Layer Chromatography(10) revealed that methanol extraction the R_f values under U.V. radiation in short U.V. 254 nm components having double bond (unstauration) presents 8 different components are separated using silica gel C_f 254 nm as stationary phase and mobile phase. Out of separated compounds, 4 are suspective to long U.V. 366 nm. Hence short U.V. range is suitable to detect more separated compounds. T.L.C. plate observed after spray the reagent Dragendorff's (mainly used to detect alkaloid) shows one spot at R_f 0.45. (Table-3)



2.1. Phd. - Phellodenn Pn. - Phellogen Co. - Cortex Ph. - Phloem. Xy. - Xylem Mdr. - Medullary Rays

2.2. Rcr - Rosette crystal. Acr - Acicular crystal

2.3. Xy - Xylem. Mdr - Medullary rays

2.4. S - Starch grains. Par - Parenchyma

Plate 2: T. S. Of Root

Table 1: physicochemical parameters root powder of *C.repanda*

Parameters	Value % w/w
Moisture content	10.85
Total ash	18.57
Acid insoluble ash	34.23
Alcohol soluble extractive	05.20
Water soluble extractive	07.01
pH	05.95

Table 2: Qualitative chemical screening root powder of *C.repanda*

Phytoconstituents	Tests	Results
Alkaloids	Mayer's Test	+ +
	Dragendorff's Test	+ +
	Wagner's Test	+ +
Saponins	Foam Test	+ +
	Froth Test	+ +
Tannins	Lead Acetate Test	+ +
	Gelatin Test	+ +
Calcium	CalciumTest	+ +

++ = Present.

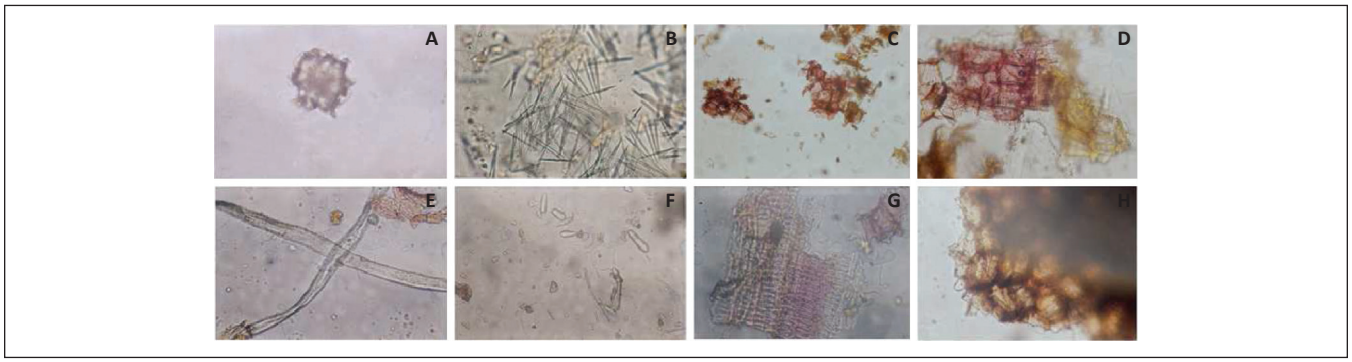


Plate 3: Powder Microscopy

Plate 3: A- Rosette Crystal. B- Acicular Crystals. C- Cork In Surface View. D- Lignified Parenchyma. E- Simple Fibres. F- Starch Grains. G- Scalariform & pitted Vessels. H- Tannin containing cells cells

Table 3: chromatography result of *C.repanda* root powder methanolic extract.

R_f 254 nm	R_f 366 nm	After spraying Dragendorff's Reagent
0.05, 0.45, 0.50, 0.56 0.69, 0.81, 0.92, 0.96.	0.05, 0.45, 0.92, 0.96	0.45

C.repanda root and its powder paste were used in the treatment of bone fractures and cuts and wounds conditions. The standardization of a crud drug is an integral part of establishing its correct identity. Before any crude drug can be included in herbal pharmacopoeia, pharmacognostic parameters and standards must be established. The results of the present investigations could serve as a basis for proper identification, collection and investigation of the plant. The macro and micro-morphological features of root described, distinguishes it from other members of the genera. The transverse section and its powder microscopy results are unique to the plant and are required in its standardization. The phytochemical evaluation revealed the presence of various secondary plant metabolites which have been claimed to be responsible for various pharmacological activities.

CONCLUSION

The Diagnostic morphological and microscopical characters were noted down for easy identification of plant material. Physico-chemical parameters have been established to identify quality and degree of purity of the plant material as per pharmacopoeial requirements. Qualitative tests indicated the presence of alkaloid, saponin, calcium, mucilage; phenolic compounds and TLC studies confirmed the same. The results are being reporting for the first time, could be useful in the identification and standardization of a crude drug the data produced in the present investigation is also helpful in the preparation of the crude drug's monograph and inclusion in various pharmacopoeias.

ACKNOWLEDGMENTS

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High Performance Thin Layer Chromatographic Method for Quantitative Determination of Quercetin in Tender Leaves of *Psidium guajava*

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ABSTRACT

A sensitive and reliable densitometric High Performance Thin Layer Chromatography method has been developed for the quantification of quercetin, a flavonoid present in leaves of *Psidium guajava*. Chromatographic analysis was performed using acetone extract of tender leaves of *Psidium guajava* on silica gel 60 F 254 TLC plates using the solvent system, toluene: acetone: formic acid (38:10:5). Detection and quantification of quercetin was done by densitometric scanning at 364 nm. The results of linearity range and correlation coefficient (0.98470) show that, within the concentration range indicated, there was a good correlation between peak area and corresponding concentration of quercetin. The proposed HPTLC method provided a good resolution of quercetin from other constituents present in acetone extract of tender leaves of *Psidium guajava* and can be used for the quantification of quercetin.

Key words: HPTLC, Psidium guajava, Quercetin, Flavonoid

INTRODUCTION

High performance thin layer chromatography, also known as planar chromatography, is a modern powerful analytical technique with separation power, performance and reproducibility superior to classic thin layer chromatography. HPTLC is very useful for qualitative and quantitative analysis of pharmaceuticals. The resolution of compounds to be separated on the chromatoplate is followed by measuring the optical density of the separated spots directly on the plate. The sample amounts are determined by comparing them to a standard curve from reference material chromatographed simultaneously under the same conditions. The original data evaluation using the conventional methods of scanning was done by measuring the optical density of the transmitted light as a function of the concentration of the sample or standard delivered on the silica gel. With the evaluation of optical density as a function of concentration, the detection device termed as densitometer.[1]

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Psidium guajava is a small tree upto 8 m in height with smooth, pale pinkish brown bark, having grey patches exfoliating in very thin woody flakes. The leaves are simple opposite light green oblong or elliptic oblong, glabrous above, pubescent beneath, pellucid-punctate, lateral nerves 10-20 pairs joined by intramarginal veins. Flowers are white, fragrant in axillary 1-3 flowered cymes. Fruits are globose or pyriform berries often varying in size and shape, pulp yellowish white or red.[2]

Almost every plant contains a characteristic pattern of flavone and flavonol glycosides in leaf or flower and thus these substances are ideal taxonomic markers for use in studying problems of plant classification, hybridization or phytogeography. The *Psidium guajava* is a rich source of phenolic compounds, including flavonoids. The leaves contain three flavonoids; quercetin, its 3-L-4-4-arabinofuranoside (avicularin) and 3-L-4-pyranoside with strong antioxidant activity.[3]

TLC methods for detection of flavonoids have been reported in literature. However, HPTLC method for quantification of quercetin from *Psidium guajava*, has not been reported in literature.[4] Densitometric HPTLC, a widely used method for the phytochemical evaluation of herbal drugs, has been developed in the present

work for the quantification of quercetin from acetone extract of *Psidium guajava*.

MATERIALS AND METHOD

Plant material

The tender leaves of guava had been collected from palai, kottayam district. The plant was identified and authenticated by the botanist Mr. Joby Paul, School of Environmental sciences, M.G University as *Psidium guajava* Linn and the voucher specimen deposited in Dept.of Pharmacognosy and has been given the code 004 dated 28-11-07.

Reagents and standard

Toluene, acetone and formic acid used were of analytical grade. Standard quercetin was procured from Sisco research and laboratories chemicals private limited, Bombay.

Preparation of sample solution

Psidium guajava tender leaves were extracted in soxhlet assembly successively with solvents of increase in polarity such as petroleum ether, benzene, chloroform, ethyl acetate, acetone and alcohol. Each time before extracting with the next solvent, the drug material was dried in hot-air oven below 50°C. Each extract was concentrated by distilling off the solvent and then evaporating to dryness.[5]

On CO-TLC with quercetin standard, the acetone extract of *P.guajava* gave similar spots. So acetone extract was selected for development of HPTLC method. About 10 mg of the acetone extract of the guava was taken and dissolved in methanol and the volume was made up to 10 ml in a standard flask (1000 µg/ml).

Preparation of standard

10 mg of the quercetin standard was taken and dissolved in methanol. This was transferred to a standard flask and the volume was made up to 100 ml to prepare 100 µg/ml Solution.

HPTLC method for the estimation of quercetin

Preparation of calibration curve of quercetin:

From the standard stock solution (100 µg/ml), 200-1000 µg/spot was prepared and checked for linearity.

Method specification

Silica gel 60 F 254 precoated plates (10×10cm) were used with toluene: acetone: formic acid (38:10:5) as solvent system. From 1000 µg/ml sample solution, 10 and 20 µl of the samples were applied as different tracks on precoated TLC plates by using Linomat 5 spotter. Ascending mode was used for the development and was developed up to 8 cm. The developed plates were then dried and scanned using TLC scanner 3 with Wincats software under 364 nm.[6]

RESULTS

Standard quercetin showed single peak in HPTLC chromatogram. The calibration curve of quercetin (figure: 1) was prepared by plotting the concentration of quercetin versus average area of the peak over the range 200-1000µg/spot (Table: 1). The correlation coefficient was found to be 0.98470.

Amount of quercetin in the sample (acetone extract of *P. guajava*) was computed from calibration curve (figure: 2 and figure: 3). Amount of Quercetin in the extract was 52.29ng/µg of extract.

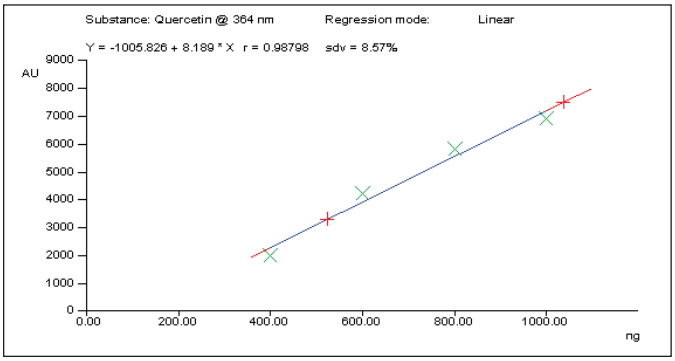

Figure 1: Calibration curve of standard Quercetin

Table 1: HPTLC profile

Sl NO:	Amount (ng)	R_f	Area (ng)
	Standard (Quercetin)		
1.	400	0.45	1979.55
2.	600	0.46	4205.63
3.	800	0.46	5820.00
4.	1000	0.46	6900.77
	Sample (Acetone extract of *P.guajava* tender leaves)		
1.	20,000	0.45	522.96
2.	40,000	0.44	1037.00

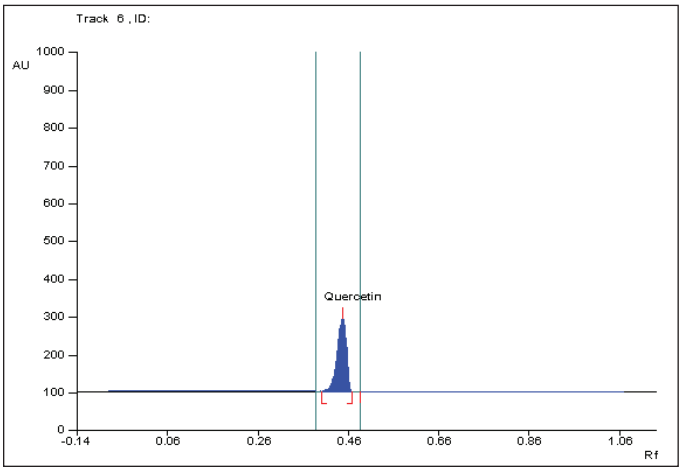

Figure 2: Chromatogram of acetone extract of *Psidium guajava* tender leaves sample

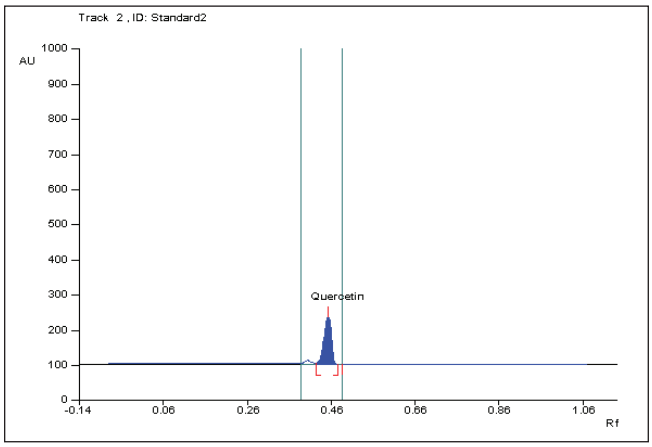

Figure 3: Chromatogram of standard Quercetin

CONCLUSION

The proposed HPTLC method was found to be rapid, simple and accurate for quantitative estimation of quercetin in *P.guajava* tender leaves extract. The results of linearity range and correlation coefficient show that, within the concentration range indicated, there was a good correlation between peak area and corresponding concentration of quercetin.

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HPTLC Finger Print Profile of Extracts from Dried Aerial Parts of *Bryophyllum Pinnatum* in Different Solvents

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ABSTRACT

Introduction: *Bryophyllum pinnatum* Kurz. is perennial herb growing widely and used in folkoric medicine in tropical Africa, America, India, china and Australia. The divine herb has potent medicinal values and used in traditional medicine for the treatment of variety of ailments and well known for its haemostatic and wound healing properties. **Methods:** Chromatographic techniques were used for separation of components from different extracts of plant parts. This study was planned to develop a HPTLC fingerprint profile of drug extracts from aerial parts of *Bryophyllum pinnatum* in different solvents such as petroleum ether, benzene, chloroform, acetone and methanol. **Results:** A High Performance Thin Layer Chromatography (HPTLC) method for the separation of the active constituents in *Bryophyllum pinnatum* extracts has been developed and TLC of these extracts on silica gel precoated aluminum plates of Merck by automatic TLC applicator and using solvent system Chloroform: Ethanol (9.8:0.2) was performed. In the present study, HPTLC finger print of various extracts of dried aerial parts of *Bryophyllum pinnatum* have been carried out and the results provide referential information for standardization. **Conclusion:** The HPTLC method for routine quality control of present species can be carried out using this method for different extracts of plant parts and serve in qualitative, quantitative and was appropriate for standardization of the drug. The HPTLC fingerprint is also suitable for rapid and simple authentication and comparision of subtle differences among samples of identical plant resource.

Key words: Authentication, bioactive molecules, HPTLC analysis, standardization.

INTRODUCTION

Bryophyllum pinnatum Kurz (syn. *B. calycium* and *Kalanchoe pinnata*) commonly known as parnbija, Zakhm-e-hyat (Hindi), life plant, love plant, air plant (Mexican), Good luck or resurrection plant.[1] It is a glabrous, ornamental, crassulescent herb, cultivated in houses and gardens. It is a perennial medicinal herb popularly used as folkloric medicine in tropical Africa, India, China, Australia and tropical America and other parts of the world to treat various inflammatory diseases. The leaves of the plant have great medicinal value and possess various properties like haemostatic, refrigerant, emollient, mucilaginous, vulnerary, depurative, anti-inflammatory, disinfectant and tonic. It is also employed for kidney stones, gastric ulcers, skin disorders and edema of the legs. It contains triterpenoids, glycosides, flavonoids, steroids, bufadienolides, lipids and organic acids.[2-4]

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It is a succulent perennial plant that grows 1-1.5 m in height and the stem is hollow four-angled and usually branched. Leaves are opposite, decussate, succulent, 10-20 cm long, distributed all over India. In traditional medicine, the leaves of this plant have been used for antimicrobial, antifungal, antiulcer, anti-inflammatory, analgesic, antihypertensive, potent anti-histamine and anti-allergic activity.[5, 6] In the recent year advancement in of chromatographic and spectral fingerprints plays an important role in the quality control of complex herbal medicines.[7] Chemical finger prints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the chemical integrities of the herbal medicines and its products and therefore be used for authentication and identification of herbal plant.[8] HPTLC is more efficient, faster method and the results are more reliable and reproducible. In combination with digital scanning profiling, HPTLC also provides accurate and precise R_f values and quantitative analysis of sample by *in situ* scanning densitometry aided by formation of easily detected derivatives by post-chromatographic chemical reactions as required, as

well as a record of the separation in the form of a chromatogram with fractions represented as peaks with defined parameters including absorbance (intensity), Rf, height and area.[9] Furthermore, the feature of a pictorial fluorescence image of HPTLC coupled with a digital scanning profile is more and more attractive to herbal analysts for constructing an herbal chromatographic fingerprint by means of HPTLC. The main objective of this study was to evaluate, develop and to optimize the HPTLC fingerprint method in standardization of *Bryophyllum pinnatum* to provide beneficial information in regarding the separation, identification and standardization of drug according to WHO guidelines. These HPTLC fluorescence images coupled with scanning profiles provided adequate information and parameters for comprehensive identification, assessment and comparision of major active constituent fingerprints in the samples studied to serve as a basis for their use in medicinal preparations.[9-11]

MATERIAL AND METHODS

Collection and Identification

The plant of *Bryophyllum pinnatum* Kurz were collected from Tau Devilal Herbal garden, Churpur and positively identified. The specimen was submitted to the A. R College of pharmacy, Vallabh Vidya Nagar, Anand. The collected plant material was made thoroughly free from any foreign organic matter. The aerial parts of the plant were separated, cut into small pieces, shade dried and powdered with mixer and sieved.

Extraction of Plant Material

The powder was extracted with different solvents ranging from non-polar to polar solvents. About 10 g of the crude drug powder was subjected for extraction (Soxhlet extraction) in round bottom flask, first with petroleum ether (60-80°C) for 2-3 hours. The extract was concentrated under reduced pressure at 50-60°C. The dried marc of *Bryophyllum pinnatum was* once again subjected to successive extraction with different solvents viz. benzene, Chloroform, acetone, methanol.

Extracts were concentrated under vacuum and finally made up to 10 ml with HPLC grade methanol and ready for HPTLC analysis.

Chromatography

A highly sensitive and accurate HPTLC method was developed and used for *Bryophyllum pinnatum* extracts. 5 µl aliquots of each of the extracts were separately applied on aluminium plates precoated with Silica gel 60 F_{254} HPTLC plates, 10 × 10 cm (Merck, Darmstadt, Germany) with the help of Camag Linomat-V applicator and eluted the plate to a distance of 7 cm at room temperature (25°) in solvent system Chloroform: Ethanol (9.8:0.2). Sample solution was applied on 6 mm wide band using Camag Linomat-V automated TLC applicator with the nitrogen flow providing a delivery speed of 150 nL/sec from syringe.

Development, Detection and Quantitation: After sample application, plates were developed in a Camag twin through glass tank pre-saturated with the mobile phase Chloroform: Ethanol (9.8: 0.2) for 20 min., the plate was developed horizontally in Camag horizontal developing chamber (10 × 10 cm) at the room temperature. After heating the plate at 100°C for 5 min., derivatization of the chromatogram was performed by Camag glass reagent spray by spraying still hot plate with 5% methanolic- sulphuric acid system. The plate was observed after 30 min. under UV-366 nm light in Camag UV cabinet and the HPTLC fluorescence image documented. The corresponding digital scanning profiling was carried out with a Camag TLC scanner III fitted with winCATS- V1.2.3 software at a single wavelength 490 nm. Documentation of chromatograms was carried out with digital camera. The components get separated by the principle of adsorption, having differential migration rates of individual component towards the phases.

RESULTS AND DISCUSSION

The various extracts of *Bryophyllum pinnatum* were subjected to HPTLC analysis by specific solvent system Chloroform: Ethanol (9.8:0.2) and detected under UV at 366 nm and 490 nm. The HPTLC images shown in Figure 1, 2 and 3 indicate that all sample constituents were clearly separated without any tailing and diffuseness. The R_f value of the corresponding component as obtained through the software system attached with the instrument. Area corresponds to each peak for the corresponding spot or component determines the concentration of the component in the solution. It is evident from Table 1 that in the Petroleum Ether extract of aerial parts of *Bryophyllum pinnatum* there are 10 spots at the following R_f 0.10, 0.18, 0.22, 0.26, 0.30, 0.38, 0.50, 0.58, 0.65, 0.76 as shown in Figure 4, indicating the occurrence of atleast 10 different components in Petroleum Ether extract. It is also clear from Table 1 and the chromatogram as shown Figure 4 that out of 10 components, the component with R_f values 0.38 (light blue, violet), 0.22 (light blue, violet), 0.76 (pinkish blue, purple), 0.18 (reddish brown, reddish brown) and 0.26 (reddish brown, light purple) at 366 nm and visible 490 nm were found to be more predominant as the percentage area is more with 30.31%, 14.73%, 12.94%, 10.63% and 8.92% respectively. And remaining components were found to be very less in quantity as the percentage area for all the spots was less than 7.5%.

It is evident from Table 2 that in the Benzene extract of aerial parts of *Bryophyllum pinnatum*, there are 7 spots at the following

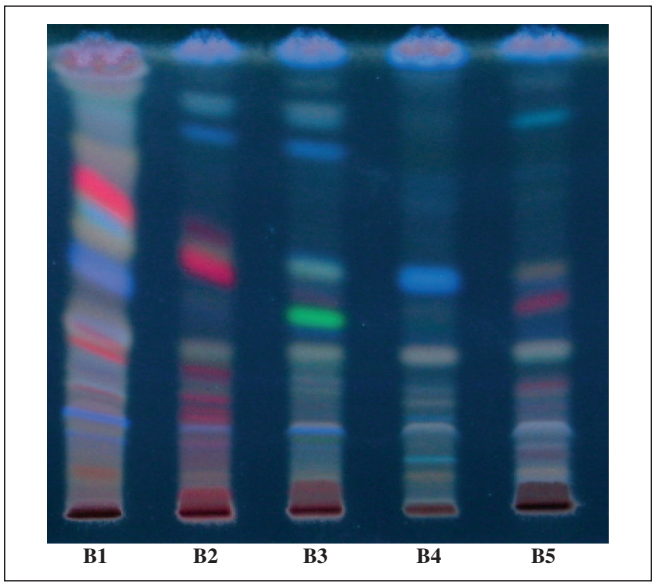

Figure 1: HPTLC fluorescence image after derivatization observed at 366 nm

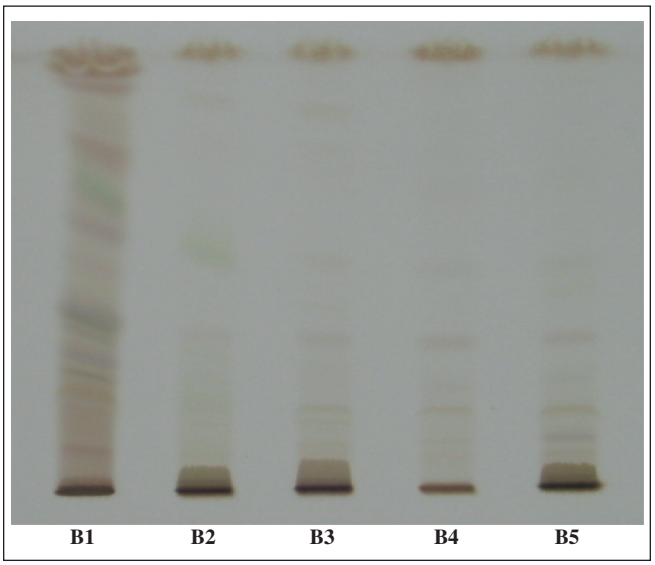

Figure 2: HPTLC plate at visible after derivatization with 5% Sulphuric MeOH solution and observed at visible range

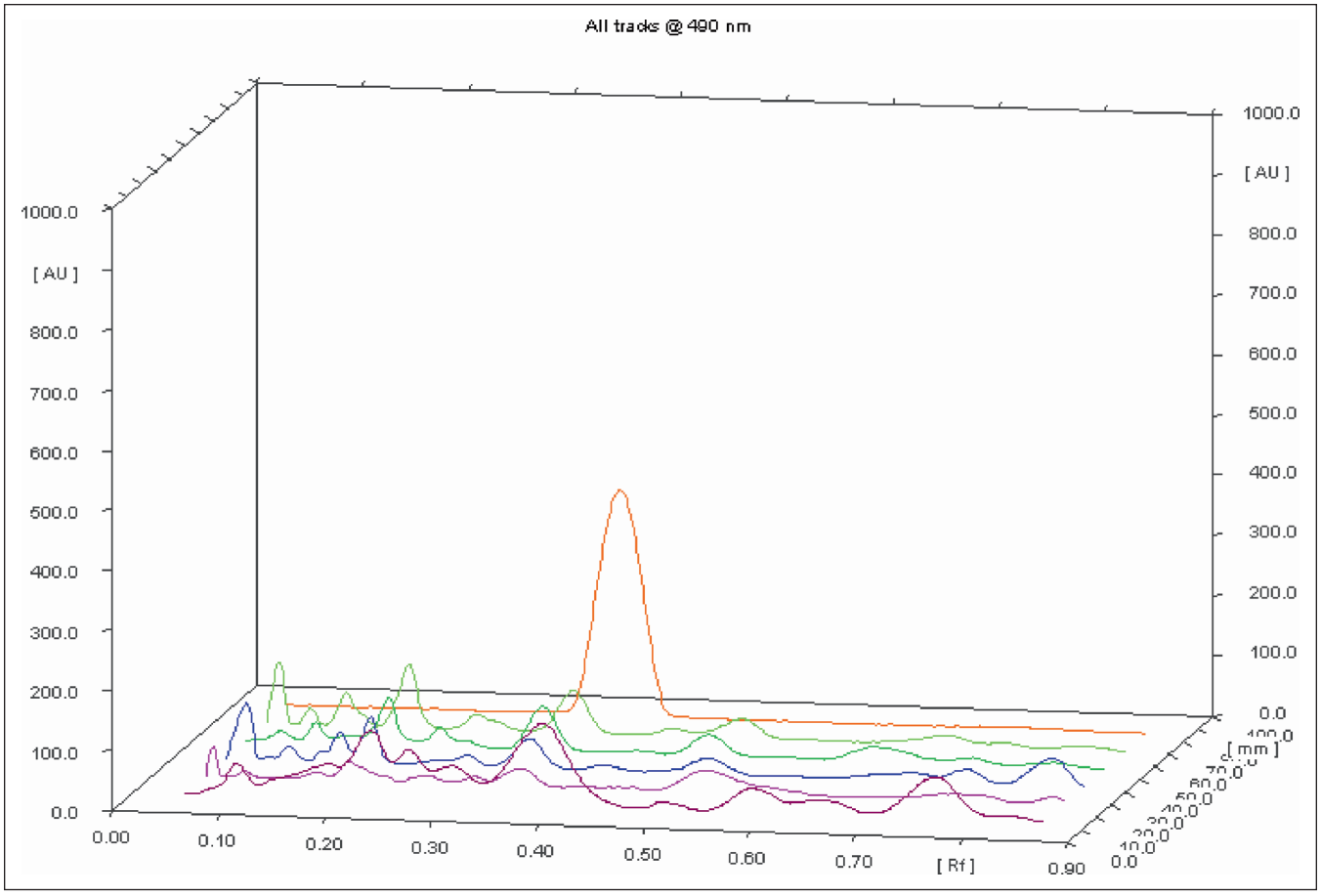

Figure 3: Densitometric Chromatogram of Various Fractions of Dried Aerial parts of *Bryophyllum pinnatum* at 490 nm (3D View).

R_f 0.15, 0.18, 0.26, 0.34, 0.52, 0.76, 0.85 as shown in figure 5, indicating the occurrence of atleast 7 different components in Benzene extract. It is also clear from **table 2** and the chromatogram as shown **figure 5** that out of 7 components, the component with R_f values 0.52 (reddish brown, violet), 0.34 (light blue, violet), 0.18 (light blue, light green), 0.76 (light blue, purple) at 366 nm and visible 490 nm were found to be more predominant as the percentage area is more with

Table 1: Peak list and Rf value of the Chromatogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Petroleum Ether Fraction (B1) after Spraying with 5% Sulphuric acid – Methanol Solution at 490 nm

Track	Peak	Max Rf	Max Height	Height %	Area	Area %
1	1	0.10	52.2	8.08	1000.0	4.81
1	2	0.18	56.5	8.74	2212.0	10.63
1	3	0.22	113.8	17.63	3065.2	14.73
1	4	0.26	84.4	13.08	1855.6	8.92
1	5	0.30	60.2	9.33	1619.0	7.78
1	6	0.38	135.4	20.98	6305.1	30.31
1	7	0.50	12.2	1.89	256.6	1.23
1	8	0.58	39.4	6.10	1323.6	6.36
1	9	0.65	23.4	3.62	474.4	2.28
1	10	0.76	68.0	10.54	2692.2	12.94

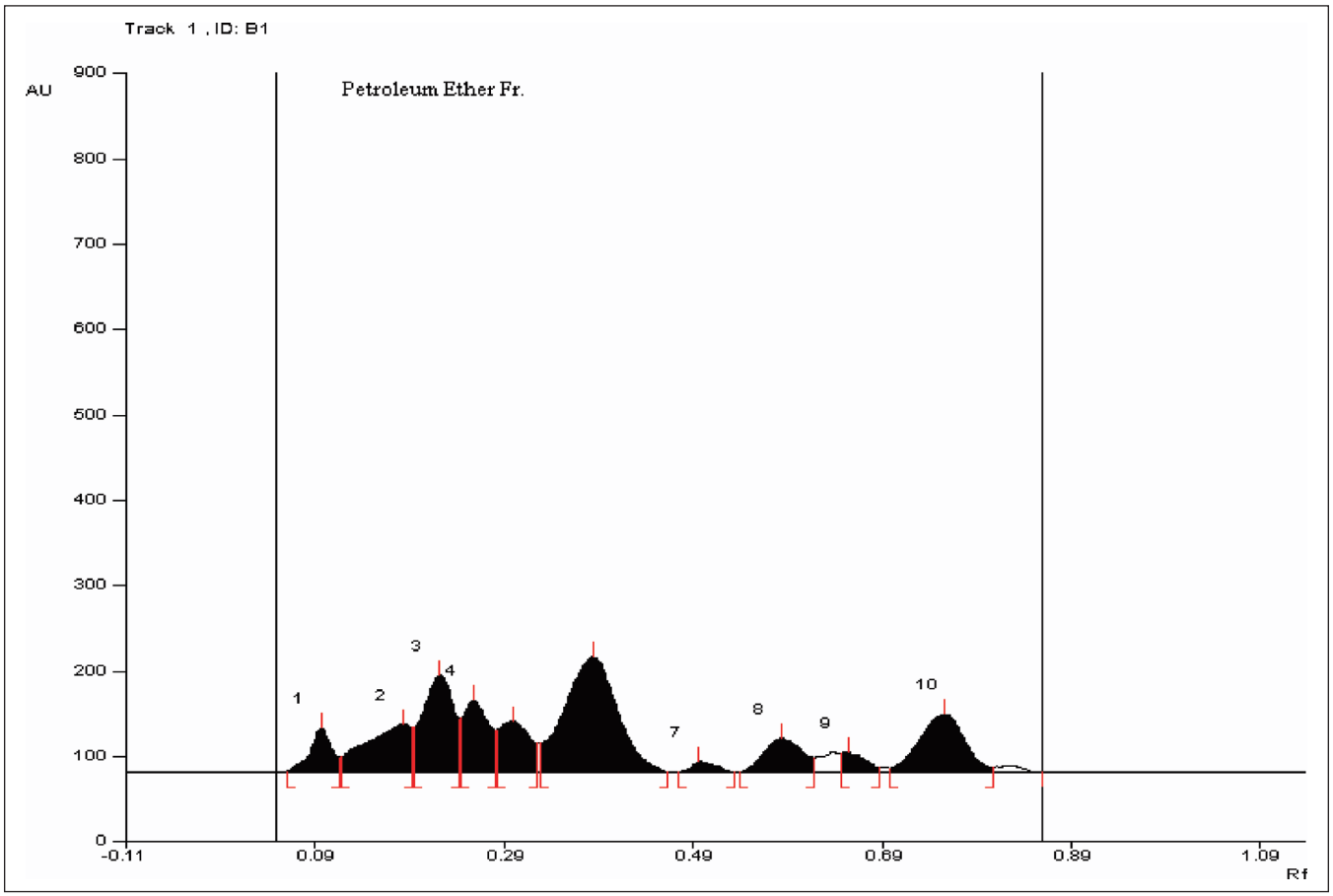

Figure 4: Typical HPTLC Densitogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Petroleum Ether Fraction (B1) after spraying with 5% Sulphuric acid – Methanol Solution at 490 nm.

Table 2: Peak list and Rf value of the Chromatogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Benzene Fraction (B2) after Spraying with 5% Sulphuric acid – Methanol Solution at 490 nm

Track	Peak	Max Rf	Max Height	Height %	Area	Area %
2	1	0.15	11.3	7.87	171.4	4.09
2	2	0.18	32.2	22.45	651.2	15.53
2	3	0.26	12.5	8.72	243.3	5.80
2	4	0.34	29.4	20.49	710.6	16.95
2	5	0.52	35.5	24.71	1729.4	41.25
2	6	0.76	10.6	7.37	521.0	12.43
2	7	0.85	12.0	8.38	165.6	3.95

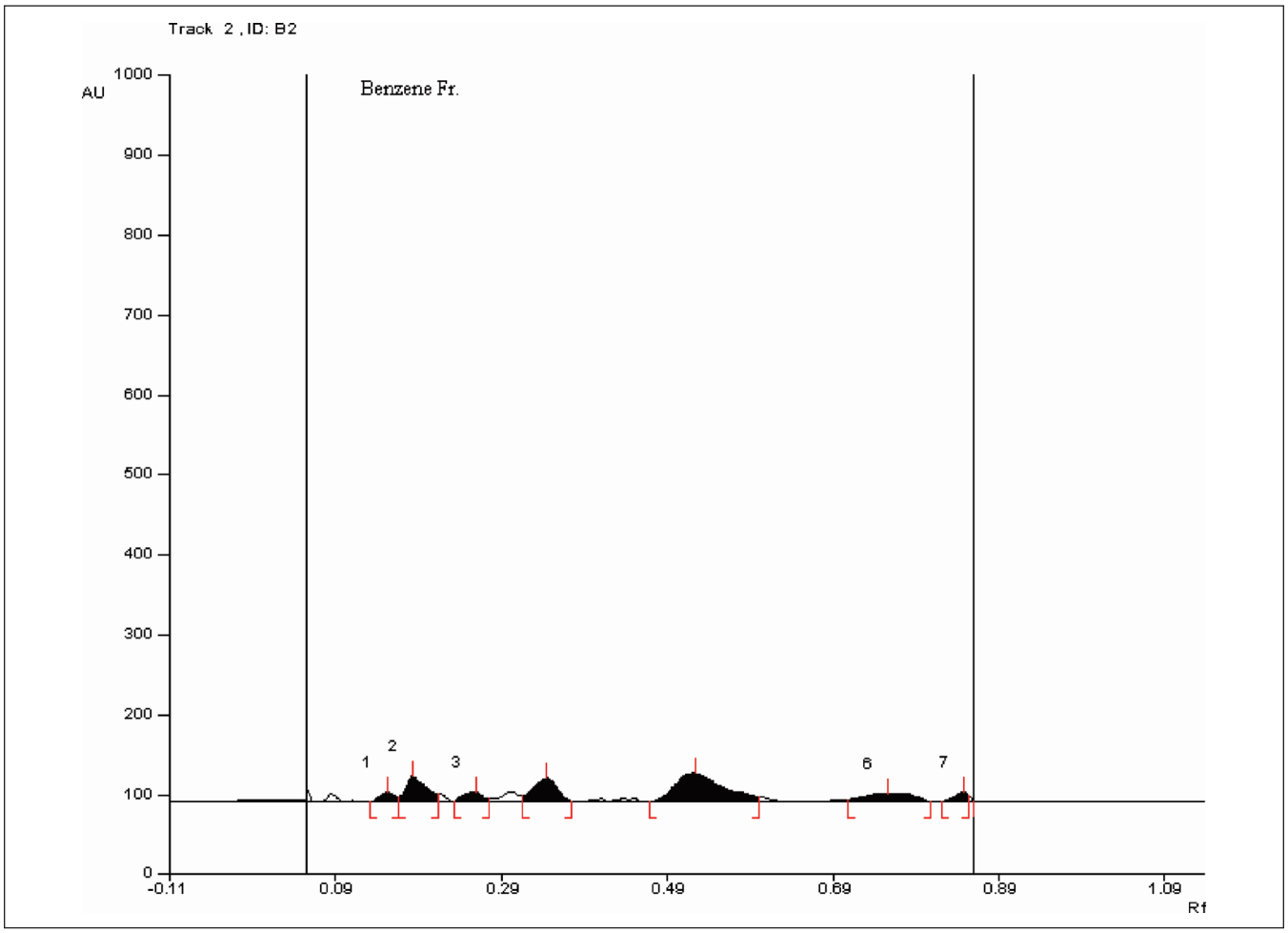

Figure 5: Typical HPTLC Densitogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Benzene Fraction (B2) after spraying with 5% Sulphuric acid – Methanol Solution at 490 nm.

Table 3: Peak list and Rf value of the Chromatogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Chloroform Fraction (B3) after Spraying with 5% Sulphuric acid – Methanol Solution at 490 nm

Track	Peak	Max Rf	Max Height	Height %	Area	Area %
3	1	0.07	78.6	19.62	684.4	9.51
3	2	0.11	24.1	6.03	259.1	3.60
3	3	0.16	49.2	12.29	710.3	9.87
3	4	0.18	76.0	18.97	971.3	13.49
3	5	0.28	17.9	4.45	441.9	6.14
3	6	0.33	48.4	12.07	1094.6	15.21
3	7	0.50	26.4	6.58	760.7	10.57
3	8	0.70	13.3	3.32	553.0	7.68
3	9	0.75	22.5	5.62	513.2	7.13
3	10	0.83	44.3	11.06	1210.0	16.81

41.25%, 16.95%, 15.53% and 12.43% respectively. And remaining components were found to be very less in quantity as the percentage area for all the spots was less than 6%.

Table 3 states that in the Chloroform extract of aerial parts of *Bryophyllum pinnatum* there are 10 spots at the following R_f 0.07, 0.11, 0.16, 0.18, 0.28, 0.33, 0.50, 0.70, 0.75 and 0.83 as shown in Figure 6, indicating the occurrence of atleast 10 different components in Chloroform extract. It is also clear from Table 3 and the chromatogram as shown Figure 6 that out of 10 components, the component with R_f values 0.83 (light blue, violet), 0.33 (light blue, violet), 0.18 (light blue, violet), 0.50 (light green, violet) and 0.16 (light blue, light green) at 366 nm and visible 490 nm were found to be more predominant as the percentage area is more with 16.81%, 15.21%, 13.49%, 10.57% and 9.87% respectively. And remaining components were found to be very less in quantity as the percentage area for all the spots was less than 7.7%.

Table 4 states that in the Acetone extract of aerial parts of *Bryophyllum pinnatum* there are 8 spots at the following R_f 0.08, 0.11, 0.18, 0.23, 0.33, 0.49, 0.64 and 0.73 as shown in Figure 7, indicating the occurrence of atleast 10 different components in Acetone extract. It is also clear from Table 4 and the chromatogram as shown Figure 7 that out of 8 components, the component with R_f values 0.33 (light blue, violet), 0.18 (light blue, violet), 0.64 (light blue, light green) and 0.23 (light blue, violet) at 366 nm and visible 490 nm were found to be more predominant as the percentage area is more with 25.36%, 20.01%, 16.49%, 10.37% respectively. And remaining components were found to be very less in quantity as the percentage area for all the spots was less than 6%.

While Table 5 states that in the Methanol extract of aerial parts of *Bryophyllum pinnatum* there are 9 spots at the following

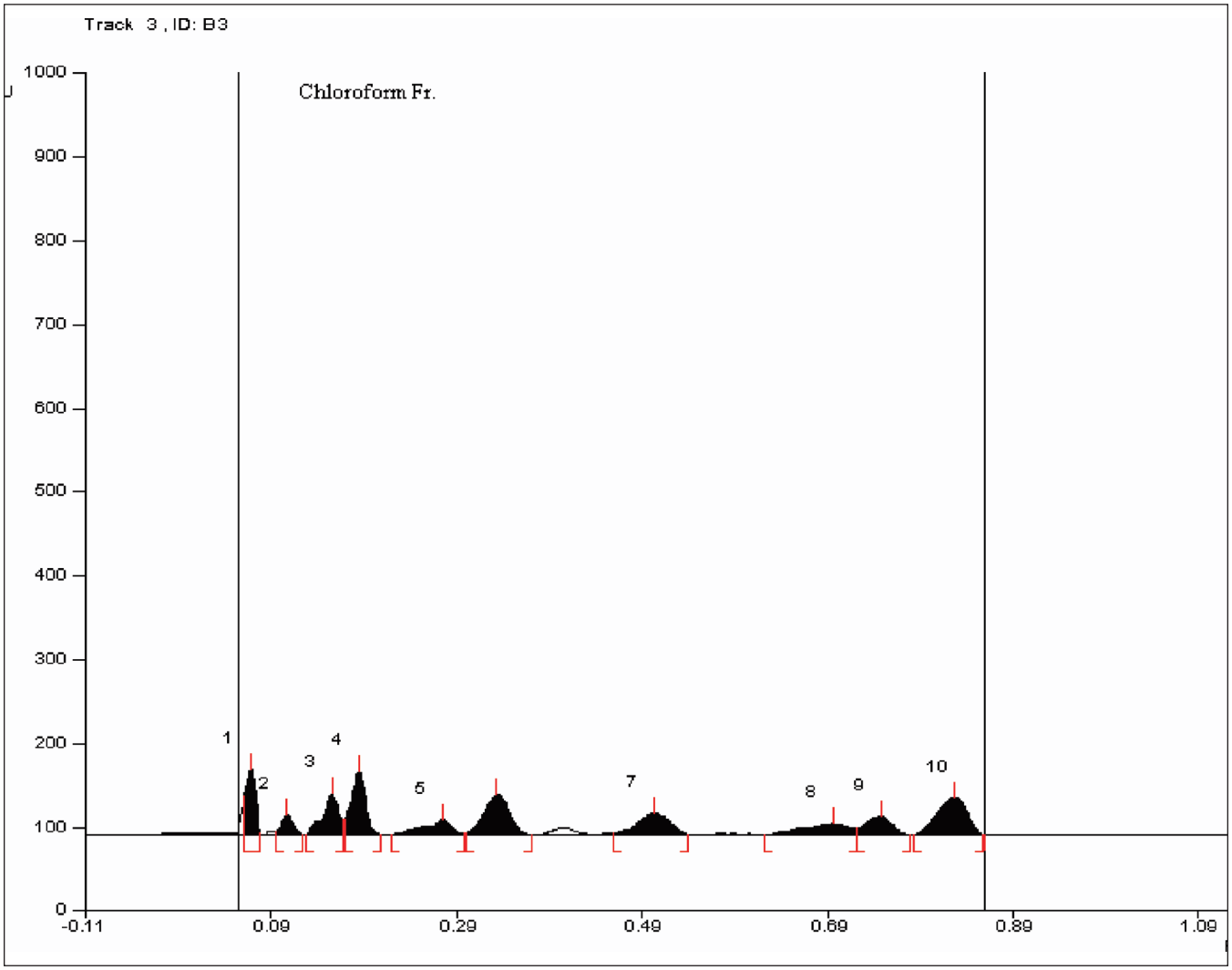

Figure 6: Typical HPTLC Densitogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Chloroform Fraction (B3) after spraying with 5% Sulphuric acid – Methanol Solution at 490 nm.

Table 4: Peak list and Rf value of the Chromatogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Acetone Fraction (B4) after Spraying with 5% Sulphuric acid – Methanol Solution at 490 nm

Track	Peak	Max Rf	Max Height	Height %	Area	Area %
4	1	0.08	16.1	5.37	224.3	3.52
4	2	0.11	32.8	10.93	370.1	5.81
4	3	0.18	78.5	26.13	1273.6	20.01
4	4	0.23	31.6	10.54	660.1	10.37
4	5	0.33	72.1	24.00	1614.3	25.36
4	6	0.49	34.4	11.45	897.5	14.10
4	7	0.64	23.6	7.87	1049.8	16.49
4	8	0.73	11.2	3.71	276.7	4.35

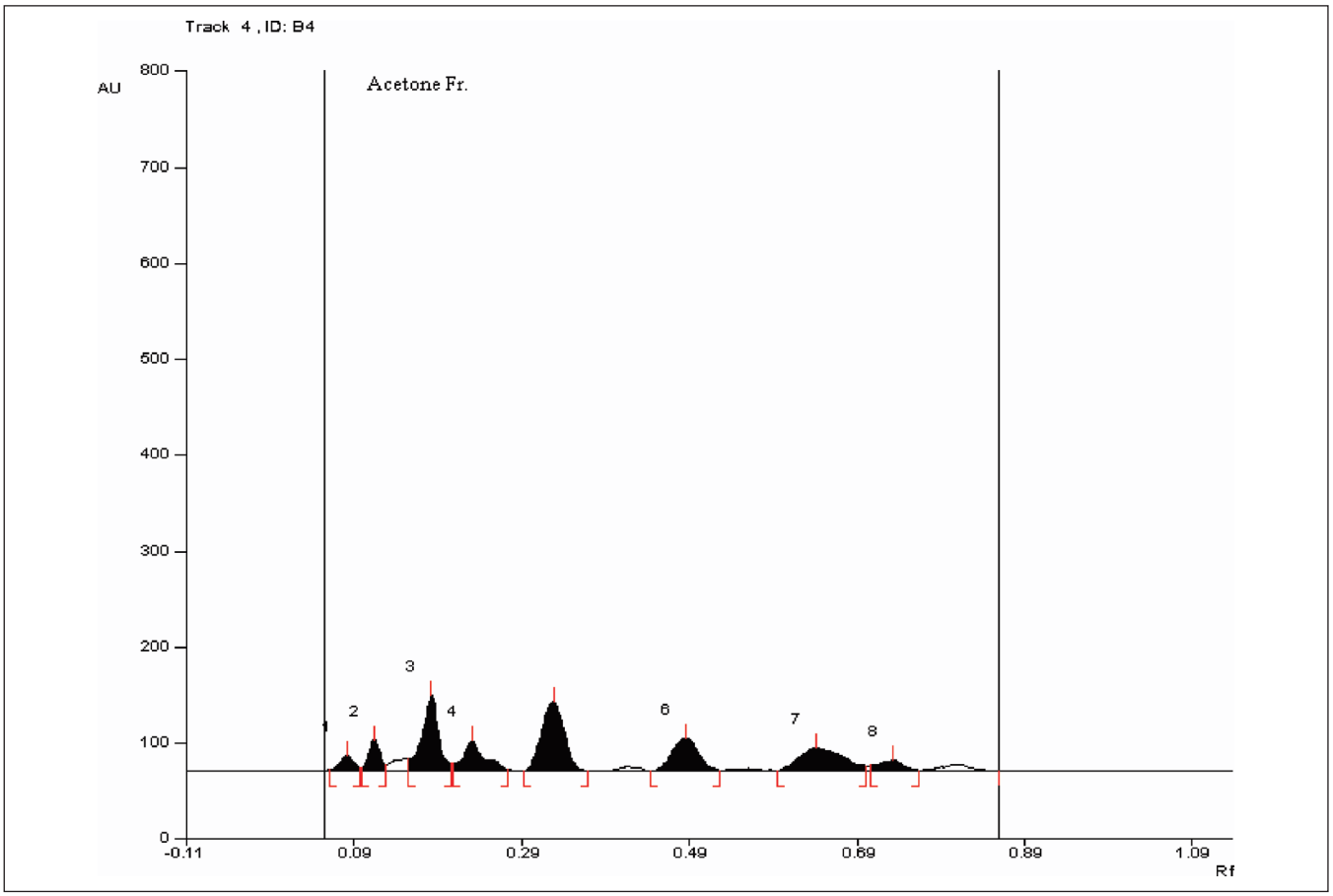

Figure 7: Typical HPTLC Densitogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Acetone Fraction (B4) after spraying with 5% Sulphuric acid – Methanol Solution at 490 nm.

Table 5: Peak list and Rf value of the Chromatogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Methanol Fraction (B5) after Spraying with 5% Sulphuric acid – Methanol Solution at 490 nm

Track	Peak	Max Rf	Max Height	Height %	Area	Area %
5	1	0.06	67.4	16.44	270.7	3.84
5	2	0.09	26.0	6.35	227.9	3.24
5	3	0.12	54.3	13.24	799.5	11.35
5	4	0.18	105.4	25.73	1597.6	22.68
5	5	0.25	25.9	6.31	574.7	8.16
5	6	0.34	70.0	17.09	1643.0	23.32
5	7	0.43	12.8	3.11	322.0	4.57
5	8	0.50	32.5	7.92	978.7	13.89
5	9	0.69	15.6	3.80	630.1	8.94

R_f 0.06, 0.09, 0.12, 0.18, 0.25, 0.34, 0.43, 0.50 and 0.69 as shown in Figure 8, indicating the occurrence of atleast 9 different components in Methanol extract. It is also clear from Table 5 and the chromatogram as shown Figure 8 that out of 9 components, the component with R_f values 0.34 (light blue, violet), 0.18 (light blue, violet), 0.50 (reddish brown, light green) and 0.12 (light blue, violet) at 366 nm and visible 490 nm were found to be more predominant as the percentage area is more with 23.32%, 22.68%, 13.89%, 11.35% respectively. And remaining components were found to be very less in quantity as the percentage area for all the spots was less than 9%.

CONCLUSION

Although TLC is a conventional method used generally in pharmacopoeias as one of the standardization methods, the HPTLC method is more practical. HPTLC is feasible for development of chromatographic fingerprints to determine major active constituents of medicinal plants. This method of HPTLC for the different solvent extracts of dried aerial parts of *Bryophyllum pinnatum* was very much helpful in determining the quality of the crude drug and also helps to separate and isolate the components using other chromatographic techniques which can be

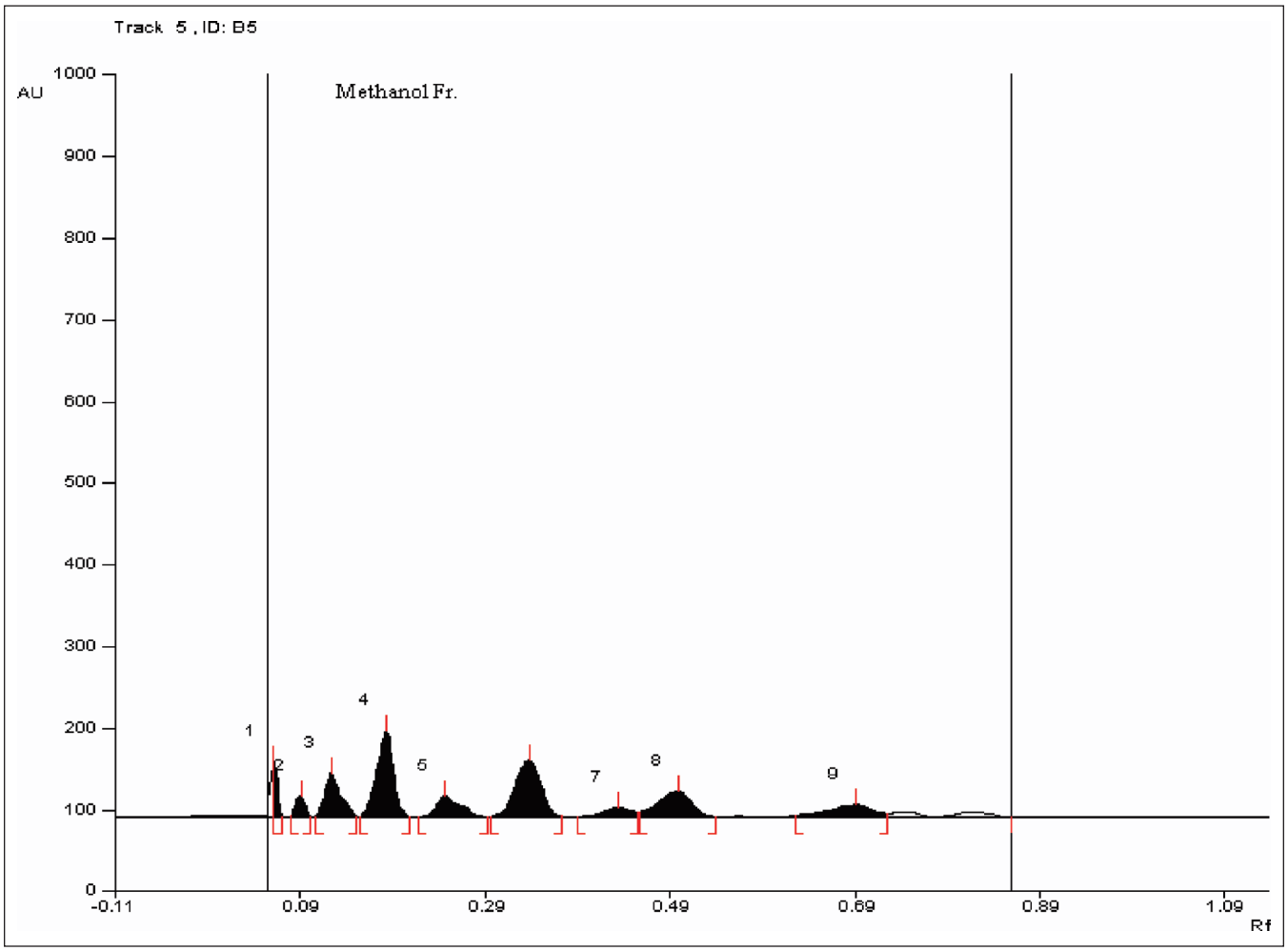

Figure 8: Typical HPTLC Densitogram of Aerial Parts of *Bryophyllum pinnatum* Extracted with Methanolic Fraction (B5) after spraying with 5% Sulphuric acid – Methanol Solution at 490 nm.

used for further studies. The separation and resolution are much better, and the results are much more reliable and reproducible than TLC. Combined with digital scanning profiling, it has the main advantage of in situ quantitative measurement by scanning densitometry. Furthermore, the colorful pictorial HPTLC image provides extra, intuitive visible color and/or fluorescence parameters for parallel assessment on the same plate. In conclusion, the results obtained from qualitative evaluation of HPTLC fingerprint images will be helpful in the identification and quality control of the drug and ensure therapeutic efficacy. HPTLC analysis of dried aerial parts of *Bryophyllum pinnatum* can provide standard fingerprints and can be used as a reference for the identification and quality control of the drug.

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Antibacterial, Antioxidant and Anti-inflammatory Properties of Margaritaria discoidea, a Wound Healing Remedy from Ghana

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ABSTRACT

Margaritaria discoidea (Baill.) Müll Arg. (Euphorbiaceae), has folkloric use in the treatment of wounds and skin infections. We report on the antibacterial, antioxidant and anti-inflammatory effects of the leaves and stem bark of *Margaritaria discoidea*. A 70% ethanolic extract of the various plant parts were tested for antibacterial activity using the agar well diffusion and micro dilution assays. Free radical scavenging, total antioxidant and phenol content were estimated. Using the carrageenan-induced foot pad oedema in chicks, the anti-inflammatory activity of the extract was assessed. The bark extract gave the higher activity with a zone of inhibition of 16.33 ± 0.88 mm against *Bacillus subtilis*. The MIC's for the bark extracts ranged from 500 µg/mL to over 1000 µg/mL against the test organisms. However, the leaf extract had no activity against all organisms tested. The leaf and bark extracts demonstrated free radical scavenging activity yielding IC_{50} values of 0.0185 and 0.0181 mg/mL respectively. In the total antioxidant assay, ascorbic acid equivalents ranged from 0.49 mg/g in the bark to 0.56 mg/g in the leaf. Both extracts had high phenolic content correlating with their antioxidant activity. The extracts showed significant anti-inflammatory activity. The ED_{50}s of the leaf and bark extracts were 12.20 and 8.27 mg/kg body weight respectively. The results were comparable to those of diclofenac and dexamethasone, the reference drugs used in this study. On the basis of the antimicrobial, antioxidant and anti-inflammatory activities observed for both extracts, the ethnomedicinal use of the plant in the mamagement of wounds and skin infections is supported.

Key words: Antimicrobial; Carrageenan-induced oedema; Free radical; *Margaritaria discoidea*; Wound healing.

INTRODUCTION

Over 80% of the world's population depends on traditional medicines for numerous skin disorders.[1] Skin disorders, primarily wounds, are the third most common causes of people seeking medical care in developing countries, including Ghana. Medical costs and lost productivity from the workforce suggest that chronic wounds cost several billions of dollars annually. Contributing to these staggering costs is treatment regimen that is expensive and/or ineffective and associated with recurrence rate.[2]

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Wound healing is the body's natural process of regenerating dermal and epidermal tissue. When an individual is wounded, a cascade of biochemical events is initiated, leading to the repair of the damaged tissues. These events overlap but can be categorized as: the inflammatory, proliferative and remodelling phases;[3] each phase is characterized by the infiltration into the wound site of specific cells, all of which interact by chemical signals to optimize repair.

Open wounds are prone to infection, especially by bacteria, and also provide an entry point for systemic infections. To arrest this, neutrophils must be released into the wound site. The over-abundant neutrophil infiltration is responsible for the chronic inflammation characteristic of non-healing ulcers. These neutrophils release the enzymes collagenase and elastase which are responsible for the destruction of the connective tissue matrix and important growth factors respectively.[4] Antimicrobial agents may prevent this occurring and may underlie their use in treating wounds.

Antioxidants counter the excess proteases and reactive oxygen species (ROS) often formed by neutrophil accumulation in the wound site and protect protease inhibitors from oxidative damage. Fibroblasts and other cells may be killed by excess ROS and antioxidant agents may reduce the possibility of these adverse events occurring.[5]

When an individual is wounded, it is accompanied within a short time by the classical symptoms of inflammation: redness, increased heat, swelling, pain and loss of function. The release of eicosanoids, prostaglandins and leukotrienes are responsible for these symptoms. Fundamentally, inflammation is a protective response, with the aim of ridding the individual of the injurious stimuli and initiates the healing process. However, in chronic inflammation, the orderly process of healing is lost and there is the formation of chronic non-healing ulcers. Healing proceeds only after the inflammation is controlled.[6] The synthesis of eicosanoids, prostaglandins and leukotrienes are processes that can be inhibited by anti-inflammatory agents.

Medicinal plants have played and continue to play an invaluable role as drugs or lead compounds in the management of diseases.[7] There has been a growing interest in medicinal plants traditionally used in the treatment of wounds, and infectious diseases.[2,5] This study involves the scientific investigation of the leaves and stem bark of *Margaritaria discoidea* (Baill.) Müll Arg. (Euphorbiaceae), which finds use in ethnomedicine in Ghana as a wound healing agent.[8]

The leaves of *Phyllanthus discoideus* (Baill.) Müll Arg. also referred to as *Margaritaria discoidea* demonstrated antibacterial[9,10] activity. The alkaloids responsible for the antibacterial activity have been isolated and characterised.[11] The present study aimed to verify the anti-inflammatory, antioxidant and antibacterial effects of the leaves and bark of the plant.

MATERIALS AND METHODS

Plant materials

The stem bark of *Margaritaria discoidea* – voucher specimen number (KNUST/HM1/2010/S003) and the leaves of *Margaritaria discoidea* – voucher specimen number (KNUST/HM1/2010/L30) were collected from Kente, Amansie Central District in the Ashanti Region of Ghana in June 2009. The plants were initially identified locally by a resident herbalist and authenticated at the Department of Pharmacognosy, College of Health Sciences, Kwame Nkrumah University of Science and Technology herbarium where herbarium specimens have been deposited.

Chemicals

All chemicals used were of analytical grade and purchased from Sigma Aldrich Co Ltd. Irvine, UK. Organic solvents were also of analytical grade and purchased from BDH Laboratory Supplies (England). Precoated aluminium-backed silica gel F_{254} TLC plates (0.25 mm thickness), product code OB 315394 were purchased from Merck KGaA, Germany. Carrageenan sodium salt was purchased from Sigma Chemicals, St. Louis, MO, USA. Diclofenac and dexamethasone were purchased from Troge, Hamburg, Germany and Pharm-Inter, Brussels, Belgium respectively.

Microorganisms used

Eight bacterial species, including four Gram-positive bacteria (*Enterococcus faecalis* ATCC 29212, *Bacillus thurigiensis* ATCC 13838, *Staphylococcus aureus* ATCC 25923 and *Bacilllus subtilis* NCTC 10073) and four Gram–negative bacteria (Salmonella *typhi* NCTC 6017, *Escherichia coli* NCTC 9002, *Proteus vulgaris* NCTC 4635 and *Pseudomonas aeruginosa* ATCC 27853) were used for the antibacterial tests.

Animals

Cockerels (*Gallus gallus*; strain shaver 579, Akropong Farms, Kumasi, Ghana) were obtained 1-day post-hatch and were housed in stainless steel cages ($34 \times 57 \times 40$ cm^3) at a population density of 12-13 chicks per cage. Feed (Chick Mash, GAFCO, Tema, Ghana) and water were available *ad libitum* through 1-quart gravity-fed feeders and water trough. Room temperature was maintained at 29° C, and overhead incandescent illumination was maintained on a 12 hour light-dark cycle. Daily maintenance of the cages was conducted during the first quarter of the light cycle. Chicks were tested at 7 days of age. Group sample sizes of 5 were used throughout the study.

Extraction of plant materials

The leaves and stem barks of *Margaritaria discoidea* were dried and coarsely powdered. 50 g (dry weight) of each powdered plant part were extracted with 70% ethanol (1.5 L) using soxhlet extraction.

Antimicrobial assays

Agar well diffusion method

The antimicrobial activities of the different extracts were determined using the agar well diffusion method as outlined by Vanden Berghe and Vlietnick.[12] Crude extracts were prepared at concentrations of 5 mg/mL using methanol. Wells of 9 mm diameter were made in 20 mL nutrient agar (Oxoid) seeded with 2 loopfuls of a suspension of test organisms under aseptic conditions. The wells were filled with 100 µL of the extracts, allowed to diffuse for 1 hour at room temperature and incubated at 37° C for 24 hours, after which they were examined for zones of inhibition. Amoxycillin was included as positive control All experiments were carried out in triplicates.

Micro dilution assay

Minimal inhibitory concentration (MIC) values of the extracts were determined based on a micro-well dilution

method.[13] The inocula of microorganisms were prepared from 12-hour broth cultures and serial dilutions were made to achieve a suspension of approximately 10^5 CFU/mL. The plant extracts were screened at concentrations of between 1000 μg/mL and 7.8 μg/mL.

The 96-well sterile plates were prepared by dispensing into each well 100 μL of double strength nutrient broth and 100 μL of test samples together with 20 μL of the inoculum. The microplates were incubated at 37° C for 24 hours. Growth of the microorganisms was determined by adding 20 μL of a 5% solution of tetrazolium salt (MTT) and incubating for further 30 minutes. Dark wells indicated the presence of microorganisms as the dehydrogenase enzymes in the live bacteria reacts to form a dark complex with the tetrazolium salt. Amoxycillin was included as positive control. All experiments were carried out in triplicates.

Antioxidant assays

Rapid screening for antioxidants

Extracts were monitored initially for antioxidant activity on TLC (solvent system: chloroform, methanol 9:1) using 20 mg/L of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol, and antioxidant compounds in the extracts gave clear zones against a purple background.[14]

Free radical scavenging activity of the ethanolic extracts

Assay was performed by the DPPH method described by Blois (15). 20 mg/L solution of DPPH in methanol was prepared and 3 mL of this solution were added to1 mL of the ethanolic test extracts at 3, 1.5, 0.75 and 0375 mg/mL. After 30 min, the absorbance was measured at 517 nm. Inhibition of radical scavenging was calculated according to the following equation.

$$\text{DPPH scavenging activity (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100$$

With A_0 being the absorbance of the control and A_1 is the absorbance in the presence of the test sample

Total phenols determination

Total phenols were determined by Folin-Ciocalteau's reagent. [16] Different doses were tested for both tannic acid and the plant extracts: tannic acid (0.03-0.1 mg/mL); extract (0.125-2.5 mg/mL). 1 mL of each plant extract or tannic acid (standard phenolic compound) was mixed with 1 mL Folin-Ciocalteau's reagent and aqueous Na_2CO_3 (1 mL, 2%). The mixtures were incubated at 25° C for 2 hours, and then centrifuged at 3000 rpm for 10 min and absorbance of the supernatant determined at 760 nm. Distilled water (1 mL) was added to 1 mL Folin- Ciocalteau's reagent, processed in the same way as the test drugs and used as blank. The standard curve was prepared. Total phenol values were expressed in terms of tannic acid equivalent (mg/g of dry mass).

Total Antioxidant Capacity

Total antioxidant capacity of extract was determined as described by Prieto.[17] Ascorbic acid served as positive control. Different doses were tested for both ascorbic acid and the plant extracts: ascorbic acid (0.03-1 mg/mL); extract (0.125-2.5 mg/mL). 1 mL of each plant extract or ascorbic acid (standard antioxidant compound) was mixed with 3 mL of reagent solution (0.6 M H_2SO_4, 28 mM Na_2HPO_4 and 4 mM ammonium molybdate) and 1 mL of aqueous Na_2CO_3 (2%) The mixtures were incubated at 95° C for 90 min, and then centrifuged at 3000 rpm for 10 min and absorbance of the supernatant determined at 695 nm. Distilled water (1 mL) was added to 3 mL Molybdate, processed in the same way as the test drugs and used as blank. The standard curve was prepared. Total antioxidant values are expressed in terms of ascorbic acid equivalent (mg/g of dry mass).

Anti-inflammatory assay

Carrageenan-induced oedema

Anti-inflammatory activity was determined by the method of Roach and Sufka[18], modified by Woode *et al.,*.[19] Chicks were randomly divided into groups of 5 and had access to food and water *ad libitum*. Foot volumes were measured by water displacement plethysmography as described by Fereidoni *et al.*,.[20] Oedema was induced by subplantar injection of carrageenan (10 μL of a 2%w/v solution in saline) into the right footpad of the chicks. Three test groups received the extracts (30, 100 and 300 mg/kg, p.o.), the standard groups received diclofenac (5, 15 and 50 mg/kg, i.p.) and dexamethasone (1, 3 and 10 mg/kg, i.p.) and the control animals received the vehicle only. All treatments were administered 30 min for i.p. route and 1 hour for oral route before carrageenan injection.

Statistical analysis

Raw scores for right foot volumes were individually normalized as percentage of change from their values at time 0 then averaged for each treatment group. The time-course curves for foot volume was subjected to two-way (treatment × time) repeated measures analysis of variance with Bonferroni's *post hoc t* test. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC). The inhibition percentage of oedema was calculated for each animal group in comparison with its vehicle-treated group.

Differences in AUCs were analyzed by ANOVA followed by Newman-Keul's *post hoc t* test. ED_{50} (dose responsible

for 50% of the maximal effect) for each drug was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

$$Y = \frac{a + (b - a)}{[1 + 10^{(LogED_{50} - X)}]}$$

Where X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

GraphPad Prism for Windows version 5.00 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis and ED_{50} determinations. $P < 0.05$ was considered statistically significant.

Phytochemical screening

The presence of phenolics, alkaloids, phytosterols, terpenoids, flavonoids, reducing sugars and saponins were detected by simple qualitative phytochemical methods.[21]

RESULTS

Antimicrobial effects

Agar well diffusion

The largest diameter of zone of inhibition, 16.33 mm was given by the 70% ethanolic extract of *Margaritaria discoidea* bark against *Bacillus subtilis NCTC 10073* (Table 1). The extract of *Margaritaria discoidea* leaves showed no activity against the test organisms used.

Micro-dilution assay

Minimum inhibitory concentrations were observed for the extracts that showed activity in the agar well diffusion assay. The MIC values ranged from 500 µg/mL to more than 1000 µg/mL (Table 2).

Table. 1. Growth inhibition activity of 70% ethanolic extracts of the plant materials against a battery of microorganisms.

Microorganisms	Zones of inhibition (mm) ± SEM MDB
Gram positive	
Bacillus subtilis NCTC 10073	16.33 ± 0.88
Bacillus thurigiensis ATCC 13838	15.00 ± 0.00
Staphylococcus aureus ATCC 25923	15.33 ± 0.67
Enterococcus faecalis ATCC 29212	15.00 ± 1.00
Gram negative	
Proteus vulgaris NCTC 4635	12.33 ± 0.33
Pseudomonas aeruginosa ATCC 27853	–
Salmonella typhi NCTC 6017	–
Escherichia coli NCTC 9002	15.00 ± 0.00

Each value in the table was obtained by calculating the average of three replicates ± standard error of the mean. Extract: MDB = Margaritaria discoidea bark.

Antioxidant effects

TLC-screening for antioxidant compounds

The active compounds were detected as yellow spots on a violet background. Both extracts were subjected to further testing.

Free radical scavenging activity

The abilities of the test extracts which were detected in the TLC screening to donate hydrogen atoms or electrons were measured spectrophotometrically. Both extracts reduced DPPH to the yellow coloured product, diphenylpicrylhydrazine, and the absorbance at 517 nm declined. The leaves and bark of *Margaritaria discoidea* showed effect with IC_{50}s of 0.0185 and 0.0181 mg/mL respectively (Table 3).

Total phenol contents

The total phenols were 23.49 ± ± 0.01 and 19.61 ± 0.02 mg/g in the leaf and bark extracts respectively as shown in Figure 1.

Total antioxidant capacity

The total antioxidant capacity of the leaf was 0.559 ± 0.00 whereas the bark was 0.488 ± 0.00 as shown in Figure 2.

Anti-inflammatory activity

Carrageenan-induced oedema

Figure 3 shows the time course curve and AUC for the effect of diclofenac, dexamethasone, *Margaritaria discoidea* leaf (MDL) and *Margaritaria discoidea* bark (MDB) on

Table 2: Mean minimum inhibitory concentration (MIC; µg/mL) of *M. discoidea* bark on various microorganisms. n=3.

Microorganisms	MIC µg/mL MDB
Gram positive	
Bacillus subtilis NCTC 10073	500
Bacillus thurigiensis ATCC 13838	>1000
Staphylococcus aureus ATCC 25923	>1000
Enterococcus faecalis ATCC 29212	>1000
Gram negative	
Proteus vulgaris NCTC 4635	1000
Pseudomonas aeruginosa ATCC 27853	NT
Escherichia coli NCTC 9002	>1000
Salmonella typhi NCTC 6017	NT

All experiments were carried out in triplicates. 200 µg/mL of amoxicillin served as positive control. NT = Not tested because extract was not active by the agar well diffusion assay. MIC readings for all wells were the same. Extracts: MDB = *Margaritaria discoidea* bark.

Table 3. IC_{50} values (mg/mL) for free radical scavenging activity by extracts.

Extract	IC_{50} DPPH
MDL	0.0185
MDB	0.0181

Extracts: MDL = *Margaritaria discoidea* leaves, MDB = *Margaritaria discoidea* bark.

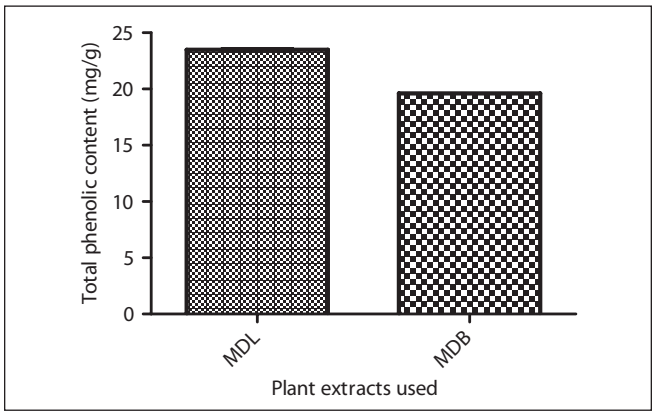

Figure 1: Total phenolic content (mg/g) in the 70% ethanolic extracts of the selected plants. Extracts: MDL = *Margaritaria discoidea* leaves, MDB = *Margaritaria discoidea* bark.

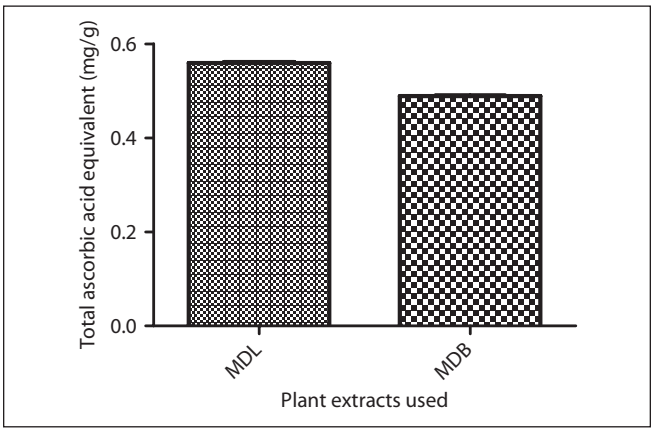

Figure 2: Total ascorbic acid equivalent (mg/g) in the 70% ethanolic extracts of the selected plants. Extracts: MDL = *Margaritaria discoidea* leaves, MDB = *Margaritaria discoidea* bark.

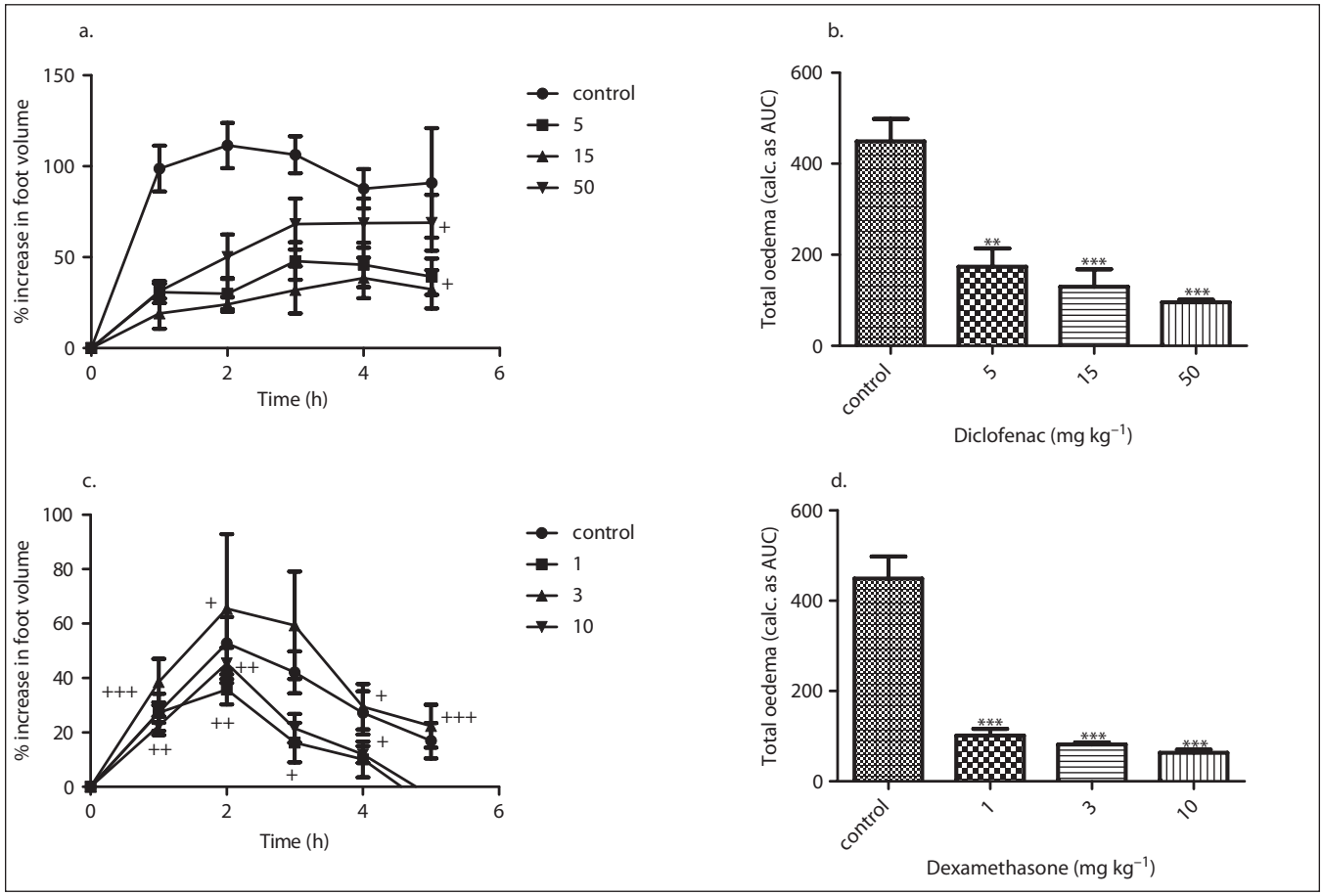

Figure 3: Effect of diclofenac (10 – 100 mg/kg; i.p.), dexamethasone (1 – 10 mg/kg; i.p.) and extract (30 – 300 mg/kg; p.o) on time course curve (a, c, e, g) and the total oedema response (b, d , f, h respectively) in carrageenan-induced oedema in chicks. Values are means ± SEM. (n =5). $^{+++}P < 0.0001$; $^{++}P < 0.001$; $^{+}P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{***}P < 0.0001$; $^{**}P < 0.001$; $^{*}P < 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test.

carrageenan-induced oedema in chicks. On the time-course curve, MDB treatment exhibited a significant effect [$F_{3,12}$ =37.04, $p < 0.0001$] with maximal inhibitory effect of total oedema by 92.64% at 300 mg/kg. MDL treatment exhibited a significant effect [$F_{3,12}$ =29.29, $p < 0.0001$] with maximal inhibitory effect of total oedema by 84.94% at 30 mg/kg. Diclofenac exhibited a significant effect [$F_{3,12}$ = 16.22, $p = 0.0002$] and a 78.75% maximal inhibitory effect of total oedema at 50 mg/kg. Dexamethasone, a steroidal anti-inflammatory agent showed a significant

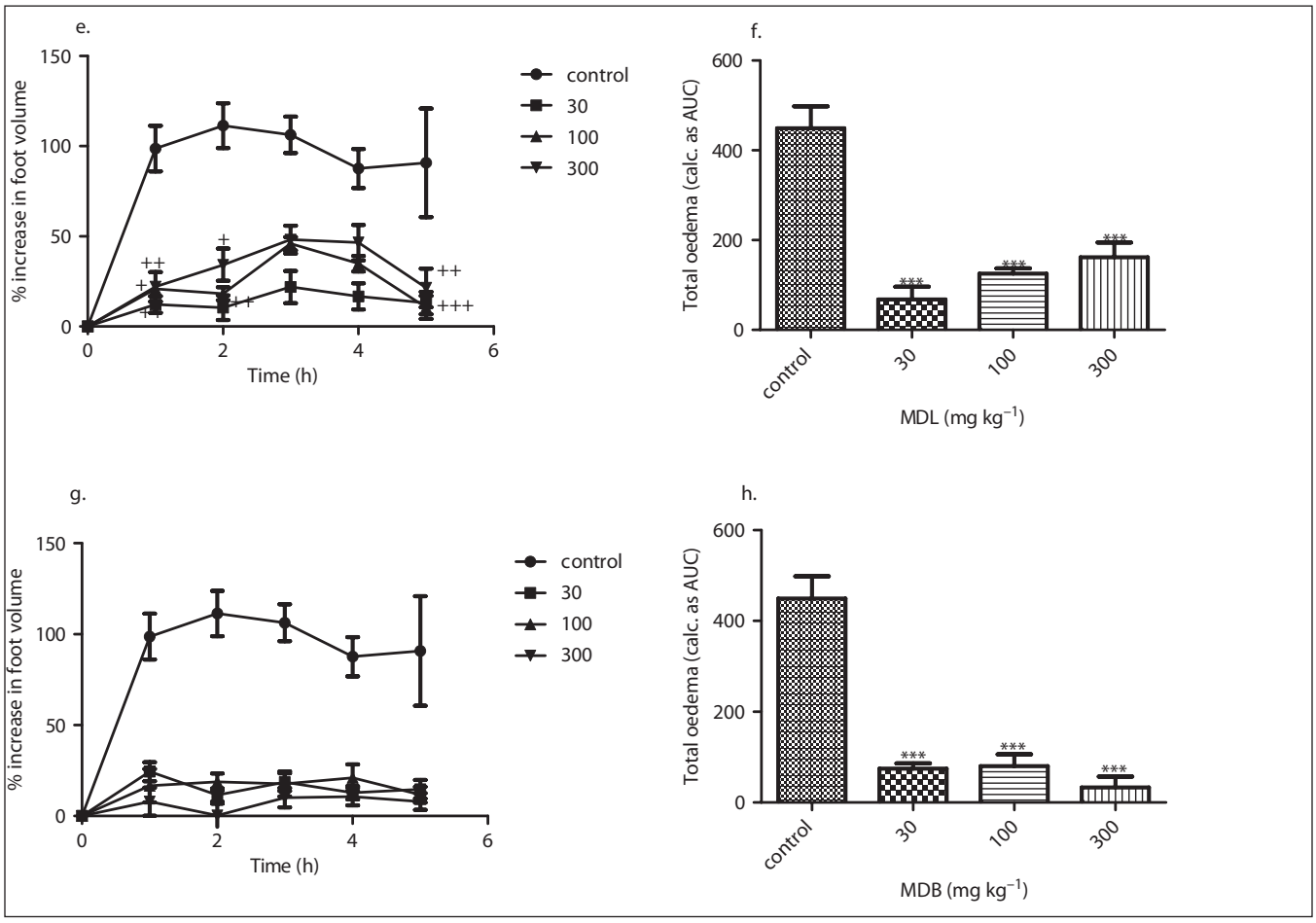

Figure 3: *Continued*

Table 4. ED50 values for the effect of MDL, MDB, Diclofenac and Dexamethasone in Carrageenan-induced oedema in chicks.

Drug	ED_{50}(mg/kg)
MDL	12.20
MDB	8.27
Dexamethasone	3.43
Diclofenac	4.42

Extracts: MDL = *Margaritaria discoidea* leaves, MDB = *Margaritaria discoidea* bark.

Table 5. Results of the phytochemical screening for all powdered plant materials.

Plant secondary metabolites	Powdered plant materials	
	MDL	MDB
Phenolic	++	++
Reducing sugar	+	+
Alkaloids	+	++
Phytosterols	+	+
Triterpenoids	–	+
Saponins	–	+
Flavonoids	+	+

– = absent, + = present Extracts: MDL = *Margaritaria discoidea* leaves, MDB = *Margaritaria discoidea* bark.

effect [$F_{3,12} = 49.25$, $p < 0.0001$] and a maximal inhibitory effect of 85.98% at 10 mg/kg.

Based on the ED_{50} values (Table 4) obtained from the dose response curves (Figure 3), MDB was more potent of the plant extracts. The standard drugs, diclofenac and dexamethasone were approximately twice as effective as MDB as an anti-inflammatory agent.

Phytochemical screening. The results of the phytochemical screening on the powdered plant parts are as shown in Table 5.

DISCUSSION

In a series of *in vitro* tests, the 70% ethanolic extracts exhibited antibacterial, antioxidant and anti-inflammatory effects. The ethanolic extracts of the stem bark of *Margaritaria discoidea* was active with MIC values ranging from 500 µg/mL to more than 1000 µg/mL against Gram-positive (*Enterococcus faecalis* ATCC 29212, *Bacillus thurigiensis* ATCC 13838, *Staphylococcus aureus* ATCC 25923 and *Bacilllus subtilis* NCTC 10073) and Gram–negative (*Escherichia coli*

NCTC 9002 and *Proteus vulgaris* NCTC 4635). The largest diameter of zone of inhibition 16.33 mm observed for the agar well diffusion method, was against *Bacilllus subtilis* NCTC 10073 (Table 1). The extract of the leaves of *Margaritaria discoidea* showed no activity against all organisms employed. However in a previous study, the alkaloid fraction obtained from the lyophilized aqueous extract of the leaves was found to show antibacterial activity.[10]

Infected wounds heal less rapidly and often result in the formation of unpleasant exudates and toxins, which would lead to the killing of regenerating cells in the healing process.[5] The most familiar pathogen to be isolated from infected wounds is *Staphylococcus aureus* which may be isolated from approximately 1/3 of all infected wounds.[22] Interestingly, the extract of the bark of *Margaritaria discoidea* showed activity against *Staphylococcus aureus* ATCC 25923. The respective MIC was >1000 µg/mL. The stem bark extract thus has inhibitory effects on *Staphylococcus aureus* and other pathogens, and may serve as anti-infectives and hence its usefulness.

Quite a number of plant secondary metabolites of different classes have been shown to possess antimicrobial effect.[23] Subsequently, the presence of one or more of these metabolites in the extracts may be responsible for the effects observed in the antimicrobial assays (Table 5).

The ethanolic extracts of the leaves and stem bark of *Margaritaria discoidea* had similar scavenging activities on DPPH with IC_{50} of 0.018 mg/mL. In the total antioxidant assay, the extract of *Margaritaria discoidea* leaf was more active with ascorbic acid equivalent being 0.559 mg/g dry weight of extract.

Phenolic compounds are a class of antioxidant agents which act as free radical terminators.[24] Phenolic compounds, due to their redox properties, act as reducing agents, hydrogen donors or singlet oxygen quenchers.[25] In this study, an attempt was made to establish the co-relationship between total phenolic content and antioxidant activity. The correlation developed between total antioxidant activity (*Y*) and total phenolic content (*X*) of all the plant extracts had correlation coefficient, R of 1.00 and 0.99 for leaf and bark respectively. Phenolic content of the extract correlated highly with their total antioxidant capacities. The results obtained suggest the potential of the extracts as antioxidant agents.

The carrageenan-induced oedema, an animal model of acute inflammation, involves the synthesis and/or release of histamine, serotonin, kinins, prostaglandins and cyclooxygenase-2.[26] The inflammatory mediators released during acute inflammation are potent vasodilator substances, which increase the vascular permeability and subsequently cause the observed oedema.[27] The extracts reduced the oedema produced. The leaf extract of *Margaritaria discoidea* showed a maximal inhibitory effect of total oedema by 84.94% at 30 mg/kg. The maximal inhibitory effect of total oedema by 92.64% at 300 mg/kg was exhibited by the bark of *Margaritaria discoidea*. The results of our work compare with that of Adedapo *et al*.[28] So it may be suggested that their anti-inflammatory activity is backed by inhibiting the synthesis, release or action of the inflammatory mediators.

Flavonoids have been identified to inhibit the action of prostaglandins which are involved in the last phase of acute inflammation.[29,30] Glycosides have also showed *in vitro* inhibition of the chemical mediators of the inflammatory process.[31] It may be suggested that the observed anti-inflammatory activity may be due to the presence of flavonoids and glycosides present in the plant extracts (Table 5).

In conclusion, the different phases of the wound healing process overlap and ideally a plant-based remedy should affect at least two different processes before it can be said to have some scientific support for its traditional use.[5] These findings, suggest that the extracts of all the plant materials possess antibacterial, antioxidant and anti-inflammatory properties which are likely to contribute to their beneficial effect in wound healing. Further isolation of the various compounds responsible for these activities is in progress in our laboratories.

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Relaxant Effect of the Extracts of *Crataegus mexicana* on Guinea Pig Tracheal Smooth Muscle

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ABSTRACT

Crataegus sp. has been used in the traditional medicine of Mexico as well as other countries for the treatment of several respiratory diseases, such as flu, cough and asthma. The tracheal relaxant effect of the leaves of *C. mexicana* is investigated here for the first time, through a bioassay-guided study by using isolated tracheal rings of guinea-pig as an experimental model. The hexane extract was the most active compared to dichloromethane and methanol. An active fraction was obtained from the hexane extract. Assays by HPLC-MS reveal that at least 14 compounds may exist in it. In addition, the results suggest that relaxant effect of the effective fraction was in part related to the activity of -adrenergic receptors and not to K^+_{ATP} channels. This study represents the first in which the relaxant effect of leaves of *C. mexicana* on tracheal rings of guinea pig was clearly demonstrated. More studies are required to correctly identify the bioactive compounds that contribute to the relaxant effects of *Crataegus mexicana*, and to know the mechanisms of action of these compounds.

Key words: Rosaceae, hawthorn, bronchodilator, respiratory diseases, traditional medicine.

INTRODUCTION

The *Crataegus* genus (Rosaceae) comprises approximately 280 species and is found in northern temperate regions of East Asia, Europe, and Eastern North America.[1] The common name for the *Crataegus* species is hawthorn, and in Mexico is known as Tejocote.[2] Fruits, leaves, and flowers of the *Crataegus* sp. contain a number of chemical compounds, such as flavonoids, oligomeric proanthocyanidins, phenolic acids, triterpene acids, organic acids, sterols and trace amounts of cardioactive amines.[1, 3]

Several biological activities for that genus, such as cardioprotective, hypolipidemic, antioxidant, anti-inflammatory, antispasmodic, diuretic, digestive and others, have been reported.[1,4,5] Throughout Europe numerous preparations with the fruit, leaf and flower are currently available alone and in combination with other herbal extracts.[3] In addition, the *Crataegus* sp. has been used in the traditional medicine of Mexico as well as other countries for the treatment of several respiratory diseases, such as flu, cough, cold, bronchitis and asthma.[6, 7]

In spite of the widespread use of the *Crataegus* plant species for purposes of medical treatment, no studies exist regarding its usefulness in respiratory diseases. Therefore, we decided to investigate the tracheal relaxant effect of the leaves of *Crataegus mexicana* Moc. & Sessé ex DC. (Rosaceae) by using isolated tracheal rings of guinea-pig as an experimental model.

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MATERIAL AND METHODS

Plant material

The leaves of *C. mexicana* were collected in Chapingo, in the state of Mexico, during February of 2009. The identification was performed by Ernestina Cedillo, from the Herbarium of the Division of Forestry Sciences, Chapingo Autonomous University, with the voucher number 62654.

Extraction and bioassay-guided fractionation

The leaves of *C. mexicana* were dried at room temperature (22 ± 2°C) in the shade. After grinding 3.2 kg of leaves, they were successively extracted by maceration at room temperature (22 ± 2°C) for 3 days, first with hexane (12 L × 3), then dichloromethane (12 L × 3) and finally methanol (12 L × 3). Evaporation of the solvents in vacuum yielded 91.0, 49.5 and 592.8 g of syrupy residues, respectively. In accordance with the bioassay-guided study of the extracts, the hexane extract (77 g) was subjected to separation over a silica gel column (0.063-0.200 mm, 770 g) by using a step gradient of hexane (2 L, F1), hexane/EtOAc (9:1, 2 L, F2), hexane/EtOAc (7:3, 2 L, F3), hexane/EtOAc (1:1, 2 L, F4), EtOAc (2 L, F5) and MeOH (2 L, F6). The F2 and F3 fractions were the most active. The F2 fraction (16 g) was chromatographed on a silica gel column (320 g), obtaining three fractions, neither one of which presented activity (data not shown). F3 fraction (11.8 g) was chromatographed on a silica gel column (220 g) and was eluted with hexane, hexane/EtOAc mixtures, EtOAc and MeOH**,** obtaining eight fractions, of which the fraction F3´ was the most active. In order to identify the chemical compounds of F3´, a sample was analyzed by the HPLC-MS technique (Figure 1).

Animals

All the experiments were performed with adult male guinea pigs (350-450 g) obtained from the animal house of the Superior Medicine School (IPN). Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. The guinea pigs were housed under standard conditions, food and water being available *ad libitum*.

Drugs

Acetylcholine chloride, histamine dihydrochloride, carbachol chloride, propranolol hydrochloride, salbutamol, glibenclamide and KCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other reagents used were of analytical grade. Glibenclamide was dissolved in dimethyl sulfoxide (DMSO) and diluted with water. The other drugs

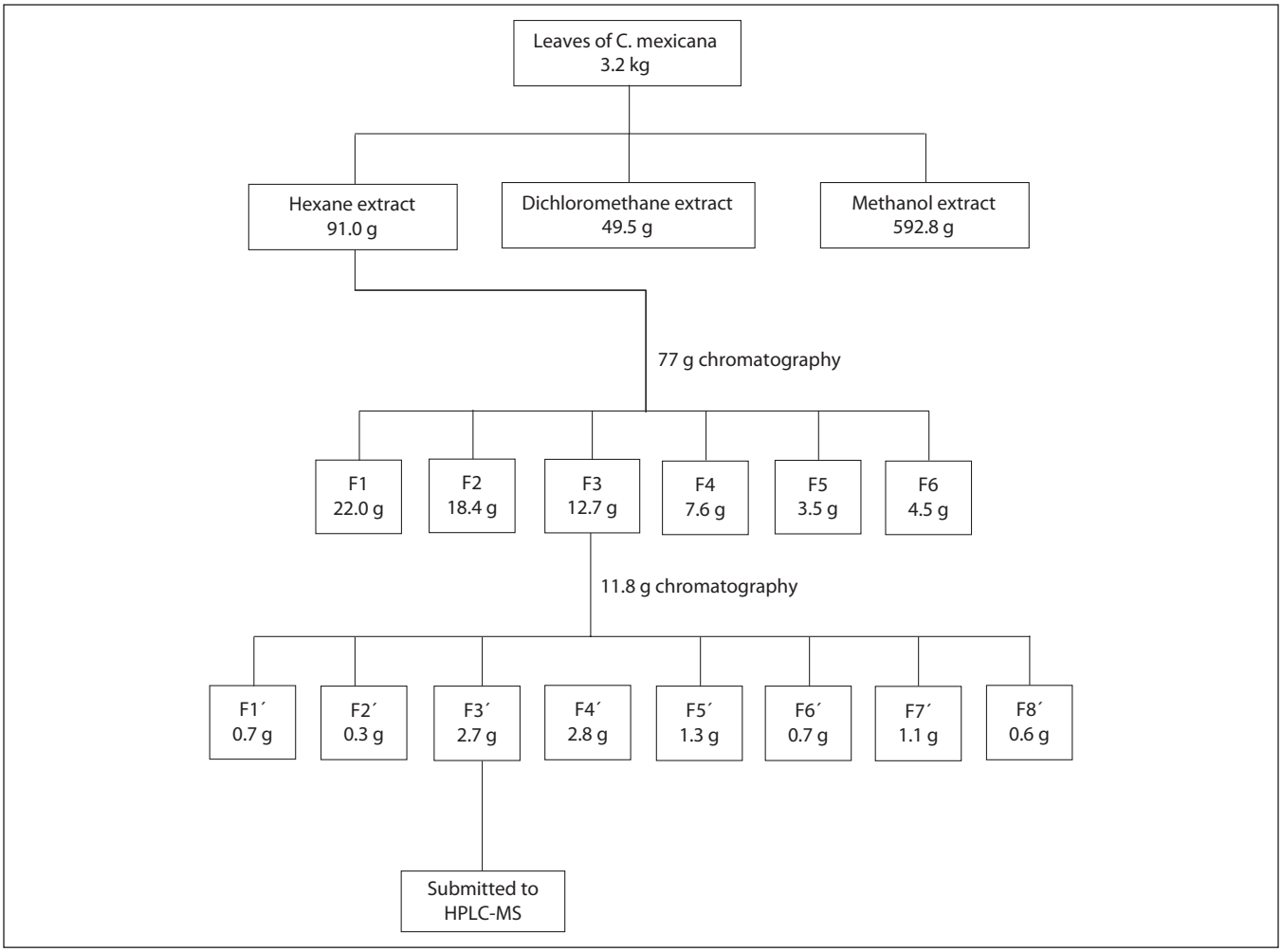

Figure 1: Scheme of biossay-guided fractionation of the hexane extract from *Crataegus mexicana*.

were dissolved in distilled water. The extracts and fractions were suspended in distilled water with traces of Tween 80. The final concentration of DMSO or Tween 80 was less than 0.1% and did not significantly affect the tracheal response.

Preparation of guinea pig trachea

The animals were euthanized by intraperitoneal injection with an overdose of sodium pentobarbital (75 mg kg^{-1}), the trachea was dissected and the connective tissue was cleaned off. Twelve tracheal rings about 2 mm in length, containing two to three cartilages each, were obtained from each guinea pig. Each tracheal ring was hung between two hooks inserted into the lumen, and placed in a 10 mL organ bath containing Krebs solution with the following composition (mM): NaCl 118.0, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2.2H_2O$ 2.5, $NaHCO_3$ 25.0, glucose 11.1. This solution was maintained at 37 ± 0.1°C and bubbled with 5% CO_2-95% O_2 mixture. Isometric tension was recorded through a twelve-channel Biopack System polygraph MP100 via a Biopac TSD 125C force transducer (Santa Barbara, USA). The data were digitalized and analyzed by means of software for data acquisition (Acknowledge 3.8.1) (Santa Barbara, USA). Tissues were placed under a resting tension of 1.5 g and allowed to stabilize for 60 min. They were washed with fresh Krebs solution at 15 min intervals before starting the experiments. After the stabilization period the tracheal rings were submitted to pre-stimulation with acetylcholine chloride (3 μM) two times at 30 min intervals, and after this stimulation they were washed with fresh Krebs solution. [8]

Effect of extracts and different fractions on the pre-contracted guinea pig trachea

Thirty minutes after stimulation with acetylcholine, the rings were contracted with carbachol (3 μM). When the plateau of the contraction was reached, 31.6, 56.2, 100.0, 133.3, 177.8, 237.1, 316.2, 421.6 or 562.3 μg/mL of the test extracts or fractions were cumulatively injected every 5 min. Then, their effective concentration thirty or fifty (EC_{30} or EC_{50}) was calculated.

Effect of the hexane extract on the histamine or KCl pre-contracted guinea pig trachea

Thirty minutes after stimulation with acetylcholine, the tracheal rings were contracted with histamine (30 μM) or KCl 40 (mM) in independent experiments. When the plateau of the contraction was reached, concentrations from 31.6 to 562.3 μg/mL of hexane extract were cumulatively injected every 5 min. Then, the percentages of the relaxation provoked in each treatment were calculated as well as the respective EC_{50}. As a relaxant standard drug we used salbutamol.

Effect of propranolol on the relaxant activity of the F3´ fraction

The relaxant activity of the F3´ fraction was studied on the carbachol chloride contracted guinea pig tracheal rings in the absence or presence of propranolol. The relaxant activity of salbutamol was used as a reference. When the plateau of the contraction was reached, 3 μM of propranolol was injected into the organ bath, and then every 5 min a concentration from 31.6 to 562.3 μg/mL of the F3´ fraction, or from 10^{-9} to 10^{-7} M of salbutamol, were cumulatively injected.[8] The percentage of the relaxation induced by each treatment was calculated as well as the respective EC_{50}.

Effect of blocking the ATP-sensitive potassium channel on the relaxant effect of the F3´ fraction

The relaxant effect of F3´ on the carbachol contracted guinea pig tracheal ring was studied in the absence or the presence of glibenclamide. For this purpose, 10 μM glibenclamide was injected in the organ bath after the plateau of the contraction was reached, and then 31.6 to 562.3 μg/mL of the F3´ fraction were cumulatively injected every 5 min[8] followed by the calculation of the EC_{50} for each treatment.

Data analysis

Values of EC_{30} and EC_{50} were calculated by linear regression.[9] All values are shown as the mean ± SEM of at least six experiments. The differences among these values were statistically calculated by one-way analysis of variance (ANOVA), and then determined by Dunnett´s test. The differences were considered statistically significant if the p-value was less than 0.05.

RESULTS

Bioassay-guided fractionation

The relaxing activities of different extracts of *C. mexicana* on carbachol (3 μM) pre-contracted tracheal rings are given in Figure 2. The hexane extract was more potent (EC_{30} = 224.9 ± 8.8 μg/mL) and more active, (its maximum effect value (Emax) was 52.9 ± 2.3%) than the dichloromethane and methanol extracts. The Emax values of dichloromethane (Emax = 36.4 ± 3.2%) and methanol (Emax = 36.9 ± 4.0%) were significantly different ($p<0.05$) than the hexane extract. Of the six (F1 to F6) fractions obtained from silica gel separation of hexane extract, F2 and F3 were found to be the most active fractions (Figure 3), with the EC_{30} = 246.2 ± 6.7 μg/mL and 253.0 ± 5.6 μg/mL, respectively. These values are not significantly different ($p<0.05$). Then F2 and F3 were each separated by silica gel column. Only sufficient quantities of three fractions were obtained from F2. However, when evaluated no activity was observed (data no shown). From F3 we obtained eight fractions (F1´ to

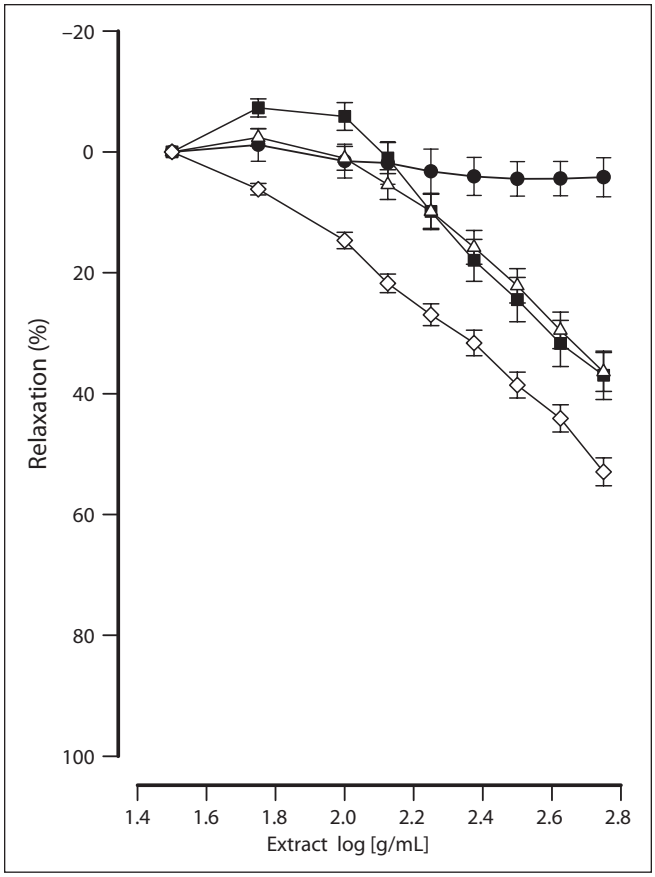

Figure 2: The relaxant effect of the (◇) hexane, (△) dichloromethane and (■) methanol extracts of Crataegus mexicana (31.62 to 562.34 µg/mL) or (●) control (vehicle) on carbachol (3 µM) induced precontraction in guinea pig tracheal rings. Each point value represents the mean ± S.E.M. (n = 6)

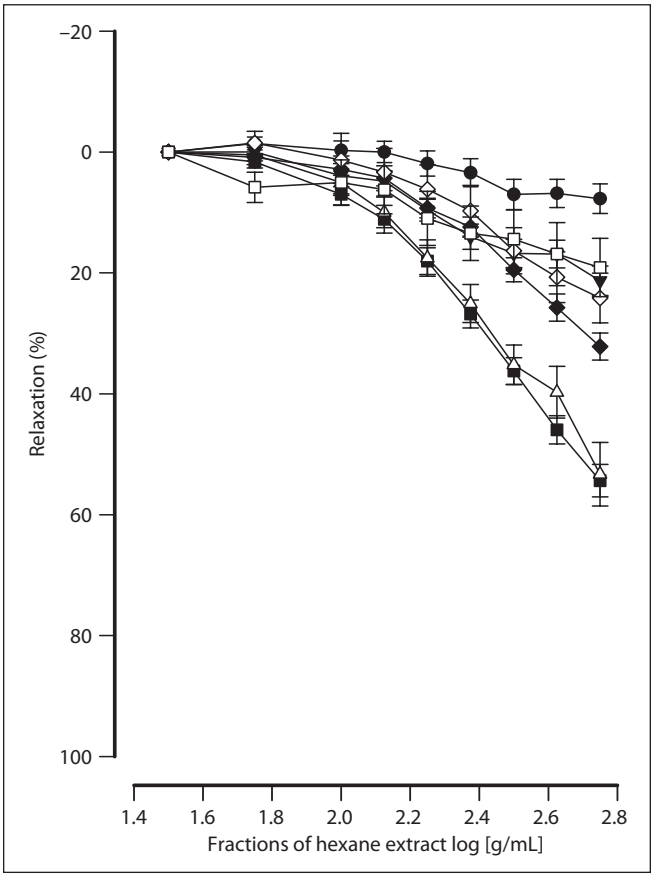

Figure 3: The relaxant effects of (◘) F1, (■) F2, (△) F3, (◆) F4, (◇) F5 and (□) F6 fractions of the hexane extract (31.62 to 562.34 µg/mL) or (●) control (vehicle) on carbachol (3 µM) induced precontraction in guinea pig tracheal rings. Each point value represents the mean ± S.E.M. (n = 6).

F8´), fraction F3´ being the most active, with a maximum relaxant effect of 97.5 ± 3.8% (Table 1) and obtained a value of EC_{50} = 285.9 ± 6.2 µg/mL. Assays by HPLC-MS of fraction F3´ reveal that at least 14 compounds may exist in it (Figure 4).

Effect of the hexane extract on the histamine or KCl pre-contracted guinea pig trachea ring

When 30 µM of histamine was used to contract the tracheal rings, the hexane extract produced a concentration-dependent relaxation on the pre-contracted organ (Figure 5A), with an Emax = 118.2 ± 6.7% and an EC_{50} = 144.0 ± 9.3 µg/mL. In the same way, when 40 mM of KCl was used to induce the contraction of the rings, the hexane extract produced a concentration-dependent relaxation (Figure 5), with an Emax = 88.5 ± 4.7% and an EC_{50} = 303.5 ± 8.2 µg/mL. Salbutamol used as relaxant standard drug was able to relax the histamine, KCl and carbachol induced pre-contraction (data no shown). The Emax values of all these evaluations were significantly different from those of the control (vehicles).

Table 1. Emax (%) values of the second fractionation against carbachol (3 µM) induced precontractions.

Fraction (562 µg/mL)	Emax (%)
Control	26.2 ± 4.3
F1´	50.4 ± 4.7*
F2´	69.3 ± 3.8*
F3´	97.5 ± 3.8*
F4´	53.0 ± 4.0*
F5´	45.5 ± 3.2*
F6´	56.5 ± 3.6*
F7´	29.7 ± 2.6
F8´	36.0 ± 3.9*

Values are presented as the mean ± SEM (n=6, n being the number of experiments). *p < 0.05 ANOVA followed by the Dunnett´s test.

Effect of propranolol and glibenclamide on the relaxant effect of the F3´ fraction

The effect of the F3´ fraction was modified significantly ($p<0.05$) by pretreatment with the β-adrenoceptor antagonist, propranolol, at 3 µM (Figure 6A). The EC_{50} values were 229.8 ± 9.0 and 278.2 ± 8.7 µg/mL in the presence or absence of propranolol, respectively. Propranolol completely blocked

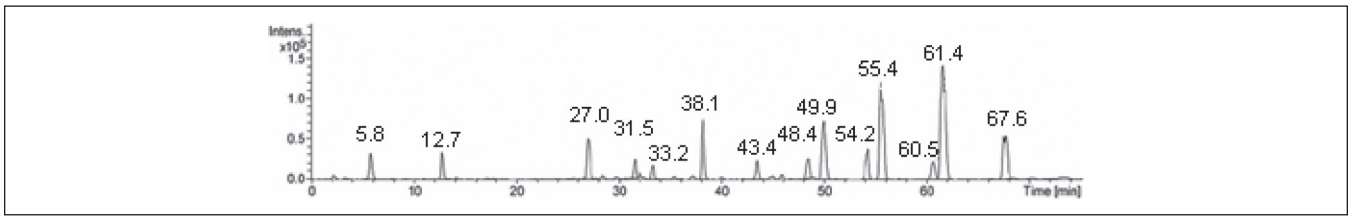

Figure 4: HPLC spectra of F3´ of *Crataegus mexicana*. Retention times of major peaks are indicated.

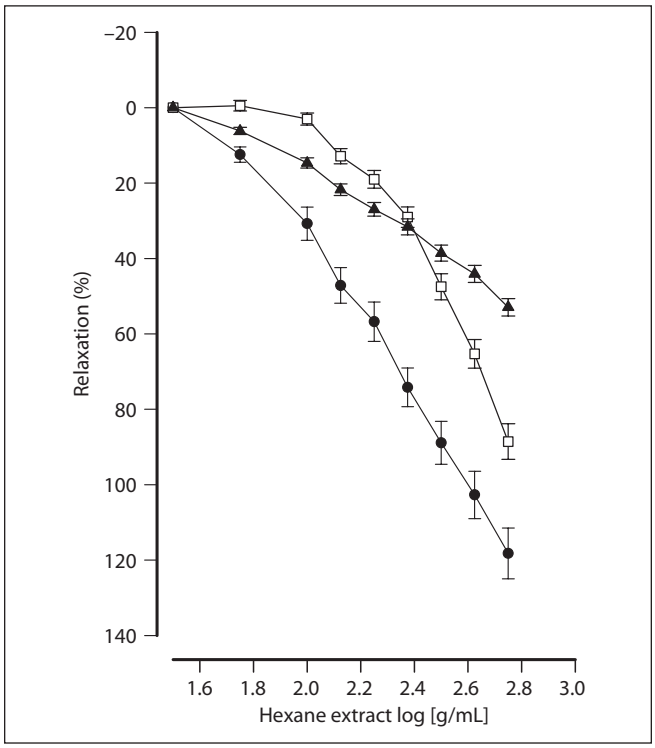

Figure 5: The relaxant effect of the hexane extract (31.62 to 562.34 µg/mL) on the (●) histamine (30 µM), (□) KCl (40 mM) or (▲) Carbachol (3 µM) induced precontraction in guinea pig tracheal rings. Each point value represents the mean ± S.E.M. (n= 6).

the salbutamol (as a relaxant standard drug) relaxant effect after the carbachol induced contraction (data are not shown). Also, glibenclamide (10 µM), an ATP-sensitive potassium channel blocker, did not affect the log concentration-response curves of the F3´ fraction (Figure 6B).

DISCUSSION

In this study we provide preliminary scientific support to the popular practice of employing *Crataegus mexicana* in the treatment of respiratory diseases. It was found that extracts obtained from the leaves of this plant have a mild relaxant effect in the tracheal smooth muscle of the guinea pig model in preparations precontracted with carbachol. The hexane extract was the most active (Figure 2), but interestingly its effect was even greater with the tracheal muscle precontracted with other contractile agents, such as histamine or KCl, than with carbachol (Figure 5). As has been reported, the contractions induced by histamine and KCl are mainly dependent on Ca^{2+} from the extracelular medium, specifically an increased Ca^{2+} influx across the membrane,[10] and can be eliminated by voltage-operated calcium channels blockers.[11] Considering the aforementioned, in the relaxant effect of the hexane extract a reduction of the Ca^{2+} influx through calcium channels could possibly be implicated. However, further studies are needed to corroborate this idea.

The bioassay-guided study was performed following the relaxation of tissues precontracted with carbachol. F2 and F3 obtained from the first fractionation of the hexane extract were active, presenting a 54.3 ± 2.6 and 53.3 ± 5.2% maximum relaxant effect, respectively (Figure 3). However, the similar relaxant effect of F2, F3 and the hexane extract (Figure 2) suggest that more than one of the compounds of the plant contribute to the relaxant effect.

On the other hand, neither of the three fractions obtained from F2 were able to produce a relaxant effect alone (data not shown), suggesting that the compounds present in F2 need to be together in order to produce the relaxant effect.

Some authors consider that the action of the *Crataegus* extract is attributable to a complex of active compounds, which can be termed the synergic effect.[12] In fact, many studies have been conducted to ascertain if hawthorn extracts can exert any therapeutic benefits in the treatment of cardiovascular disease or delay its onset. Frequently whole plant extracts and/or flavonoid combinations have been used as opposed to specific isolated classes of phytochemicals. It has been found that the separation of phenolic extracts of *Crataegus* into individual compounds does not appear to be beneficial regarding their anti-oxidant effects, as mixtures of compounds tend to exert stronger effects than those of individual compounds at the same concentrations.[12] Interestingly, when F3 was separated in the second fractionation, we found that F3´ presented the maximum relaxant effect (Table 1). The HPLC-MS (Figure 4) results showed that this active fraction contained a highly complex mixture. Further separation of F3´ will certainly be necessary in order to correctly identify bioactive compounds that contribute to the relaxant effect

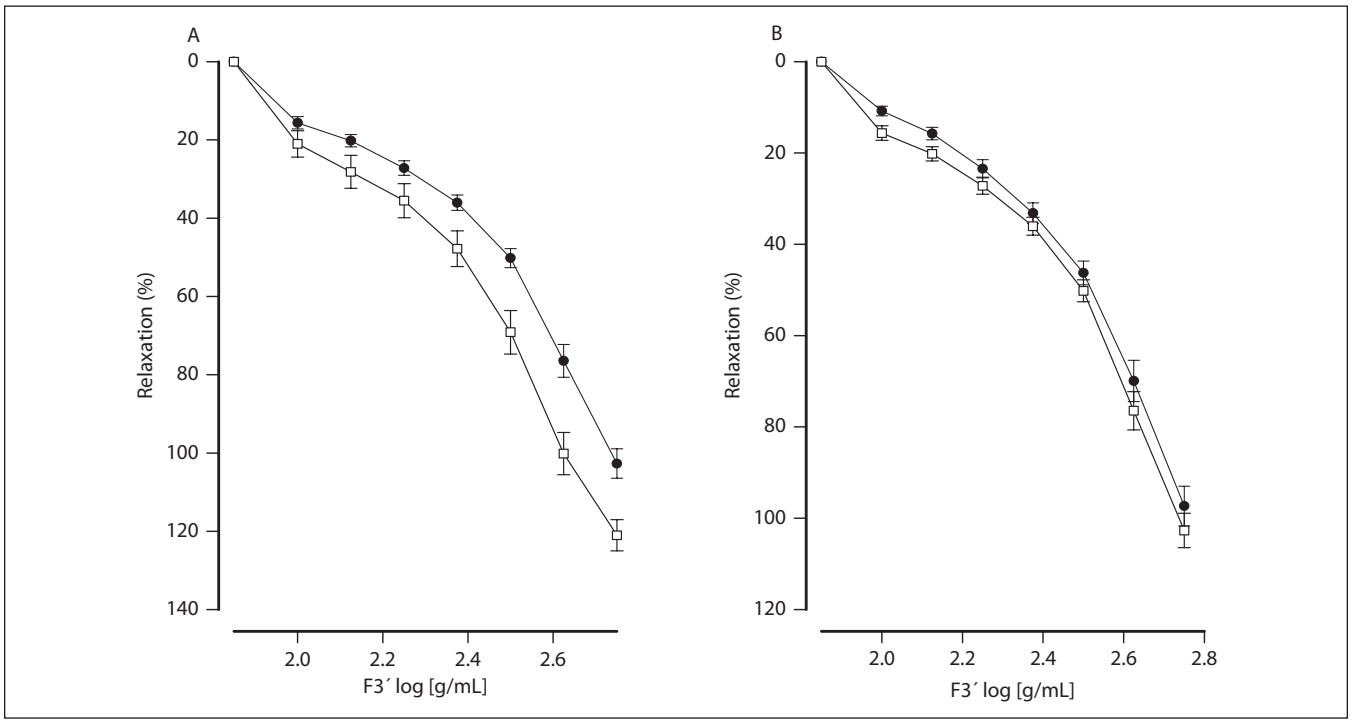

Figure 6: The relaxant effect of F3´ (31.62 to 562.34 µg/mL) on carbachol (3 µM) induced precontraction A) in the (□) absence or (●) presence of propranolol. B) in the (□) absence or (●) presence of glibenclamide. Each point value represents the mean ± S.E.M. (n = 6).

of *Crataegus mexicana*. Nonetheless, the results described in the present study provide a starting point for further investigation of multiple relaxant compounds in the *Crataegus* species.

In an attempt to provide information about the mechanism of the relaxant effect of F3´, the participation of the β-adrenergic receptor was evaluated by pretreating the rings with propranolol (a β-adrenoceptor antagonist). The results show that the EC_{50} of F3´ increased by the pretreatment with propranol, which suggests that the effect is in part related to the activation of β_2-adrenergic receptors (Figure 6A). Since the pretreatment with glibenclamide did not affect the relaxant effect of F3´ (Figure 6B), the participation of ATP-sensitive K^+ channels can be discarded.

CONCLUSION

This study represents the first in which the relaxant effect of leaves of *C. mexicana* on tracheal rings of guinea pig was clearly demonstrated. Of the extracts of *C. mexicana*, hexane was the most active, its relaxant effect being independent of the contractile agent used. In the biossay guided study, F3´ was identified as the main relaxing fraction, with an activity related to a mild β-adrenergic participation and unrelated to K^+ channels. More studies are required to correctly identify the bioactive compounds that contribute to the relaxant effects of *Crataegus mexicana*, and to know the mechanisms of action of these compounds.

ACKNOWLEDGEMENTS

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Protective Responce of Methnolic Extract of *Garcinia Indica* Fruits on CCl_4 Induced Liver Damage

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ABSTRACT

Garcinia indica commonly known as "kokum" is widely used in different parts of India for the treatment of obesity. The present study was to evaluate the protective response of methanolic extract of *Garcinia indica* fruits on CCl_4 induced liver damage. Chronic liver diseases commonly result in liver fibrosis. Carbon tetra chloride (CCl_4) is widely used for experimental induction of liver fibrosis. It is a potent heptatoxin producing centrilobular necrosis which causes liver injury. Five groups each of 6 rats were used. First group (normal control) was given 1 ml of liquid paraffin /kg b.wt. daily for 60 days and kept as normal control. Rats of the second group were given the same dose of liquid paraffin, in addition CCl_4 (30% in liquid paraffin) was given in a single oral dose of 1 ml /kg b.wt, for every 72 hours. Rats of the third, fourth and fifth groups were pre-treated orally with 200,400 mg/kg of methanolic extract of *Garcinia indica* suspended in 1% CMC and 25 mg/kg b.wt of silymarin Serum was separated and used for various biochemical estimations. Liver and kidney were collected in ice-cold containers, washed with saline, homogenized with appropriate buffer and used for the estimation of protein, liver enzymes (SGPT & SGOT), ALP, hepatic TBARS, Glycogen content, catalase activity, liver Na^+-K^+ ATPase activity and glutathione. Results of this study revealed that *Garcinia indica* could afford a significant protection in the alleviation of CCl_4 induced hepatocellular injury.

Key words: *Garcinia indica*, hepatoprotective activity, silymarin and ccl_4

INTRODUCTION

Liver is the most vital organ concerned with the biochemical activities in human body. The main role is to detoxicate the toxic substances.[1] Liver diseases remain one of the serious health problems. The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8 - 30%) compare to that of developed countries (2 – 3 %) with a similar dose schedule.[2] Medicinal plants are backbone of Indian traditional system of medicine. However only a small portion of hepatoprotective plants as well as formulations used in traditional of medicine of pharmacologically evaluated for their safety and efficacy.[3] In India, about 40 polyherbal commercial formulations are reputed to have hepato protective action it has been reported that 160 phytoconstituents from 101 plants have hepatoprotective activity. Herbal drugs are prescribed widely even when their biologically active components are unknown because of their effectiveness, fewer side effects relatively low cost.[4]

Garcinia indica, also known as kokum, is a plant native to tropical Asian, African and polynesian countries.[5] kokum is an underexploited fruit tree species found in tropical rain forests of Western Ghats of India, Konkana, North Kanara, South Kanara, Bombay, Goa and Coorg.[6] The extract obtained from *Garcinia indica* fruits is an herbal preparation that has been reported to have many medicinal properties[7] including antiulcer activity. This gastroprotective effect seems to be related an ability to decrease acidity and increase mucosal defence[8]. Additionally the extract is reported antioxidant,[8,9] anticancer, antibiotic, suppressed colonic abrrant cypt foci formation,[10] induction of apoptosis in human leukemia HL-60 cells and anti inflammatory[8] in experimental animals. It is traditionally home remedy in case of flatulence, heart strokes, liver disorders and infections.[11] *Garcinia indica* or kokum contains other compounds with potential anti oxidant properties. These include citric acid, malic acid, polyphenols, carbohydrates,[8] ascorbic acid and anthocyanin pigments.[13] In view of the reported hepatoprotective activity of *Garcinia indica* and traditionally claims the fruits of *Garcinia indica* fruit

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was evaluated against CCl_4 induced hepatic damage in rats with the aim of developing a natural protective drug.

MATERIALS AND METHODS

Materials: Fresh kokum (*Garcinia indica*) fruits were procured from the orchards near Mangalore in the month of April 2009 and identified and authentified by Botanical Science of India, Coimbatore, and Tamil Nadu and voucher submitted for the herbacium.

Preparation of photochemical extract: The fresh fruits are washed and cut into four equal pieces (runds) parallel to the major axis, then ground after the removal of seeds. Then fruits were dried under sunshade for 6-7 days and coarsely powdered. The powder was extracted using soxhelt apparatus with methanol 2000 ml.The methanol was distilled condensed using rotatory vacuum evaporator and stored in desicator. The powder of the extract was suspended in appropriate solvent system and was subjected for qualitative phyto constituents and indicated the presence of carbohydrates, flavonids, citric acid and malic acid.[14,15]

Chemicals and Drugs: Silymarin was purchased from Micro labs, Hosur, Karnataka, India. Carbon tetra chloride(CCl_4), 1-chloro-2, 4-dinitrobenzene(CDTNB), Di thio bis-2-nitrobenzoic acid (DTNB), Trichloro acetic acid (TCA) were purchased from SICCO Research Laboratory, Bombay, India. Bovine serum albumin (BSA) was purchased from Sigma Chemicals, St.Louis , USA and Thiobarbituric acid from Loba Chime ,Bombay, India.

Animals: Male wistar albino rats (200-250 gm) procured from the National Institute of Nutrition, Hyderabad. Housed in clean poly propylene cages and maintained at standard environmental conditions. They were fed with standard pellet diet (Hindustan liver, Bangalore) and water ad *libitum* during quarantine period. All procedures completed with the norms of the Animal Ethics Committee of our institution.

Toxicity studies: *Garcinia indica* in the dose range 200-2000 mg/kg were administered orally to different groups of rats comprising of 6 rats in each group. Mortality was observed after 72 hrs. Acute toxicity was determined according to the method Litchfield and Wilcoxon.[38]

Carbon tetra chloride induced hepato toxicity: The experiment was carried out following the method[16] with some modifications. The rats were divided into five groups (n = 6). First group (normal control) was given 1 ml of liquid paraffin /kg b.wt. daily once in every 72 hrs for 60 days were administered in animals from Group II-V. Group II served as CCl_4 and was not treated with any drug methanolic extract of *Garcinia indica* (MEGI) at the dose of 200 and 400 mg/kg once daily were administered orally to the animals in Group III and Group IV respectively for 60 days. Standard drug Silymarin at the dose of 25 mg/kg was administered similarly to the animals in Group V. After 24 hrs of the last dose blood was collected from retro orbital plexus under ether anesthesia. The blood samples were allowed to clot and the serum was separated by centrifugation at 2500 rpm at 37°c and used for the assay of bio chemical marker enzymes. Immediately after collecting blood the animals were sacrificed and liver dissected out for biochemical studies SGOT, SGPT, alkaline phosphate (ALP)[17,18] and bilirubin[19] were determine by using commercially available kids (Span diagnostic limited, surat, India). Liver tissues were analyzed for content of glutathione, level of catalase activity,[20] glycogen content,[21] Total protein, Liver Na^+- K^+ ATPase Activity[22] and Thiobarbituric acid reactive substances.[23]

Histopathological studies: The tissues of liver were fixed in 10% formalin and embedded in paraffin wax. Sectoins of 4-5 microns and stained with haematoxylin. Eosin and histopathological observations were made under light microscope.[24]

Statistical analysis: The results are expressed as mean ± S.D. The difference between experimental groups were compared by one-way ANOVA (Toxic control Vs treatment. Bonferrioni's method; using Jandal Scientific, Sigmastat statistical software, version 1.0) and were considered statistically significantly when $p< 0.005$.

RESULTS

The acute oral toxicity study of methanolic extract *Garcinia indica* showed no mortality up to 2000 mg/kg.

The effect of MEGI on serum transaminases (SGOT), serum phosphatases (SGPT), alkaline phosphatases (ALP), bilurubin, total serum protein and TBARS level in CCl_4 intoxicated rats are summarized in Table 1.The effect of MEGI on Glycogen (GLY), catalase activity, Na^+- k^+ ATPase activity and Glutathione content (GSH) were summarized in Table 2.

Histological studies also provided Supportive evidence for bio chemical analysus. Histology of the liver section of normal control animals showed normal hepatic cells each with well preserved cytoplasm, prominent nucleus and nucleolus and well bought out central vein (Figure 1). The liver sections of CCl_4 intoxicated mice showed massive fatty changes, necrosis, ballooning

Table 1: Effect of MEGI on some serum bio chemical parameters of CCl_4 intoxicated rats.

Parameters	Normal group	CCl_4 treated group	MEGI (200 mg/kg)	MEGI (400 mg/kg)	Silymarin (25 mg/kg)
Liver wt (gm)	6.95 ± 0.3	5.78 ± 0.5	6.30 ± 0.2	6.57 ± 0.32	7. 01 ± 0.9
SGOT (IU/l)	61.13 ± 10.03	210.56 ± 14.87	164.00 ± 13.37	106.02 ± 12.49	92.28 ± 10.96
SGPT (IU/L)	48.26 ± 8.53	110.16 ± 11.19	84.05 ± 8.79	69.76 ± 7.92	60.08 ± 6.57
ALP (IU/L)	129.18 ± 8.62	392.49 ± 12.29	223.51 ± 14.89	197.52 ± 12.62	163.25 ± 10.08
Bilurubin (mg/dl)	0.90 ± 0.24	2.91 ± 0.38	1.62 ± 0.25	1.13 ± 0.46	1.05 ± 0.22
Total protein	7.03 ± 0.60	5.93 ± 0.72	7.10 ± 0.88	7.25 ± 0.99	7.49 ± 0.85

Values are mean ± S.D (n = 6). CCl_4 control group compared with all the treatment groups: p<o.oo5

Table 2: Effect of MEGI on Glycogen, Lipid peroxidation, Catalase activity, Na^+-K^+ATP ase and glutathione content of CCl_4 intoxicated rats.

Parmeters	Normal group	CCl_4 treated group	MEGI (200 mg/kg)	MEGI (400 mg/kg)	Silymarin (25 mg/kg)
Glycogen (mg./gwet tissue)	69.98 ± 2.6	52.21 ± 2.16	55.15 ± 6.21	57.76 ± 0.32	66.29 ± 1.29
Lipid peroxidation (µ moles MDA/g liver)	40.75 ± 4.34	115.29 ± 10.86	59.84 ± 6.95	49.84 ± 5.85	40.93 ± 4.38
Catalase activity (Unit/g liver)	2.28 ± 0.31	0.92 ± 0.29	1.62 ± 0.71	2.96 ± 0.28	2.10 ± 0.23
Na+-K+ATP ase (µ/mg protein)	7.96 ± 0.45	5.21 ± 0.91	6.01 ± 2.92	6.92 ± 0.15	7.62 ± 0.90
GSH(µg/g of liver)	22.56 ± 4.65	10.16 ± 1.28	13.92 ± 1.89	29.79 ± 3.61	39.76 ± 16.58

Values are mean ± S.D (n = 6). CCl_4 control group compared with all the treatment groups: p<o.oo5.

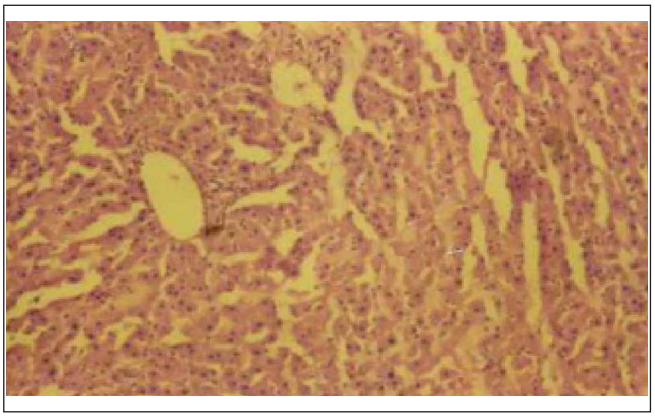

Figure 1: Hepatocytes of the normal control group showed a normal lobular architecture of the liver.

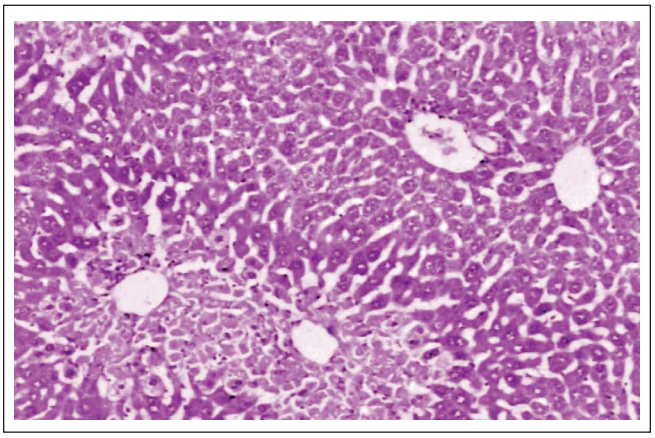

Figure 2: Hepatocytes of the CCl_4 treated group showed liver cell necrosis and inflammation also observed in the centrilobular region with portal triaditis.

degradation , broad infiltration of the lymphocytes and kuffer cells around the central vein and the loss of cellular boundaries(Figure 2). The animals treated with 200 mg/kg dose of methanolic extract of *Garcinia indica* exhibited only mild to moderated necrosis and lymphocyte infilteration (Figure 3). However, moderate accumulation of fatty globules (Figure 4) was noticied in the sections of animals treated with 400 mg/kg dose of methanolic extract of *Garcinia indica*. The sections of liver taken from the animals treated with standard drug silymarin showed the hepatic architecture, which was similar to that of control group (Figure 5).

DISCUSSION

Carbon tetrachloride is one of the most widely used chemical for the screening of hepato protective drugs.[25] It is well documented that carbon tetra chloride is biotransformed under the action of cytochrome P_{450} in the microsomal compartment of liver to trichloro methyl radical (CCl_3.).[26,27] Radical which readily reacts with molecular oxygen to form trichloro methyl peroxy radical[27] attack the cell membrane and leads to membrane damage, alteration in the structure and function of cellular membrane by forming covalent bonds with macro molecules and induce peroxidative

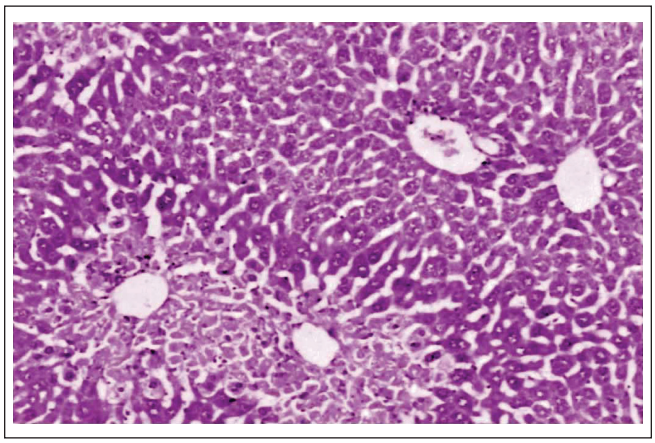

Figure 3: Hepatocytes of the CCl_4 treated group showed liver cell necrosis and inflammation also observed in the centrilobular region with portal triaditis.

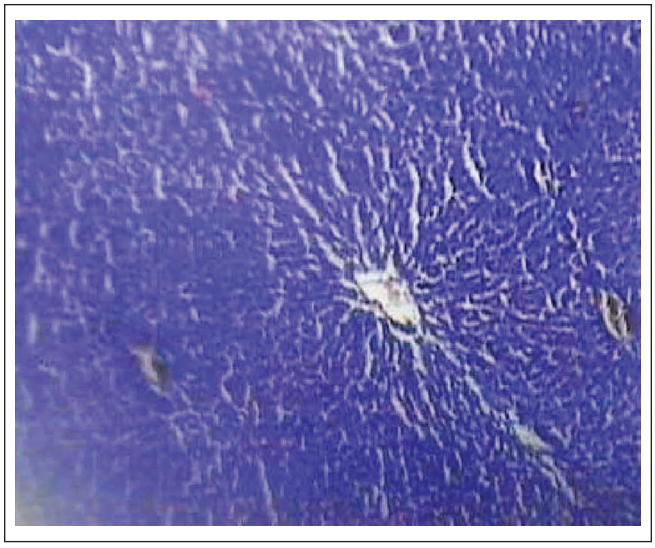

Figure 4: Hepatocytes of the MEGI pretreated group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal.

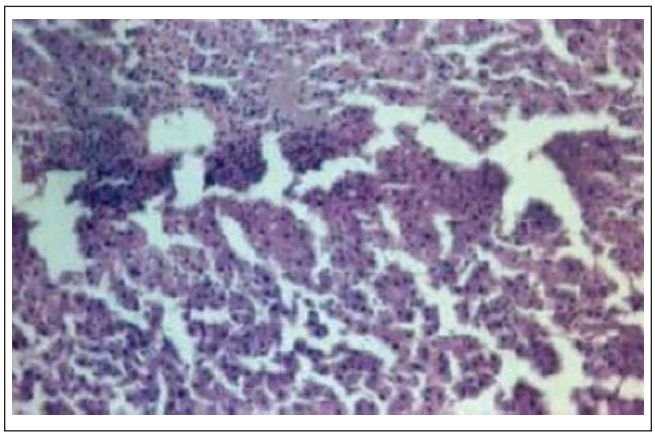

Figure 5: Microphotograph of liver section from Silymarin and CCl_4 treated rat.

degradation of the lipids of endoplasmic reticulum rich in poly unsaturated fatty acids.[28] This leads to the formation of lipid peroxide followed by pathological changes such as depression of protein synthesis,[29] elevated levels of serum marker enzymes such as SGPT, SGOT, ALP[30] and bilirubin[31] and released into circulation after cellular damage. Depletion of glutathione content, catalase activity[32] and increased in lipid peroxidation[28] is a better markers for the hepato cellular damage.[34,33]

The significant of SGPT, an enzyme found primarily in liver, is far greater enhanced and released into the blood stream is the result of liver abnormality. If therefore serve as a fairly specific indicator of liver status and it's elevated levels in serum indicates liver damage. MEGI reduces the SGPT levels indicating its protective effect over liver and important in liver functional efficiency.

SGOT is an enzyme found primarily in the cells of the liver, heart, skeletal muscles, kidneys, and pancreas and to a lesser extent in red blood cells. Its serum concentration is in proportational to the amount of cellular leakage or damage. It is released into serum in larger quantities when any one of these tissue is damaged. Its increased levels are usually associated with liver disease or heart attacks. MEGI decreased the SGOT level, which is an indication of the protective effect on liver and heart.

The raise in the levels of serum bilirubin is most sensitive and confirms the intensity of Jaundice.[35] MEGI decreased the serum bilirubin level. It is an indication that MEGI fruit has liver protective response.

Liver is damaged with CCl_4 indicates that increase the level of lipid peroxidation values because free radicals induced peroxidation. MEGI decreases the level of lipid peroxide values when compared to CCl_4 toxicated rats. The content of lipid peroxidation value is increased in MEGI extract treated groups when compare to silymarin treated group.

An increased level of ALP indicates bones disease, liver disease or bile tract blockage. Increase in serum ALP is due to increased synthesis, in presence of increasing biliary pressure[36] in CCl_4 toxicated rats. MEGI has reduces the level of increased of serum ALP

CCl_4 causes the decreases in the number of hepatocytes which in turn may result into decreased hepatic capacity to synthesize protein, glycogen and consequently reduces in liver weight. But, when the MEGI was given along with CCl_4 the significant increase in total protein and glycogen was observed indicating the hepato protection in the liver.

The activities of Na^+ -K^+ ATPase are decreased in CCl_4 induced animals. MEGI prevented this effect of CCl_4. Therefore; MEGI may be useful agent for normalization of CCl_4 induced impaired membrane function and adrenal cortex.[37]

Glutathione is an important endogenous antioxidant system that is found in particularly high concentration in liver and it is known to have key functions in protective processes. The reduced form of GSH becomes readily oxidized to GSSG on interacting with free radicals. Excessive production of free radicals resulted in the oxidative stress, which leads to damage of macromolecules e.g. lipids, and can induce lipid peroxidation *in-vivo*. In our study, CCl_4 treatment produced the depletion in glutathione (GSH). Post-treatment of the rats with MEGI significantly increased the concentration of GSH. These results suggest that the hepato protective action of MEGI might be due to the presence of antioxidants like Poly phenols.

However, this is also proved by measuring catalase activity in different groups. MEGI at the dose of 400 mg/kg not only shows better improvement in catalase activity than the silymarin treated group but also increase catalase activity even more than the normal animals (Table 2).

A comparative histopathalogical study of the liver from different groups further corroborated the hepatoprotective potential.

Possible mechanism that may be responsible for the protection of CCl_4 induced liver damage by free radical scavenger intercepting those radicals involved in CCl_4 metabolism by microsomal enzymes.

CONCLUSION

MEGI is a promising hepato protective agent. The hepatoprotective action combined with antioxidant activity has a synergistic effect to prevent the process of initiation, oxidative stress and progression of hepatocelluar damage.[37]

ACKNOWLEDGEMENT

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Antioxidant and DNA Damage Preventive Properties of *Centella asiatica* (L) Urb.

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ABSTRACT

Centella asiatica (L.) (Apiaceae) is commonly known as Mandukaparni. It is distributed in South America and all around Asia. It is a well known medicinal plant in Ayurveda system of medicine being used for various ailments like inflammation, diarrhea, asthma, tuberculosis, depression, memory loss and psoriasis. The aim of the present study was to assess the antioxidant potential *in vitro* of extracts of *Centella asiatica* in different solvents like hexane, chloroform, ethyl acetate, acetone, methanol and water. Highest polyphenols content was found in chloroform extract followed by methanol extract (9.04 µg/mg, 7.7 µg/mg gallic acid equivalents) and flavonoid content was found to be highest in water followed by chloroform extract (2.19 µg/g, 2.00 µg/g) respectively. The IC_{50} value of the DPPH and hydroxyl radical scavenging activity of methanol extract showed 0.07 mg/ml and 500 µg/ml respectively. Reducing power assay results also followed in the same way. Methanol extract was comparatively effective in preventing more DNA damage. The results obtained in this study clearly indicate that *C.asiatica* has a significant potential as a natural anti-oxidant and DNA damage preventing agent.

Key words: Anti-oxidant; DPPH; *Centella asiatica*; DNA damage; Reducing power

INTRODUCTION

Medicinal plants have been a useful source for the research of new biologically active compounds. Different approaches are used to select a plant for research, specially the ethno-medical data approach. Apart from the medicinal effects of traditional herbs, exploratory researches have been made and a wide variety of new biological activities from traditional medicinal plants have recently been reported, including anticancer activity.[1]

Centella asiatica (L.) Urb., syn. *Hydrocotyle asiatica* (L.) popularly known in Brazil as *Cairuçu-asiático, Centelha, Codagem and Pata-de-mula,*[2] is a cosmopolitan member of the Umbelliferae family. In India, it is commonly known as 'Mandukaparni'. In Srilanka and Indonesia it is given the name 'Thankuni Sak'. In classical Indian Ayurveda literature, it is considered to be one of the 'Rasayana' (rejuvenator) drugs.[3] *C. asiatica* has also been reported to be useful in the treatment of inflammations, diarrhea, asthma, tuberculosis and various skin lesions and ailments like leprosy, lupus, psoriasis and keloid. In addition, numerous clinical reports verify the ulcer-preventive and antidepressive sedative effects of *C. asiatica* preparations, as well as their ability to improve venous insufficiency and microangiopathy.[4-5] *Centella asiatica* contains triterpene glycosides such as centellasaponin, asiaticoside, madecassoside, sceffoleoside,[6] asiatic acid and madecassic acid.[7-8] Asiaticoside is the most abundant triterpene glycoside in the water extract and it is transformed into asiatic acid *in vivo* by hydrolysis. Although the asiatic acid has shown cytotoxic activity on fibroblast cells[9] and induces apoptosis in different sorts of cancer.[10-14]

Free radicals are known as being capable of damaging a lot of cellular components such as proteins, lipids and DNA.[15] To protect the cells from oxidative damages by free radicals, produced during oxygen metabolism, an antioxidant system is used by aerobic organisms. The main antioxidant enzymes and agents such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), glutathione, ascorbic acid and tocopherol are important for cellular protection, due to their ability to eliminate free radicals, such as reactive oxygen species (ROS).[16]

Nowadays, there is an increasing interest in the biochemical functions of natural antioxidant extracts from vegetables,

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fruits, and medicinal plants, which can become candidates to prevent oxidative damage, promoting health. The phenolic constituents found in vegetables have received considerable attention for being the main components of antioxidant activity, in spite of not being the only ones. The antioxidant activity of phenolic constituents has been attributed to its oxide-reduction properties, which play an important role in the adsorption or neutralization of free radicals.[17]

Based on its reputation to improve health condition, *Centella asiatica* has been selected for the present study and evaluated for its antioxidant properties and DNA damage protection using various *in vitro* systems.

MATERIALS AND METHODS

Plant material

Centella asiatica (L.) Urb. Leaves were collected from local market and identified with the help of herbarium collection, Department of Botany, University of Mysore, Mysore. The leaves were allowed to dry in shade for three days. The shade dried leaves were taken for further studies.

Chemicals and reagents

Hexane, chloroform, ethyl acetate, acetone, methanol and distilled water were used as solvent for extraction of antioxidant compounds. Other solvents and chemicals used in this study were dimethyl sulphoxide (DMSO), petroleum ether (40-60° C), H_2SO_4, NaOH, HCl, H_3BO_3, DPPH (1,1,Diphenyl 1-2,picryl hydrazyl), BHA, Gallic acid, Folin-Ciocalteu reagent, $FeCl_2$, ferrozine, potassium ferricyanide, NaEDTA, ascorbic acid, TCA, $FeCl_3$, Na_2CO_3, catechin, deoxyribose, H_2O_2, thiobarbituric acid (TBA). All the chemicals and reagents were of analytical grade and were stored at prescribed conditions in the laboratory.

Sequential extraction

Fifty grams of crushed leaf sample was taken for extraction procedure. Cold extraction method was followed for sequential extraction of *centella* from non polar to polar solvents viz., hexane, chloroform, ethyl acetate, acetone, methanol and water. The extract was filtered through Whatman No.1. The filtrate was flash evaporated using a round bottom flask of known weight. Each of the extract was weighed and total yield was calculated for each solvent system. A known weight of each dried extract was dissolved in the respective solvents to prepare a stock solution of 100 mg/ml. All the stock solutions were kept at 4° C until further use.

Total polyphenols estimation

Total polyphenols content of each extract was determined using folin-ciocalteu reagent (FC reagent) method.[18] Briefly, sample in different amounts was mixed with distilled water to make up final volume (3 ml). Then, 0.5 ml FC reagent was mixed and incubated for 10 min at room temperature. Two milliliters of 7% Na_2CO_3 was added and boiled the content in a boiling water bath for one minute. After cooling, absorbance was measured at 650 nm (there is no distinct absorbance maximum wavelength for polyphenols, so 650 nm wavelength was used). Gallic acid was used as a standard and amount of total polyphenols content was expressed as µg gallic acid equivalent per milligram (µg GAE/mg) extract.

Total flavonoids estimation

Estimation of total flavanoids by the method of Delcour and Varebeke.[19] Catechin was used as a standard and total flavonoids content was expressed as microgram catechin equivalents per milligram (µg CE/mg) extract. In brief, sample volume was make up with methanol to 1 ml. Then, 5 ml of Chromogen reagent (HCl + CH_3OH + Cinnamaldehyde in 1: 3: 0.004 ratio) was added to each test tube and absorbance was measured at 640 nm.

DPPH radical scavenging activity

The antioxidant activity of different extracts was checked on the basis of 1, 1 diphenyl–2–picrylhydrazyl (DPPH) free radical scavenging activity. DPPH assay was performed as per the method described by Eberhardt *et al.,*.[20] DPPH (500 µl, 0.5 mM in methanol) solution was mixed with different amounts of sample and volume was made to 3.5 ml with methanol. The mixture was incubated in dark for 45 min at room temperature. Absorbance was recorded at 515 nm in a spectrophotometer. BHA was used as standard antioxidant compound. A positive control was prepared by mixing 3 ml of methanol and 0.5 ml of DPPH solution. Sample blanks were prepared in methanol without DPPH solution to eliminate the absorbance of crude extracts. Methanol was used as blank. The DPPH radical scavenging activity percentage was calculated by using the formula as given below:

$$\text{DPPH° scavenging activity (\%)} = \left[\frac{A_c - A_S}{A_c}\right] \times 100$$

Where A_c is the absorbance of positive control solution and A_s is the absorbance of test solution. IC_{50} value, the concentration of sample or extract required to scavenge 50% of the DPPH free radicals in the mixture, was calculated using a linear regression equation derived from the graph of % DPPH scavenging activity and sample concentration.

Hydroxyl radical scavenging activity

Deoxyribose degradation assay was performed as per the method of Halliwell *et al.,*[21] with slight modification. Briefly, different concentrations of extracts were mixed with 200 mM $FeCl_3$ and 1.04 mM EDTA (0.2 ml, 1:1), 1 mM H_2O_2 (0.1 ml), 28 mM deoxyribose (0.1 ml) and 1 mM ascorbic acid (0.1 ml) and the final volume was made to 1.1 ml with phosphate buffer (0.2 mM, pH 7.2). The mixture was

incubated at 37° C for 1 hr. Then, 1 ml of thiobarbituric acid (1% in 50 mM NaOH) and 1 ml of 5% TCA was added followed by boiling in a boiling water-bath for 20 min. After cooling, absorbance of the mixture was measured at 532 nm and the percentage inhibition was calculated.

Total reducing power estimation

The total reducing power of different extracts was determined following the method of Oyaizu.[22] Different amount of extract was mixed with equal volume of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50° C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture followed by centrifugation at 3000 rpm for 10 min. A 2.5 ml portion of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

DNA damage protective activity

DNA damage protective activity of *Centella asiatica* was checked using pRSETA plasmid grown in *E. coli*. Plasmid DNA was isolated using QIA prep Spin Mini prep kit. Plasmid DNA was oxidized with H_2O_2 + UV treatment in presence of plant extract (PE) and checked on 1% agarose gel according to Russo *et al*.,[23] with minor modifications. In brief, the experiment was performed in a 10 µl volume in a micro centrifuge tube containing 200 ng of pRSETA plasmid DNA in TE buffer (10 mM Tris-Cl and 1 mM EDTA) pH 8.0. H_2O_2 was added at final concentration of 10 mM/ml with various concentrations of plant extract (1-3 µl of 10 mg/ml concentration). The reactions were initiated by UV irradiation and continued for 5 min on the surface of a UV transilluminator (312 nm) under room temperature. After irradiation the reaction mixture along with gel loading dye (6X) was loaded on to 1% agarose gel and run at 200 V for 1 hr. Untreated pRSETA plasmid DNA was used as a positive control in each run of gel electrophoresis.

RESULTS AND DISCUSSION

Plant biochemicals are gift from nature and antioxidant compounds are typical representative of these botanical gifts. Antioxidants are the substances which can protect the human body from free radicals and the ROS effects and retard the progress of many chronic diseases.[24-25] Apart from their biological functions in plants, these antioxidants are widely present in food products and agricultural raw materials. As the name antioxidant indicates that these compounds participate in oxidation-reduction processes, which have complex reaction mechanisms, so there is no single testing method capable of providing a comprehensive picture of the antioxidant profile of a studied sample. Many compounds in food have antioxidant properties, which are capable of interacting with reactive molecules. Polyphenols and flavonoids are the powerful antioxidants within bioactive constituents.

Total polyphenols and Flavonoids

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Polyphenolic compounds have a wide range of protective effects such as anti-inflammatory responses, prevention of low density lipoprotein oxidation and anti-hypertensive, anti thrombic and carcinostatic actions.[26] In this study highest polyphenols content was found in chloroform extract of *C.asiatica* followed by methanol extract, followed by water (9.04 µg/mg, 7.7 µg/mg, 6.76 µg/mg Gallic acid equivalents respectively) (Figure-1). Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties.[27] Therefore, it is valuable to determine the total flavonoids content of the extracts under study. As shown in Figure-2, maximum amount of flavonoids was found in water followed by chloroform fractions, respectively. Several studies have shown that many flavonoids contribute significantly to the total antioxidant activity of plants. There is abundant evidence that flavonoids are effective in blocking oxidant induced neuronal injury.[28]

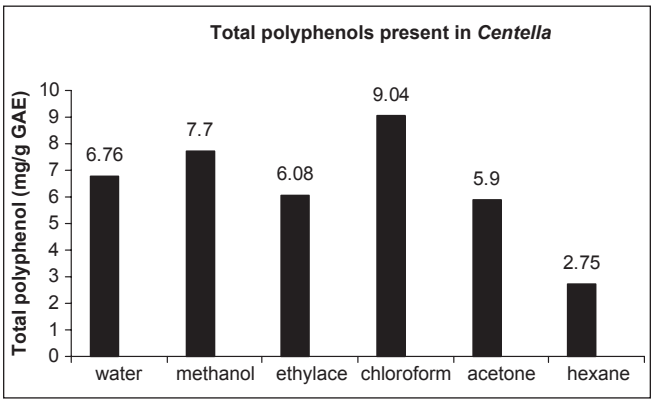

Figure 1: Total polyphenols in different extracts of *C.asiatica*

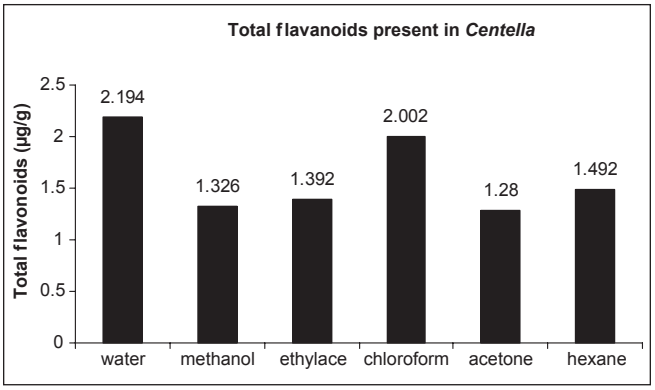

Figure 2: Total Flavonoids in different extracts of *C.asiatica*

Antioxidant activity

Determination of antioxidant activity of different solvent extracts of *Centella* was based on DPPH radical scanvenging activity and Hydroxyl radical scavenging activity (TBARS). The antioxidant activity of different extracts was calculated as their capacity to scavenge free radicals of DPPH, which

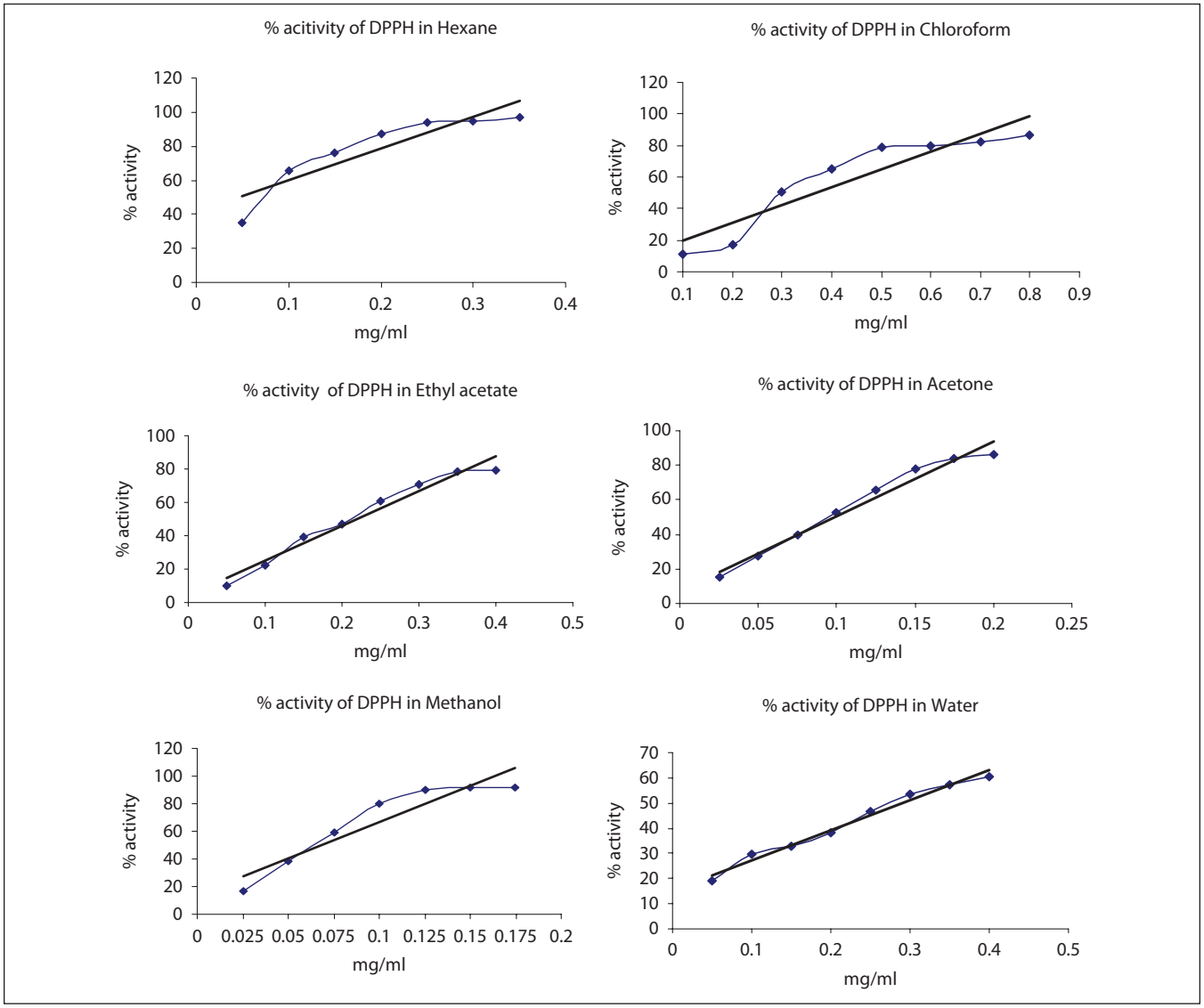

Figure 3: DPPH radical scavenging activity of different extracts of *C.asiatica*

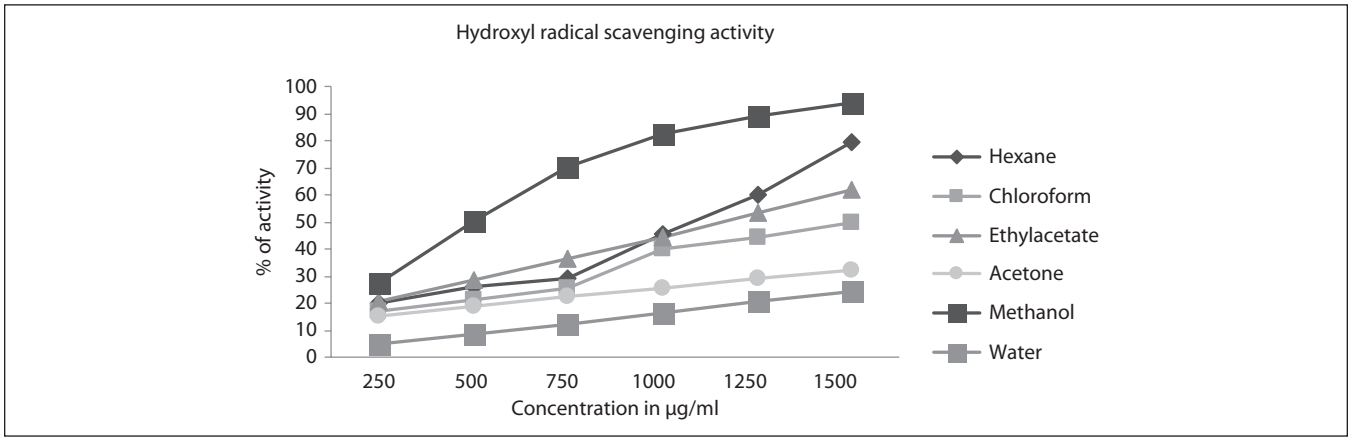

Figure 4: Hydroxyl radical scavenging activity of different extracts of *C.asiatica*

has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources.[29] Although radical scavenging activity should not be considered as being synonyms with antioxidant activity, it is a fact that almost all of the powerful natural antioxidants, such as tocopherol, carnosal and ascorbic acid are also strong scavengers of the DPPH radical. Results depicted in Figure-3 showed DPPH activity of sequential extracts of *Centella* (Hexane, Chloroform, Ethylacetate, Acetone, Methanol and Water respectively). Methanol extract was observed with highest percentage of inhibition of free radicals with IC_{50} values (0.07 mg/ml), while hexane fraction is least potent. The highest free radical scavenging activity of methanol extract can be attributed to the presence of polyphenols and flavonoids as this fraction contains maximum amount of these secondary metabolites.

Hydroxyl radicals are most reactive ROS, capable of attacking most of the biological substrates. The prevention of such deleterious effect is very necessary in terms of both human health and the shelf-life of food, cosmetics and pharmaceuticals. Therefore, it was considered important to assess the protective ability of the sample extract against ·OH radicals. In the ·OH radical – mediated 2-deoxy-D-ribose degradation assay, ·OH radicals are generated by Fenton chemistry using EDTA, Fe^{3+} ions, ascorbic acid and H_2O_2. Six sample extracts (Hexane, Chloroform, Ethyl acetate, Acetone, Methanol and Water) were taken for estimation of OH radicals scavenging activity. IC_{50} values were calculated using linear regression equations. Methanol extract showed highest hydroxyl radical scavenging activity (IC_{50}=500 µg/ml). Graphical presentation of the data has been shown in Figure-4. Ferric ion reduction to ferrous ion reducing power was observed more in methanol extract followed by water extract (Figure-5).

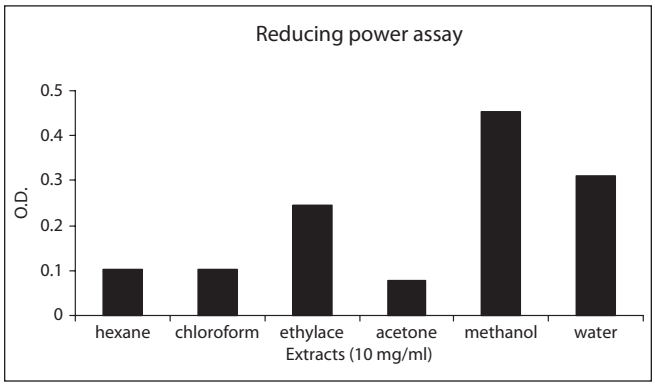

Figure 5: Reducing power activity of different extracts of *C.asiatica*

DNA damage protective activity

DNA damage protection studies were performed using methanol and water extracts. Protection of DNA strand breaks was observed more in methanol extract followed by water extract showed protection of supercoild plasmid is directly proportional to the activity of plant extract (Figure-6).

The faster moving prominent band (lane. 1) corresponded to the native supercoild circular DNA (Sc DNA) and the slower moving very faint band was the open circular form (Oc DNA). The UV irradiation of DNA in the presence of H_2O_2 (lane. 2) resulting the cleavage of Sc DNA to give prominent Oc DNA and a faint linear DNA (Lin DNA) indicating that OH- generated from UV-photolysis of H_2O_2 produced DNA stand scission. The *C.asiatica* extract was able to prevent this damage to a large extent.

CONCLUSIONS

Results of the present study prove that methanolic extract of *C.asiatica* possess very pronounced biological properties such as anti oxidant activity, reducing power and DNA damage protection activity. Furthermore these results of total polyphenols and flavonoids presence in hexane and methanolic extracts strengthen the biological activity.

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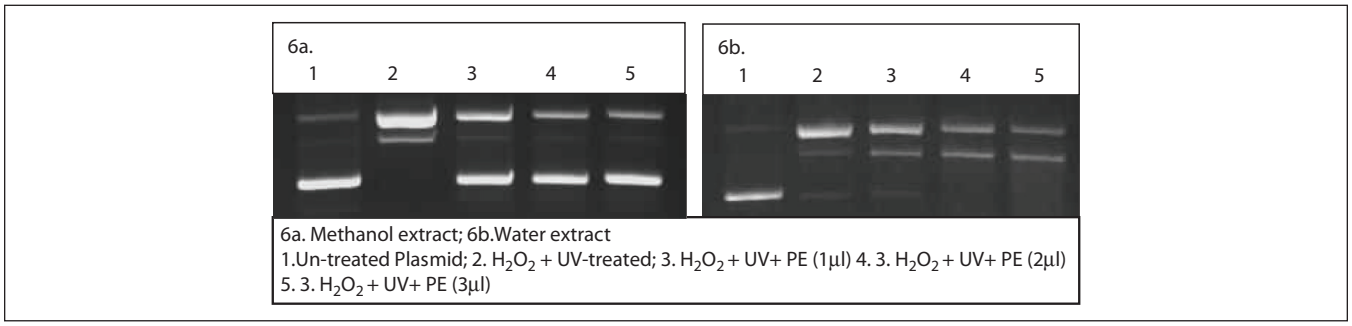

Figure 6: DNA damage protection by *C. asiatica* methanol and water extracts

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Phytopharmacological Investigation of *Aerva Lanata* Flowers with Special Emphasis on Diuretic Activity

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ABSTRACT

The hydro-alcoholic extract of flowers *Aerva lanata* was screened for its diuretic, analgesic and anti-inflammatory activities in rats. Diuretic activity was carried out as per Lipschitz et al. The 5 and 24 hrs. study of extract showed increase in urine volume and Na^+, K^+ and Cl^- ions as compared to normal saline. Urinary levels of sodium, potassium (by flame photometry) and chloride (by titrimetry) were estimated. The extract also showed analgesic and anti-inflammatory activities. The analgesic activity was studied by tail immersion method and anti-inflammatory activity was studied by carrageenan-induced paw edema in rats. The hydro-alcoholic extract of flowers of *Aerva lanata* upon phytochemical investigation revealed the presence of flavanoids, glycosides, carbohydrates, alkaloids and phytosterols.

INTRODUCTION

Aerva lanata (Linn.) Juss.(Family Amaranthaceae), commonly called 'Gorakhganja' in Hindi is distributed throughout plains of India in wastelands. It is one the most popular herb and possess many curative properties and prevent many kinds of illnesses and conditions.

It is an erect or prostrate herb, 30-60 cm in height, found in waste lands.[1] Leaves simle, alternate, short-petioled, densely tomentose, usually smaller in the flowering branches.[2,3] Flowers are very minute, pale greenish white, sessile, often bisexual, without any characteristic odour and taste. Fruits greenish, roundish, compressed utricle, seeds kidney shaped with shining black, coriaceous testa.[4]

The plant is astringent, bitter, cooling emollient, vermifuge, suppurative, diuretic and lithotriptic. It is used to treat boils, cephalagia, cough, strangury, diabetes and lithiasis. Flowers used for removal of kidney stones.[5]

The pharamacological studies have confirmed that the roots possess diuretic, anti-inflammatory, [15] anthelmintic, antibacterial and analgesic activities.[6]

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The chemical constituents are alpha-Amyrin, Campesterol, β–sitosterol, β–sitosteryl palmitate, Chrysin and four flavanoids glycosides have been reported in the plant.[7]

MATERIAL AND METHODS

The flowers of Aerva lanata were collected from Rajasthan College of Agriculture (RCA), Udaipur, Rajasthan. A specimen were submitted for authentification to Department of Botany, University of Rajasthan, Jaipur, Rajasthan. The authentification no. of Aerva lanata plant is RUBL-20543.

Preparation of extract

For extraction , first the flower powder was soaked in solvent (70% alcohol) for 2 days with occasionly shaking. After 2 days extract was filtered using muslin cloth . It was distilled with a distillation assembly to obtain the extract and solvent separation. The marc of first extraction was dried for 24 hrs. so as to remove the alcohol completely from it. Now 70% alcohol was added to dried powder to soak it, and kept for 2 days . After 2 days excess of solvent (70% alcohol) was mixed; and kept for 24 hrs. After 24 hrs. the extract was filtered using muslin cloth. Solvent was distilled to obtain the extract.

Animals

All studies were approved by the Institutional Animal Ethics Committee, B.N.Collge of Pharmacy, Udaipur (Rajasthan). Healthy rats weighing 120-150 gm were maintained on

standard rodent fed and water *ad libitum*. Animals were periodically weighed before and after the experiment. Animals were closely observed for any infection and those showing signs of infection were excluded from the study and replaced.

Pharmacological activities

Diuretic activity[8]

The Lipschitz method[14] may be employed for the assessment of diuretic activity. In this method, male albino rats weighing between 120-150 g, deprived of food and water for 18 hr prior to the experiment, were divided in 6 groups of 6 rats in each. On the day of the experiment, all the animals were administered orally, normal saline at a dose of 25 ml/kg. The Group-I of animals served as control, received only normal saline; the Group-II received furosemide (25 mg/kg) in saline; and Groups-III, IV, V and VI received the extract at dose levels of 200 mg/kg, 400 mg/kg, 800 mg/kg and 1600 mg/kg respectively in normal saline. Immediately after administration, the animals were placed in metabolic cages, specially designed to separate urine & faeces. The volume of urine collected was measured at the end of 5 hr and 24 hrs., during this period, no food and water was made available to animals. The parameters taken were total urine volume, concentration of $Na,^+$ K^+ and Cl^- in the urine. The Na^+ and K^+ concentrations were determined by flame photometry[11] and Cl^- concentration was estimated by titrimetrically[12] with silver nitrate solution (N/50) using drops of potassium chromate solution as indicator.

All results are expressed as Mean ± SEM . The data was analyzed statistically using ANOVA followed by Dunnett's Multiple Comparison test. $P < 0.05$ implies significance.

Analgesic activity[9]

The Tail Immersion method may be employed for the assessment of analgesic activity. All the animals were divided into 4 groups of 6 animals in each group and were given the following treatment. Group-I (Control) received vehicle only, Group-II received 30 mg/kg, p.o. of standard Diclofenac sodium. Group-III and IV received 400 and 800 mg/kg, p.o. of extract respectively. The animals were placed into restraining cages leaving the tail hanging out freely. The lower 5 cm portion of the tail were marked. This part of the were immersed in a cup of freshly filled water of exactly 55°C. Within a few seconds the rat reacts by withdrawing the tail. The reaction time were recorded in seconds by a stopwatch. After each determination the tail were carefully dried. The reaction time were determined periodically after oral administration of the test substance, e.g. after 30 min., 1, 2, 3, 4 and 6 hr. The cut off time of the immersion was 15 sec. A withdrawal time of more than 6 sec. were regarded as a positive response.

All results are expressed as Mean ± SEM. The data was analyzed statistically using ANOVA followed by Dunnett's Multiple Comparison test. $P < 0.05$ implies significance.

Anti-inflammatory activity[10]

The Paw edema method may be employed for the assessment of anti-inflammatory activity. All the animals were divided into 4 groups of 6 animals in each group and were given the following treatment. Group I (Control) received vehicle only, Group II received 30 mg/kg, p.o. of standard Diclofenac sodium. Group III and IV received 400 and 800 mg/kg, p.o. of extract respectively. After 1 hr. of drug treatment, all the respectively grouped animals were administered with a subcutaneous injection of 0.1 ml of 1% solution of carrageenan into the sub planter aponeurosis of the left hind paw. The paw was marked with ink at tibio tarsal joint and immersed in mercury up to this mark. The paw volume was measured plethismographically immediately after 30 min. after injection followed by 1, 2, 4 and 6 hr. in each group. The difference between the initial and subsequent reading gave the actual edema volume.

The percentage inhibition of inflammation was calculated using the formula :

$$\text{Percentage inhibition} = 100 \times \left(\frac{1 - Vt}{Vc}\right)$$

Where 'Vt' represents edema volume in test compound and 'Vc' represents edema volume in control. All the results are expressed as Mean (ml) ± SEM. The difference in paw volume at different time between test and control groups were analyzed for statistical significance by performing one-way ANOVA followed by Dunnett's Multiple Comparison test. $P < 0.05$ implies significance.

RESULTS

Table 1: Phyto-Constituents Test[13]

Hydro-alcoholic extract of Aerva lanata flowers was investigated for following constituents as tabulated below:

Phyto-Costituents	Inference
Carbohydrates	+
Glycosides	+
Alkaloids	+
Phytosterols	+
Tannins	–
Flavanoids	+

+ shows Present
– shows Negative

Table 2: Effect of *Aerva lanata* flowers extract on Excretion Parameters (5 hrs.)

Group	Treatment	Dose	Urine Volume (ml)	Na^+(mEq/lit.)	K^+ (mEq/lit.)	Cl^-(mEq/lit.)
I	Normal Saline	25 ml/kg	0.40 ± 0.005	31.03 ± 0.40	25.23 ± 0.47	28.27 ± 0.40
II	Furosemide	25 mg/kg	1.10 ± 0.011*	67.14 ± 0.39*	60.22 ± 0.67*	62.60 ± 0.49*
III	AL Extract	200 mg/kg	0.467 ± 0.008*	35.14 ± 0.33	30.17 ± 0.36	32.31 ± 0.28*
IV	AL Extract	400 mg/kg	0.683 ± 0.009*	41.06 ± 0.34*	36.18 ± 0.38	40.39 ± 0.73
V	AL Extract	800 mg/kg	0.867 ± 0.016*	51.20 ± 0.58*	43.13 ± 0.38*	44.42 ± 0.52*
VI	AL Extract	1600 mg/kg	1.017 ± 0.039*	58.93 ± 0.80	46.20 ± 0.51	48.46 ± 0.53*

Values are represented as Mean ± SEM (n = 6), * Indicates $P < 0.05$

Table 3 : Effect of *Aerva lanata* flower extract on Excretion Parameters (24 hrs.)

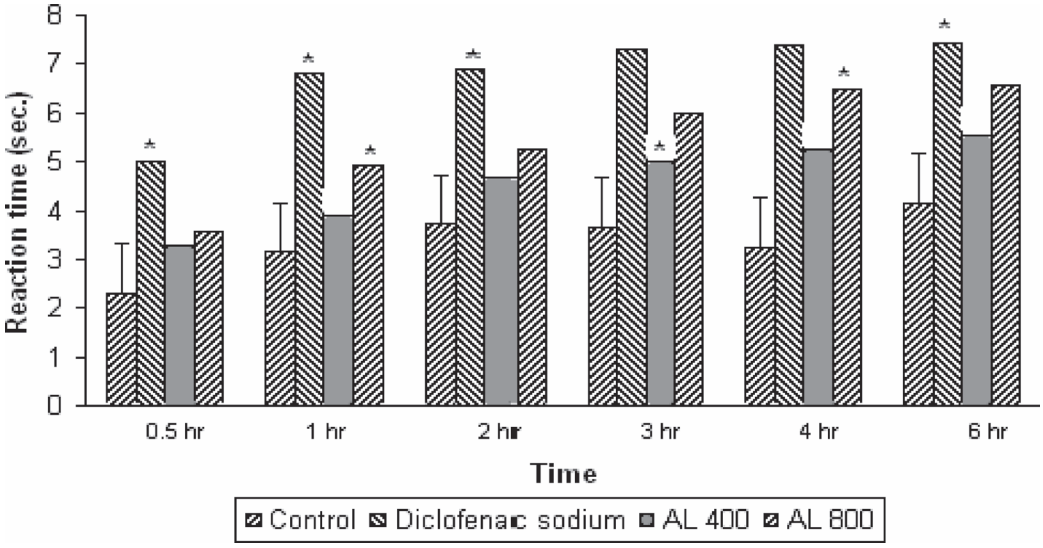
Group	Treatment	Dose	Urine Volume (ml)	Na^+ (mEq/lit.)	K^+ (mEq/lit.)	Cl^- (mEq/lit.)
I	Normal Saline	25 ml/kg	0.518 ± 0.02	38.16 ± 0.27	28.17 ± 0.40	36.35 ± 0.75
II	Furosemide	25 mg/kg	1.40 ± 0.04*	71.25 ± 0.30*	63.16 ± 0.51*	68.66 ± 1.05*
III	AL Extract	200 mg/kg	0.583 ± 0.01	39.24 ± 0.44	31.10 ± 0.71*	38.37 ± 1.36
IV	AL Extract	400 mg/kg	0.820 ± 0.03*	47.21 ± 0.65	37.12 ± 0.50	42.40 ± 1.71
V	AL Extract	800 mg/kg	1.013 ± 0.01*	63.28 ± 0.72	44.86 ± 0.96*	48.46 ± 1.69*
VI	AL Extract	1600 mg/kg	1.172 ± 0.02*	69.19 ± 0.49*	51.14 ± 0.48*	58.56 ± 0.54*

Values are represented as Mean ± SEM (n = 6), * Indicates $P < 0.05$

Table 4 : Analgesic activity of *Aerva lanata* flower extract

			Reaction time at different time interval					
Group	Treatment	Dose	½ hr	1 hr	2 hr	3 hr	4 hr	6 hr
I	Control	–	2.31 ± 0.30	3.1 ± 0.29	3.72 ± 0.40	3.67 ± 0.21	3.25 ± 0.21	4.15 ± 0.25
II	Diclofenac sodium	30 mg/kg	4.99 ± 0.70*	6.82 ± 0.36*	6.93 ± 0.33*	7.32 ± 0.34	7.39 ± 0.24	7.42 ± 0.27*
III	Extract	400 mg/kg	3.28 ± 0.24	3.89 ± 0.24	4.69 ± 0.32	5.02 ± 0.39*	5.24 ± 0.38	5.54 ± 0.36
IV	Extract	800 mg/kg	3.57 ± 0.36	4.93 ± 0.37*	5.25 ± 0.22	5.99 ± 0.52	6.48 ± 0.53*	6.57 ± 0.44

Graph (ix) : Analgesic activity of *Aerva lanata* by tail immersion method

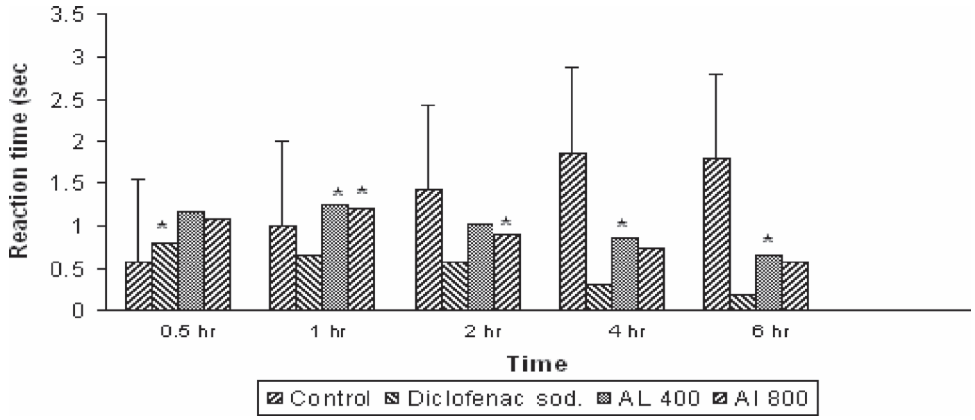


Values are represented as Mean ± SEM (n = 6), * Indicates $P < 0.05$

Table 5 : Anti-inflammatory activity of *Aerva lanata* flower extract in carrageenan –induced paw edema

Group	Treatment	Dose	Mean Paw Volume ± SEM (ml) at diff. time interval					
			½ hr	1 hr	2 hr	4 hr	6 hr	% Inhibition at 6 hrs.
I	Control	–	0.57 ± 0.07	1.00 ± 0.10	1.43 ± 0.07	1.87 ± 0.09	1.80 ± 0.10	–
II	Diclofenac sodium	30 mg/kg	0.80 ± 0.03*	0.67 ± 0.06	0.57 ± 0.03	0.33 ± 0.05	0.20 ± 0.03	88.89
III	Extract	400 mg/kg	1.16 ± 0.02	1.23 ± 0.02*	1.03 ± 0.05	0.86 ± 0.03*	0.66 ± 0.01*	63.34
IV	Extract	800 mg/kg	1.10 ± 0.08	1.20 ± 0.05*	0.90 ± 0.04*	0.73 ± 0.05	0.57 ± 0.06	68.34

Graph (x): Anti-inflammatory activity of *Aerva lanata* on Carrageenan-induced paw edema



Values are represented as Mean Paw Vol. (ml) ± SEM (n = 6), * Indicates P < 0.05

DISCUSSION

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Antibacterial and antioxidant activities of ethanol extracts from trans Himalayan medicinal plants

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ABSTRACT

The antibacterial and antioxidant activity of the ethanol extracts from some trans Himalayan medicinal plants being used in '*Amchi system of medicine*'. These plants were assessed towards selected bacteria as well as in different antioxidant models. Extracts, at concentration between 8 and 250 µg/ml, showed a significant antibacterial effect expressed as minimum inhibition concentration (MIC) against both Gram-negative and Gram-positive bacteria. In particular, samples of *Podophyllum hexandrum* leaf, *Verbascum thapsus* stem against *Bacillus subtilis* and *Salvia sclarea* flower against *Pseudomonas aeruginosa* (MIC = 8 µg/ml) were highly effective. The antioxidant activity was determined by the 2,2- diphenyl -1 picryl hydrazyl (DPPH) method, *Ephedra gerardiana* leaf (13.30 ± 0.6 µg/ml) and *Salvia* sclarea flower (14.97 ± 2.9 µg/ml) were observed with maximum activity. Conclusion of the study supports the use of these plants in traditional medicine to treat various ailments like stomach complaints, wound infections and intestinal disorders etc.

Key words: Antibacterial activity; Anti-oxidant activity; Ethnopharmacology; trans-Himalaya; Amchi system of medicine; Cold desert medicinal plants.

INTRODUCTION

Numerous useful drugs from plants have been discovered by their traditional uses.[1] Medicinal plants contain physiologically active principles include antimicrobial and anti-oxidant properties.[2] Nature is a treasure of diversified plant species possessing multiple usages to mankind. Globally, there is an increasing interest of herbal usages in the living hood set-ups.[3] Today, people all over the world are trying to keep away from chronic stress, pollution and synthetic drugs.[4] The most commonly used anti-xodant at the present time are butylated hydroxyamisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG) and tert-butylhydroxytoluene (TBHQ).[5] However there are suspected of being responsible for liver damage and carcinogenesis in laboratory animals.[6-7] Therefore the developement and utilization of more effective anti-oxidants of natural origin are desire.[8]

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The Himalayas represent the largest mountain chain in the world, covering about one million sq. km. 'Himalaya' the youngest mountain range of world is famous for its rich plant diversity and varied ecosystem, containing large number of plants. The Trans-Himalayas of Indian cold desert covers under alpine and high alpine zones with peculiar climatic condition featuring and snow covered mountains. The use of plants in curing and healing is as old as man himself.[9] Plants containing beneficial and medicinal properties have been known and used in some form or other by primitive people. Biodiversity of Trans-Himalayas is our natural wealth and its conservation is important for economic, ecological, scientific and ethical reasons. The flora of cold desert areas and their ethnobotanical importance were studied earlier by several authors.[10-12] They focused on plant diversity, its documentation and scattered ethnobotanical uses by the tribal communities. Biodiversity provides us with goods and services fundamental to our survival including food, fodder and medicine. The selection of the species used in this study was mainly based on their ethnomedicinal uses include diarrhea, dysentery, cold, cough, skin infections, healing of wounds, dandruff, respiratory infections, kidney complaints, anti-septic, etc. Some plants without ethnomedicinal prudent but not previously studied were included too.

MATERIALS AND METHODS

Study area

The field work was carried out in Nubra valley, cold desert of Himalaya's in Ladakh region. The Nubra comprises the valley of Shyok river from its acute-angled bend down to its confluence with Nubra and further towards Indus. It is northern most valley of Ladakh. Khardung La pass (18,380 ft) is the gate way of the valley and it includes Siachin glacier. The area lies between two great mountain ranges, i.e. Ladakh (on the south) and Karakoram (on the north). Approximately, 34°15' 45 to 35°30' N latitude and 76°55' to 78°05' E longitude. The topography of the valley is entirely different from other valleys of Ladakh. There is a great variation in altitude and ranges approximately between 8000 ft to 24000 ft mean sea level. The climate of the region is extreme cold desert and characterized by high wind velocity continues throughout the year causes great variation in temperatures. Winter temperatures go below zero (minimum -25°C) and summers as high as 38°C. Precipitation is scanty with less than 80 mm per annum. The valley remains cut off from other parts of country during winter months due to extreme weather conditions.

Collection and identification of plants

Information on the plants used for treatment of several complaints / diseases, other uses were obtained through interviews with traditional healers and conformed by group discussion with local medicinal men called 'Amchi'. The system of medicine being used in Ladakh is Tibetian system of medicine and also called Amchi system of medicine. The plants were identified comparing with authentic specimens at Defence Institute of High Altitude Research (DIHAR) herbarium. Various plant parts such as leaf, stem, flower and roots having ethnobotanical value were separated and shade dried for further analysis. The plants selected were washed thoroughly and dried in shade. Aerial parts of the dried plant material was ground to fine powder and about 250 g of the plant material was collected, and extracted repeatedly in ethanol. The extracts were filtered, concentrated, dried at room temperature and used for determination of antibacterial and antioxidant acitivity.

Determination of antibacterial activity

Four bacterial strains, obtained from the Department of Microbiology, RTM Nagpur University, Nagpur, India. They included the Gram positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus* and the Gram negative bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*). The bacterial strains were grown on Mueller Hinton (MH) agar plates and suspended in MH broth. The MIC values against bacterial strains were performed using the Ericcson and Sherris[13] broth dilution method (MH broth). Inoculum suspensions were prepared form 6 h broth cultures. The extract was sterilized by Millipore filtration (0.45 μm) and added to MH broth medium. The bacterial suspensions were aerobically incubated for 24 h at 37°C. The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth. Negative control cultures, containing only sterile physiological Tris-buffer, were also prepared, while the discs soaked in standard broad spectrum antibiotics i.e. Streptomycin and Amoxicillin were used as positive controls.

Determination of anti-oxidant activity

The anti-oxidant activity was assessed using quantitative 2,2- diphenyl -1 picryl hydrazyl (DPPH).[14] The solution of DPPH was prepared with methanol. The absorbances were read at 517 nm after 30 min of incubation and then percentage of decolourisation determined. Vitamin C was used as the positive control. The IC_{50} values (concentration at which 50% of decolourisation was obtained). DPPH radical's concentration was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample (Oktay et al., 2003).

RESULTS AND DISCUSSION

The Amchi's (medicine men) are still looking after more than 60% public health of tribal communities[15] and they are totally depend upon natural resources for collection of plants and their parts. Traditional and indigenous system of medicine persists in all over the world.[16] The present investigation has highlighted the therapeutic value of some plant species of cold desert Nubra valley to cure cold, cough, fever, stomach problems, kidney disorders, urinogenital complaints, skin diseases, diarrhea, dysentery and problems in menstrual cycle etc. Our findings on antibacterial activity of cold desert plants of Nubra valley justify some ethnobotanical uses such as against diarrhea, dysentery, wounds because we demonstrated strong activity of these plants against some pathogens of the digestive tract as well as septic. Due to the continuous emergence of antibiotic-resistant strains there is continual demand for new antibiotics. In many developing countries about 80% of available drugs come from medicinal plants and in industrialized countries plants make up the raw material for processes, which synthesize pure chemical derivatives.[17] The antibacterial activity results are shown in Table-1. Present findings showed that the ethanol extracts of *Salvia sclarea* flower; *Arnebia euchroma* root against *Pseudomonas aeruginosa* and

Table 1: Minimum inhibitory concentrations (MICs) in µg/ml of antibacterial activity

Leaf samples	*B.s*	*S.a*	*E.c*	*P.a*	Aerial part	*B.s*	*S.a*	*E.c*	*P.a*
Achillea millefolium	32	>250	32	n.a.	*Allium przewalskianum*	>250	>250	32	n.a.
Artemisia dracunculus	128	64	>250	n.a.	*Allium ramosum*	n.a	128	64	>250
Artemisia tournefortiana	>250	n.a	32	>250	**Flower**				
Bidens pilosa	n.a	128	64	16	*Dracocephalum heterophyllum*	128	n.a	64	>250
Ephedra gerardiana	n.a	128	32	64	*Salvia sclarea*	>250	n.a	n.a	8
Mentha royleana	32	32	>250	64	**Underground**				
Podophyllum hexandrum	8	64	16	32	*Allium przewalskianum*	32	32	64	>250
Salvia sclarea	64	64	32	n.a	*Allium ramosum*	n.a	64	32	n.a
Verbascum thapsus	32	32	32	>250	*Arnebia euchroma*	32	n.a	64	8
Stem samples					*Arnebia guttata*	32	128	32	16
Bidens pilosa	n.a	n.a	32	n.a	*Artemisia tournefortiana*	64	128	32	n.a
Mentha royleana	16	>250	32	n.a	*Bidens pilosa*	n.a	32	64	64
Salvia sclarea	n.a	64	n.a	>250	*Dactylorhiza hatagirea*	32	32	32	32
Verbascum thapsus	8	16	32	>250	*Mentha royleana*	>250	n.a	n.a	n.a
					Salvia sclarea	64	n.a	32	32
					Verbascum thapsus	32	128	>250	32

Note: *B.s = Bacillus subtilis; S.a = Staphylococcus aureus; E.c= Escherichia coli; P.a =Pseudomonas aeruginosa*; n.a. = absence of inhibition at 1000 µg/ml.

Table 2: IC 50 values in µg/ml (mean + S.D.; n=3) of anti-oxidant activities

Plant material	Anti-oxidant activity (n=3) µg/ml	Plant material	Anti-oxidant activity (n=3) µg/ml
Leaf		**Aerial parts**	
Achillea millefolium	54.50 + 3.1	*Allium przewalskianum*	18.24 + 0.21
Artemisia dracunculus	98.20 + 7.9	*Allium ramosum*	>100
Artemisia tournefortiana	>100	**Flower**	
Bidens pilosa	>100	*Dracocephalum heterophyllum*	89.76 + 5.1
Ephedra gerardiana	13.30 + 0.6	*Salvia sclarea*	14.97 + 2.9
Mentha royleana	>100	**Underground parts**	
Podophyllum hexandrum	15.94 + 0.2	*Allium przewalskianum*	>100
Salvia sclarea	96.70 + 12.1	*Allium ramosum*	>100
Verbascum thapsus	>100	*Arnebia euchroma*	33.27 + 1.1
Stem		*Arnebia guttata*	15.15 + 0.22
Bidens pilosa	>100	*Artemisia tournefortiana*	>100
Mentha royleana	>100	*Bidens pilosa*	72.33 + 4.61
Salvia sclarea	>100	*Dactylorhiza hatagirea*	97.40 + 7.3
Verbascum thapsus	33.16 + 6.9	*Mentha royleana*	>100
		Salvia sclarea	>100
		Verbascum thapsus	35.21 + 7.5

Podophyllum hexandrum leaf; *Verbascum thapsus* stem against *Bacillus subtilis* had interesting Minimum inhibitory concentrations (MICs) with 8 µg/ml. Inhibiting activity of *Pseudomonas aeruginosa* is particularly interesting from a medical point of view because these microbial agent is responsible for sever opportunistic infections. These findings were also shown in other plants extracts.[18-21]

The anti-oxidant activity of the methanol extracts were analysed for IC_{50} values are displayed in Table-2. (15.15 ± 0.22) *Ephedra gerardiana* leaf, *Salvia sclarea* flower and *Arnebia guttata rhizome* showed highest free radical scavenging activity with 13.30 ± 0.6 µg/ml, 14.97 ± 2.9 µg/ml and 15.15 ± 0.22 µg/ml respectively. Most of samples showed 50% DPPH inhibition in less than 75 µg/ml material. The growing interest in the substitution of synthetic food antioxidants with natural ones has fostered research on plant sources and screening of raw materials to identify new antioxidants. Interest in oxidation reactions is not confined to the food industry, as antioxidants are widely needed to prevent deterioration of other perishable goods, such as cosmetics, pharmaceuticals and plastics. In addition, other biological properties such as anticarcinogenicity have been reported for natural and synthetic antioxidants.[22-23] From these results it can concluded that the crude extracts of cold desert medicinal plants of Nubra valley are promising medicinal value like antibacterial and anti-oxidant activities. Further phytochemical work need to be done on these extracts

including fractionation to isolate active constituent and subsequent pharmacological evaluation.

CONCLUSION

Present results showed interesting antibacterial and anti-oxidant activity of the ethanol extracts from cold desert medicinal plants. The contemporary presence of these medicinal activities suggests that these plants may be source of bioactive substances with multifaceted activity. Further phytochemical work need to be done on these extracts including fractionation to isolate active constituent and subsequent pharmacological evaluation. Farmers should be involved in the cultivation of medicinal plants at least in their barren and fallow land; this would augment their income and in turn help in the conservation of the species. Appropriate research should be carried out in institutions in the hills to develop agro-techniques for the cultivation of medicinal plants on priority basis

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Phytochemical profile and Antibacterial activity of stem bark of *Anogeissus latifolia*

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ABSTRACT

In- vitro antibacterial potential of Apical, meddle and mature stem bark was assayed by employing the agar-well diffusion method against three Gram '+'ve bacteria *Bacillus subtilis, Staphylococcus aureus,* and *Micrococcus sp.* and five Gram '–'ve bacteria *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoneae, Salmonella typhi,* and *Proteus mirabilis.* The extract of apical bark was effective than the inner bark and mature outer bark in controlling the growth of all bacteria. The bacterium *Salmonella typhi* was most sensitive than other bacterial species. Preliminary phytochemical analysis revealed the presence of alkaloids, glycosides, flavonoids, flavanols, phenols, saponins and terpenoids. The concentrations secondary metabolites was found higher in the apical stem bark than the inner and mature outer stem bark.

Key words: Antibacterial activity, *A. latifolia,* phytochemical analysis and secondary metabolites.

INTRODUCTION

Medicinal plants have occupied an important position in the socio-cultural, development of rural people of India. Crude drugs are usually dried parts of the medicinal plants that form an essential raw material for the production of traditional remedies of Ayurveda, Siddha, Unani, Homeopathy etc. It has been estimated by WHO that 80% of the people living in the developing countries rely upon the traditional health practices for their primary health care needs.[1] Chemical compounds found in low concentrations in other plant parts are highly concentrated in bark.[2]

Anogeissus latifolia (DC.) is medium sized deciduous tree belonging to the family combretaceae and it is commonly known as gahtti. is effective in anaemic conditions and urinary discharges, piles.[3] Bark is remedy for chronic cough called 'Dangya Khokala'.[4] Tribal people residing in the forest of Gundlabranhmeswaram wild life sanctuary apply paste of stem bark on scorpion sting.[5] Tribals in Udaipur district of Rajasthan, use the bark of this tree in the treatment of fever.[6] Decoction of bark, two spoons daily is useful as remedy against cough and leaf decoction is effective in epileptic fits.[7] In this study, we evaluated the antimicrobial activity of methanolic extracts of *Anogeissus latifolia* against several pathogenic microorganisms.

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MATERIALS AND METHODS

Collection and processing of the plant material

Different bark samples (Apical bark, inner bark and mature outer bark) of *A. latifolia,* were collected from the hilly regions of Kolhapur district. The bark was collected in the month of May2009. The bark samples were cut into pieces, sun-dried then oven dried at 60° C. Dried bark samples were ground into powder and stored in an air tight plastic container.

Microorganisms

Bacillus subtilis, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoneae*, *Salmonella typhi*, *Proteus mirabilis and Micrococcus sp.* were used for testing antibacterial activity of bark extracts. The test organisms used in this study were obtained from the department of Microbiology, Shivaji University, Kolhapur, Maharashtra, India. The bacterial strains were cultured on nutrient agar slants. The cultures were maintained by subculturing periodically and preserved at 4° C until further use.

Preparation of the extract

Oven dried 10 g of powdered bark material was weighed accurately and placed in soxhlet extraction chamber which

was suspended above the flask containing 100 mL of 80% methanol and below a condenser. The flask was heated and the methanol evaporated and moved into the condenser where it was converted into a liquid that trickled into the extraction chamber containing the plant material. The extraction chamber was designed so that when the solvent surrounding the sample exceeded at certain level it overflowed and trickled back down into the boiling flask. At the end of the extraction process, the flask containing the methanol extract was removed and methanol was evaporated by using rotary evaporator. The weight of the residual extract was measured and percent yield was calculated. The residue of the extract was dissolved in 25 ml of pure methanol and stored in air tight glass vials at 4° C until further use.[9]

$$\text{Extract yield } \% = \frac{W1}{W2} \times 100$$

Where

W1= Net wt of powder in grams after extraction

W2= total wt of wood powder in grams taken for extraction.

Preparation of the media

Accurately weighed 28 g of nutrient agar (Himedia) was dissolved in the 1000 ml of distilled water. The medium was sterilized under 15 Lb pressure for 15 minutes in an autoclave. 30 ml of this sterilized semisolid nutrient agar medium was poured in pre-sterilized 90 mm glass petriplates under aseptic conditions in laminar flow. The plates were allowed to cool at room temperature to solidify the medium.

Determination of antibacterial activity by agar well diffusion method

Agar well diffusion method was employed to determine antibacterial activity.[8] Well of 10 mm diameter was prepared with sterilized cork-borer. Standared antibiotic Chloramphenicol at 50 µg/ml were served as positive control and Methanol as negative control. The plates inoculated with different bacterial species were incubated at 37°C in incubator for 24 h and the zone of inhibition was measured (Diameter in mm). All of the experiments were performed in triplicate. The results are reported as the average of 3 experiments.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis (Table No. 1) of methanolic extract of bark showed presence of phytoconstituents alkaloids, glycosides, flavonoids, flavanols, phenols, saponins and terpenoids. The concentration of secondary metabolites was found higher in the apical stem bark than the inner and mature outer bark. The percet extract yield of three bark samples was determined (Table No. 2) and it was noticed that the yield was maximum in apical bark than the inner and mature outer bark on main trunk.

Antibaterial activity of bark extract of *A. latifolia* against different bacterial organisms is shown in table No. 3. As shown in Table No. 3 apical stem bark extract displayed maximum antibacterial activity against than inner and mature outer bark against all the bacterial species studied. The bacterium *Salmonella typhi* was highly inhibited by apical stem bark extract (14.33 ± 0.52 mm) at 300 µL followed by *Escherichia coli* (12.67 ± 0.82 mm), *Pseudomonas aeruginosa* (12.33 ± 0.52 mm), *Staphylococcus aureus* (11.83 ± 0.75 mm) respectively. The bacteria *Klebsiella pneumoneae*, *Proteus mirabilis* and *Micrococcus sp* showed inhibition zone in the range of 6 mm at 300 µL by apical stem bark extract whereas the inhibition of bacterium *Bacillus subtilis* by all the bark extract was in the range of 8 mm. The inhibition by negative control methanol was zero while the standard antibiotic Chloramphenicol was inhibited the growth of all the bacterial species effectively at low concentration of 50 µg/ml with the zone of inhibitions ranging from 11.17 mm to 21.50

Table No. 1. Percent Extract yield

Plant name	Bark sample	Summer
A. latifolia	Apical bark	25.56%
	Inner bark	24.30%
	Mature outer	21.33%

Table No. 2 Phytochemical analysis of methanolic extract of bark of *A. latifolia*.

Sample	Phenols	Flavones	Flavonoid	Tannin	Terpenoids	Saponin	Alkaloids	Cardiac glycosides
1	+++	+++	+++	+++	+++	+++	+++	+++
2	++	++	++	++	++	++	++	++
3	+	+	+	+	+	+	+	+

1-Apical bark; 2-Inner bark and 3- Mature outer +++ : Present in high concentration , ++ : Present in moderate concentration and + : Present in low concentration

Table No : 3 Antibacterial activity of stem Bark of *Anogeissus latifolia* (DC.)

Microorganisms	Bark sample	Minimum inhibitory concentration Zone of inhibition (Diameter in 'mm')* 25 µL	50 µL	100 µL	200 µL	300 µL	M	C(50 µg/ml)
Bacillus subtilis	1	o.oo	2.13 ± 0.31	4.33 ± 0.54	6.5 ± 0.54	8.66 ± 0.51	o.oo	
	2	o.oo	1.98 ± 0.28	3.66 ± 0.51	5.67 ± 0.51	8.50 ± 0.83	o.oo	21.50 ± 1.05
	3	o.oo	1.13 ± 0.78	3.83 ± 0.40	5.66 ± 0.51	8.33 ± 0.56	o.oo	
Staphylococcus aureus	1	o.oo	2.59 ± 0.32	8.00 ± 0.63	9.17 ± 0.98	11.83 ± 0.75	o.oo	
	2	o.oo	2.18 ± 0.12	8.17 ± 0.63	10.67 ± 0.52	11.83 ± 0.73	o.oo	11.17 ± 0.98
	3	o.oo	1.45 ± 0.54	5.33 ± 0.82	7.33 ± 0.87	9.67 ± 0.80	o.oo	
Escherichia coli	1	o.oo	2.77 ± 0.71	6.83 ± 0.75	10.00 ± 0.63	12.67 ± 0.82	o.oo	
	2	o.oo	2.18 ± 0.21	5.17 ± 0.75	9.50 ± 0.55	11.50 ± 0.84	o.oo	16.50 ± 0.55
	3	o.oo	1.45 ± 0.54	3.83 ± 0.75	7.67 ± 0.52	11.50 ± 0.84	o.oo	
Pseudomonas aeruginosa	1	o.oo	2.22 ± 0.34	7.83 ± 0.41	10.17 ± 0.40	12.33 ± 0.52	o.oo	
	2	o.oo	2.88 ± 023	6.00 ± 0.63	8.17 ± 0.75	11.50 ± 1.52	o.oo	14.33 ± 0.5
	3	o.oo	2.45 ± 0.52	7.33 ± 0.52	8.50 ± 0.55	10.33 ± 0.52	o.oo	
Salmonella typhi	1	o.oo	2.42 ± 0.17	6.67 ± 0.52	11.50 ± 0.84	14.33 ± 0.52	o.oo	
	2	o.oo	2.00 ± 0.62	5.50 ± 0.84	10.83 ± 0.98	14.00 ± 0.89	o.oo	16.67 ± 1.21
	3	o.oo	1.47 ± 0.67	3.67 ± 0.52	7.33 ± 0.49	10.83 ± 0.98	o.oo	
Klebsiella pneumoneae	1	o.oo	3.10 ± 0.13	4.01 ± 0.25	6.38 ± 0.71	8.01 ± 0.55	o.oo	
	2	o.oo	2.57 ± 0.27	3.54 ±1.22	5.77 ± 0.43	6.78 ± 0.79	o.oo	14.85± 1.17
	3	o.oo	1.71 ± 0.66	2.36 ± 0.11	4.42 ± 0.55	5.81 ± 0.09	o.oo	
Proteus mirabilis	1	o.oo	2.81 ± 0.99	3.78 ± 0.46	5.72 ± 0.00	7.65 ± 0.93	o.oo	
	2	o.oo	2.15 ± 0.15	2.81 ± 0.51	3.99 ± 0.80	4.60 ± 0.31	o.oo	13.70 ±0.49
	3	o.oo	1.33 ± 0.59	2.44 ± 0.28	3.11± 0.63	3.91 ± 0.40	o.oo	
Micrococcus sp	1	o.oo	2.67 ± 0.26	3.49 ± 0.31	5.76 ± 0.55	7.58 ± 0.71	o.oo	
	2	o.oo	2.13 ± 0.78	3.23 ± 0.40	4.37 ± 0.52	6.41 ± 0.36	o.oo	16.63 ± 0.23
	3	o.oo	1.74 ± 0.63	2.45 ± 0.17	3.83 ± 0.37	4.96 ± 0.11	o.oo	

1: Apical Bark, 2 : Inner Bark and 3 : Mature outer C : ChloramPhenicol M : Methanol *: Agar well difusion method Values are mean ± *SD of three replicates*

mm. The inhibitions of all the bacterial species by inner and mature outer bark extracts was lower than the apical stem bark extract.

Several researchers have studied the bactericidal potential of bark of many taxon. Stem bark extract of *Pterocarpus santalinus* showed maximum activity against *Bacillus subtilis* (17.0 mm).[10] The methanolic extract of stem bark of *Tetracarpidium conophorum* inhibited the growth of *B. subtilis* (12.3 mm).[11] According to Manjunatha[10] the stem bark extract of *Pterocarpus santalinus* inhibits the growth of *S. aureus* (16.05 mm). Methanolic extracts of stem bark of *Vitex doniana* also noticed to posses bactericidal potential against *S. aureus*.[12] Stem bark extract of *Holarrhena antidysenterica* posses antibacterial potential against enteric pathogen *E. coli*.[13] Doughari *et al*.[14] noticed that stem bark of *Cochlospermum planchoni* inhibited the growth of *P. aeruginosa* (26 mm). Sangetha *et al*.[15] noticed that methanolic extracts from the stem of *Cassia fistula* and *Cassia surattensis* arrested the growth of *S. typhi* (19 mm). Phenolics and polyphenols present in the plants are known to be toxic to the microorganism.[16] Sangetha *et al*.[15] also noticed the bactericidal potential of *Cassia* fistula against the bacteria *Klebsiella pneumoneae, Proteus mirabilis* and *Micrococcus sp.* Tamokou *et al*.[17] isolated xanthones, physcion, friedelin and friedelanol, of these, xanthones and physcion exhibited the antimicrobial activities against bacteria *S. typhi*, *K. pneumonae*, *P, aeriginosa*, and *B. subtilis* and four yeast species *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Cryptococcus neoformans* respectively. Phenolics and polyphenols present in the plants are known to be toxic to the microorganism.[16] Flavonoids have been reported to have both antibacterial and antifungal activities.[18] Tannin from *Dichrostachys cinerea* root bark possesses antibacterial activities against *S. aureus*, *E. coli* and *P. aeruginosa*.[19] The bark extract was found to be containing tannin glycosides, alkaloids, steroids and Flavonoids which are biologically active.[20] In our study all the three bark samples revealed the presence of secondary metabolite. The reasons for the differential sensitivity pattern between Gram negative and Gram positive bacterial strains could be due to the outer phospholipids membrane with structural lipopolysaccharide components which make their cell wall impermeable to antimicrobial agents[21] while the Gram positive bacteria are more susceptible having only an outer peptidogylcan, which is not effective permeability barrier.[22]

The high inhibitory potential of methanolic extract might be due to the high solubility of the phytoconstituents in the polar organic solvent like methanol. The phytoconstituents might be present in higher concentration in the apical bark along with some new microbicidal agents reflecting its higher

bactericidal potential. Presence of these phytoconstituents in the stem barks points towards the pharmacological activities of this plant and supports the claim of the traditional users. Further research is needed to study the pharmacological potential of apical stem bark.

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Reduced Coniferin and Enhanced 6-Methoxypodophyllotoxin Production in *Linum flavum* Cell Cultures

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ABSTRACT

Treatment of cell suspension cultures of *Linum flavum* L. with Na_2EDTA reduced the coniferin and enhanced the 6-methoxypodophyllotoxin (6-MPT) production in a concentration-dependent way, in a range of 0.1–5 mM. On day 14 after treatment with Na_2EDTA, an inhibition of the coniferin production up to 88% was found. The maximum enhancement of the 6-MPT production was 400% on day 7 after treatment with 5 mM Na_2EDTA. The reduction in coniferin accumulation in the suspension cultures correlated with and inhibition of coniferyl alcohol glucosyltransferase (CAGT) activity as determined in cell homogenates. On day 14 after treatment with 2 and 5 mM Na_2EDTA, the CAGT activity was inhibited up to >89 %. The inhibitory effect of Na_2EDTA on CAGT was also shown in a partially purified enzyme preparation. Several metal ions and the elicitors nigeran and salicylic acid had no significant effect on the production of coniferin and 6-MPT.

Key words: coniferin; *Linum flavum* L.; 6-methoxypodophyllotoxin; Na_2EDTA; CAGT inhibition.

INTRODUCTION

Podophyllotoxin and podophyllotoxin-derived lignans possess cytotoxic and antiviral activities. Teniposide and etoposide are semi-synthetic derivatives of podophyllotoxin that are clinically used as anticancer drugs.[1] Podophyllotoxin is also the starting compound for the rheumatoid arthritis drug CPH 82 (Reumacon).[2] For the production of semi-synthetic podophyllotoxin derivatives on an industrial scale, podophyllotoxin is isolated from the rhizomes of *Podophyllum* plants from wild habitats, which are counted as endangered species.[3]

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The use of biotechnological approaches to improve the production of podophyllotoxin or related lignans with plant cell and organ cultures, including the biotransformation of suitable precursors and the modification of biosynthetic pathways is considered to be suitable and economically attractive.[4] Several investigations to enhance the production of podophyllotoxin-derived lignans by manipulation of cell and organ cultures have been carried out.[3,5,6,7,8] The production of podophyllotoxin, 6-MPT and its glucoside could be enhanced in cell cultures of *Podophyllum hexandrum* Royle,[7] *Linum flavum* L.[5,9,10] *Callitris drummondii* F. Mueller,[11] and *Linum album* Kotschy.[3]

Based on the close chemical resemblance with podophyllotoxin (see Figure 1), 6-MPT is considered also as an interesting starting compound for the preparation of new semi-synthetic derivatives with antitumor properties. Cell cultures of *L. flavum* produce 6-MPT and its glucosides. The cytotoxicity of 6-MPT *in vitro* against tumor cell lines was comparable with that of podophyllotoxin.[12]

Coniferyl alcohol is an early precursor of both lignins and lignans. The glucosylation of coniferyl alcohol yields coniferin that is accumulated endogenously in *L. flavum*

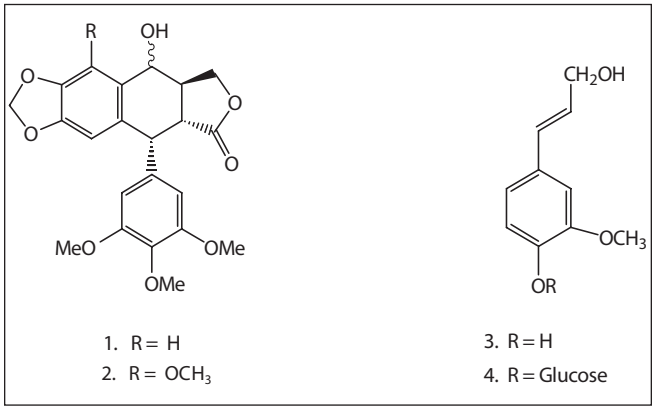

Figure 1: Chemical structures of podophyllotoxin (1), 6-methoxypodophyllotoxin (2), coniferyl alcohol (3) and coniferin (4)

cultures up to 12 % on a dry weight basis.[13] This reaction is catalysed by CAGT. Lignans are formed through radical-mediated dimerisation of two coniferyl alcohol units. Blocking the branch leading to the formation of coniferin by inhibiting CAGT could result in an enhanced production of lignans, such as 6-MPT in the cell suspension cultures of *L. flavum*. High coniferin contents in the cell suspension culture correspond with low 6-MPT levels, as was demonstrated in feeding experiment with cell cultures of *P. Hexandrum*.[3] It is known that Na_2EDTA,[14] metal ions such as Cu^{2+}, Zn^{2+} , Li^{+} and Fe^{2+}.[15,16,17] are able to inhibit the glucosyltransferase activity. Addition of one of these compounds may interfere with CAGT.

The aim of this paper is to explore the effect of Na_2EDTA and several inorganic salts on the production of coniferin and 6-methoxypodophyllotoxin (6-MPT) in cell suspension cultures of *L. flavum*. This effect is compared to that of the elicitors salicylic acid and nigeran. These compounds elicitate various biosynthetic pathways, but so far there is little evidence of their effect on the lignan biosynthesis.[18,19]

MATERIALS AND METHODS

Plant material and culture conditions

Cell suspensions of *Linum flavum* L. (Linaceae) leaves have been initiated and are maintained at the Department of Pharmaceutical Biology, University of Groningen. The cell suspensions are cultured routinely every two weeks by transferring 100 ml of fully grown suspension aseptically into 200 ml of fresh liquid medium. The medium contains MS medium.,[20] 0.2 mg of indole-3-acetic acid (IAA) and 0.2 mg of 6-benzylaminopurine (BAP), purchased from Duchefa, Haarlem, the Netherlands. The cell suspensions were incubated on a rotary shaker (175 rpm) at 26° C under a day/night regime (16/8 h: 3,000 lux, day light L 36W/10, OSRAM, Germany).

Treatment of cell suspensions

Ethylenediamine tetra acetate disodium (Na_2EDTA) was purchased from Duchefa, copper (II) sulphate pentahydrate, zinc chloride, and lithium chloride from Merck, Darmstadt, Germany, iron (II) sulphate pentahydrate and salicylic acid from Sigma-Aldrich, Zwijndrecht, the Netherlands. Nigeran from Sigma, St. Louis (USA).

These compounds were added to the culture media used for the cell suspension cultures of *L. flavum* yielding the following final concentrations: Na_2EDTA : 0.01, 0.5, 1, 2 and 5 mM, Cu^{2+}, Zn^{2+} and Li^{+}: 0.1, 1 and 5 mM, Fe^{2+}: 0.01, 0.1 and 1 mM, salicylic acid: 0.1, 0.5, 1 and 5 mM; nigeran 20 mg l^{-1}. Suspension-grown cells were harvested each 2 days during the growth cycle of 14 days. Samples of about 10 ml were taken aseptically and transferred into a calibrated conical tube and centrifuged for 5 min at 1,500 g. In order to monitor the viability and growth of the cell cultures, the medium pH and the conductance were routinely measured in the supernatant. The cells were filtered using Buchner funnel. Fresh weight (FW) was determined and put overnight in the freezer and then freeze dried. Dry weight (DW) was also determined. Coniferin and 6-MPT contents were subsequently analysed by HPLC.

Extraction

About 100 mg, accurately weighed of freeze dried and powdered cell material were extracted by ultrasonification in 2 ml methanol (80%; v/v) during 1 hour. Dichloromethane (4.0 ml) and water (4.0 ml) were added. The mixture was vortexed and centrifuged (5 min; 1,500 g). For the determination of the 6-MPT concentration, 2.0 ml of the dichloromethane phase were taken and evaporated to dryness. The residue was redissolved in 1.0 ml methanol and centrifuged. For the determination of coniferin 50 µl water phase were diluted with water until 1.0 ml and centrifuged (2 min; 10,000 g).

Treatment of aqueous phase with β-glucosidase

To confirm the coniferin production, the water phase was submitted to enzymatic hydrolysis. A 3.5% (w/v) solution of β-glucosidase (Sigma G-0395) was prepared in 0.1 M phosphate buffer, pH 5.0. To 2.0 ml samples of the water phase 0.5 ml was added, followed by incubation during 5 h at 37° C. The aglucone formed was extracted with 2.0 ml dichloromethane. Of the dichloromethane phase 1.5 ml were taken and evaporated to dryness. The residue was redissolved in 1.0 ml methanol and centrifuged (2 min; 10,000 g). Coniferyl alcohol was determined by HPLC.

Protein purification

Cells were harvested on day 1 after subculturing and stored at –20° C overnight. Frozen cells were suspended in an equal volume of the homogenisation buffer that consisted

of 0.2 M Tris-HCl, pH 7.5, 5% polyvinylpyrrolidone, 0.2 % DOWEX®- 1*2 – 100, 0.1 % DTT (w/w) and 10% ethylenglycol. The mixture was homogenised in using an ultraturrax (Janke & Kunkel, IKA-WERK, Staufen, Germany). The homogenate was filtered through miracloth and clarified by centrifugation for 20 min at 20,000 g. Proteins dissolved in the supernatant were then fractionated by $(NH_4)_2SO_4$ precipitation. The fraction obtained between 40 and 80% saturation was desalted on a HiPrep 26/10 desalting column (Amersham Biosciences, Uppsala, Sweden) previously equilibrated with 0.02 M Tris-HCl buffer, pH 7.5. The protein eluting from the desalting column was applied to a HiTrap DEAE FF column (Amersham Biosciences, Uppsala, Sweden) which had been equilibrated with 0.02 M Tris-HCl buffer, pH 7.5. The protein was eluted first with 100 ml of 0.02 M Tris-HCl buffer, followed by a linear gradient from 0.02 to 0.2 M Tris-HCl buffer and finally with 100 ml of 0.4 M Tris-HCl buffer, all at pH 7.5. Fractions of 5 ml each were collected at a rate of 1 ml min^{-1} and assayed directly for CAGT activity. Fractions containing the highest activity were combined and concentrated by vivaspin 6 ml concentrator (Vivasciences, Hannover, Germany). The combined fractions with the highest CAGT activity resulting from the desalting column (the third step of the purification procedure, see Table 2) were exposed to Na_2EDTA. The Na_2EDTA was added to 5 ml of the partially purified CAGT yielding final concentrations of 0.1, 0.5, 1, 2 and 5 mM. The mixtures were incubated at 4° C for 14 days. 200 µl (3x) of the mixture were taken at day 1, 6 and 14 and submitted for CAGT assay.

CAGT assay

The enzyme assay for CAGT was developed from the methods used by Ibrahim et al. (1976) and Schmid et al. (1982). Cells were treated with Na_2EDTA in the range of 0.1-5 mM and harvested at different time points during the growth cycle and stored at –20° C overnight. Frozen cells, 2-3 g, were suspended in homogenisation buffer and the mixture was homogenised using an ultraturrax. The homogenate was centrifuged (3,000 g; 25 min, 2° C). The supernatant was separated from the pellet. The assay buffer was prepared containing 0.2 M Tris-HCl pH 7.5, DTT 0.1 %. The standard assay mixture consisted of 0.32 µmol of coniferyl alcohol in 40 µl of ethylenglycol monomethylether, 0.32 µmol of UDP-glucose in 40 µl of assay buffer, 200 µl of protein homogenate or partially purified CAGT and assay buffer in a total volume 320 µl. The reaction was started by the addition of protein and vortexed for 5 sec immediately followed by incubation for 30 min at 30° C. The reaction was stopped by adding 2.0 ml dichloromethane followed by vortexing the mixture for 20 s and centrifugation (5 min; 1,500 g). The dichloromethane and water layers were used for HPLC analysis of coniferyl alcohol and coniferin, respectively. The protein determination was done using the Bradford assay.[21]

Analysis of coniferin, coniferyl alcohol and 6-MPT

Coniferin, coniferyl alcohol and 6-MPT were analysed by HPLC. The HPLC system consisted of an ISCO Model 2350 pump, a Shimadzu photodiode array detector (Shimadzu, 's-Hertogenbosch, the Netherlands), UV absorbance at 230 and 290 nm and LiChrocart RP-18 column (250 × 4.6 mm i.d.) (Merck, Darmstadt, Germany). The mobile phase for coniferyl alcohol and 6-MPT analysis was acetonitrile (LAB-SCAN Analytical-sciences, Dublin, Ireland) /water (40:60 v/v; 0.1% phosphoric acid) and for coniferin, methanol/water (30:70; 0.1 % phosphoric acid). Calibration curves were made using coniferyl alcohol (Sigma), coniferin and 6-MPT, which were isolated from *L. flavum* cell suspension cultures as published previously.[9,13] For the statistical evaluation of the data the student's t-test was used. A p-value <0.05 was considered as significant.

RESULTS

The effect of Na_2EDTA on cell growth was determined on the basis of dry weight accumulation as shown in Figure 2. The growth period of cell suspension cultures was 14 days. From day 1 after inoculation the cells grew until day 8. The stationary phase was reached between day 8 and 12. At the end of the period (day 14) the cell suspension was refreshed. There was no significant effect of Na_2EDTA on the growth of the cell suspensions or on the viability parameters at concentrations of 0.1, 0.5 and 1 mM (dry weight and conductivity). At a concentration of 2 and 5 mM, Na_2EDTA inhibited cell growth up to 22% and 59% respectively on day 8.

In Figure 3 the effect of treatment with Na_2EDTA on the coniferin production in *L. flavum* cells is shown. Untreated cells (control) contained up to 12.0 % coniferin on a dry weigh basis on day 14 of the growth cycle. After treatment with Na_2EDTA, the coniferin production was reduced in a concentration dependent way by 18-88% (Figure 5) on day 14, although it should be noted that the higher concentrations of Na_2EDTA (2 and 5 mM) also inhibited the cell growth.

To confirm the coniferin production, the water phase that contained coniferin was submitted to enzymatic hydrolysis using β-glucosidase. This enzyme catalyses the hydrolysis of monolignol glucosidases, that lead to the release of the corresponding alcohols.[22] The coniferyl alcohol formed fully correlated with the coniferin content as found after hydrolysis.

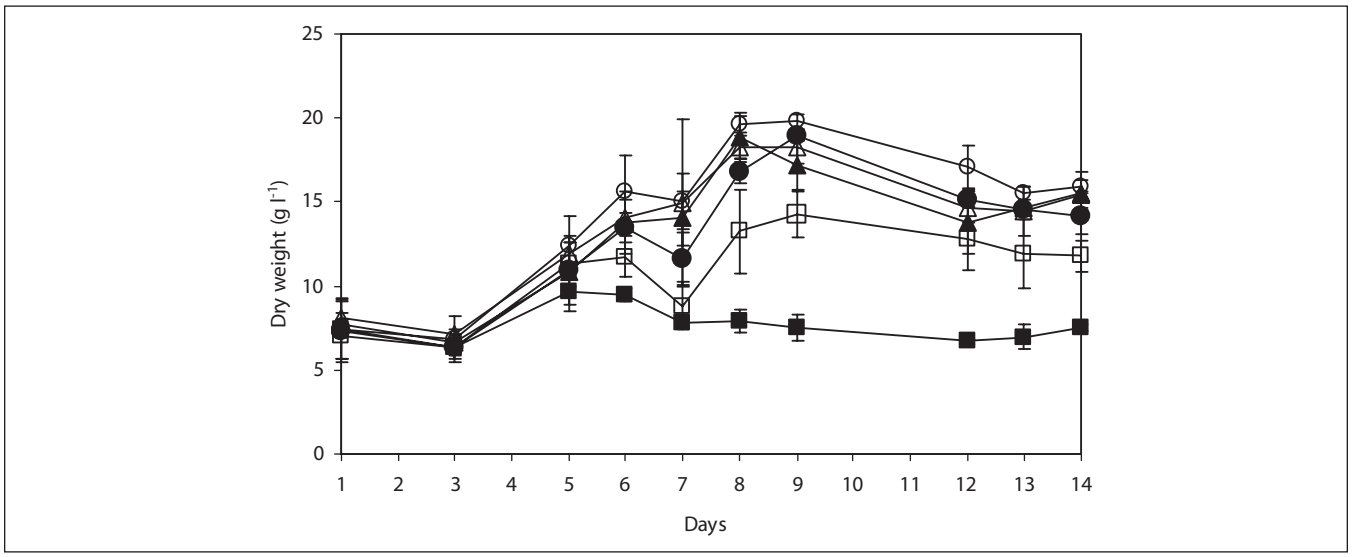

Figure 2: Growth of *L. flavum* cell suspensions culture after treatment with Na_2EDTA 5 mM (■), 2 mM (□), 1 mM (●), 0.5 mM (○), 0.1 mM (▲) and without Na_2EDTA as a control (△). Individual values expressed in g l^{-1} are averages of three independent experiments as means ± standard deviation

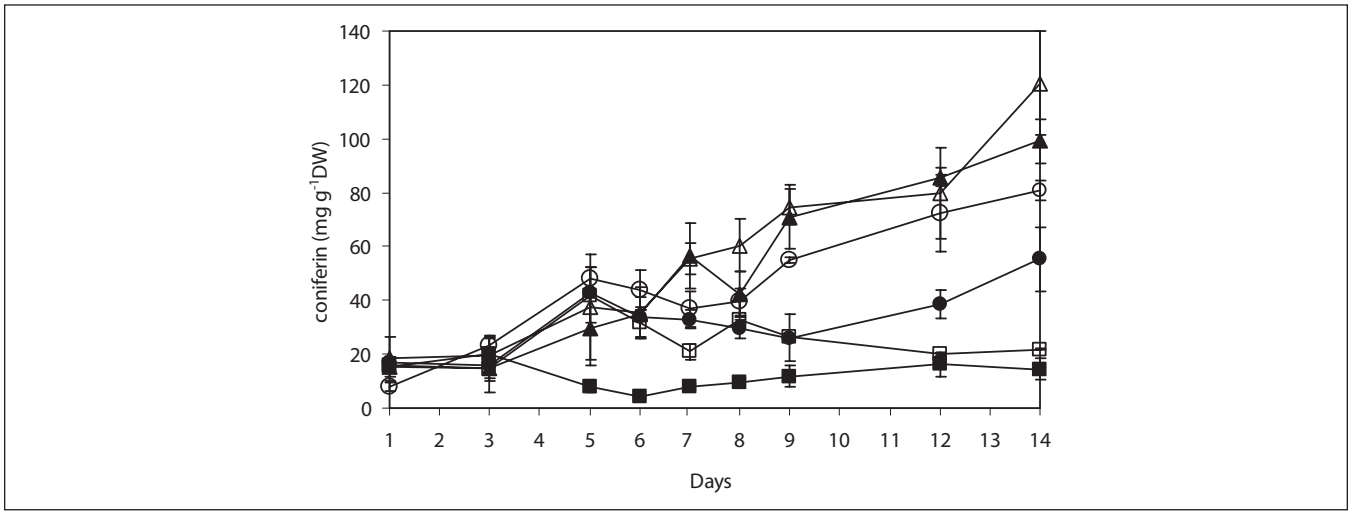

Figure 3: Coniferin production in *L. flavum* cell suspension culture after treatment with Na_2EDTA 5 mM (■), 2 mM (□), 1 mM (●), 0.5 mM (○), 0.1 mM (▲) and without Na_2EDTA as a control (Δ). Individual values expressed in mg g^{-1} of dry weight are averages of three independent experiments as means ± standard deviation

The accumulation of 6-MPT was enhanced 1.2, 1.9 and 4 fold at a concentration of Na_2EDTA of 1, 2 and 5 mM respectively on day 7 in the cell suspensions of *L. flavum* (Figure 4, Figure 5). Adding 0.1 mM and 0.5 mM Na_2EDTA did not enhance the 6-MPT production.

In the concentrations used, none of the metal ions (Cu^{2+}, Zn^{2+}, Li^{+} and Fe^{3+}) inhibited the coniferin or enhanced the 6-MPT production in cell cultures of *L. flavum*. These salts inhibited growth of cell suspension cultures. Nigeran had no effect on the growth of the cell cultures, nor on the production of 6-MPT or coniferin. Salicylic acid was lethal to the cell cultures at concentrations of 1 and 5 mM. The concentrations of 0.1 and 0.5 mM salicylic acid had no effect on the growth of the cell culture and neither on the production of 6-MPT or coniferin either.

The highest CAGT activity in cell suspension cultures was found on day 1 after inoculation. Untreated cells (control) had an activity 13.7 μkat g^{-1}. The activity was reduced by 30-62 % 1 day after inoculation with 0.1-5 mM Na_2EDTA. On day 6 the control cells had a CAGT activity of 2.5 μkat $g^{-1}$and the concurrent inhibition of Na_2EDTA 0.1–5 mM was 12–60%. A significant decrease of the enzyme activity was found on day 13 and 14 after inoculation. Enzyme activity of untreated cell was 6.6 (day 13) and 8.8 μkat g^{-1} (day 14) and the inhibition by Na_2EDTA 0.1–5 mM was 4-80% and 14-89%, respectively (Table 1).

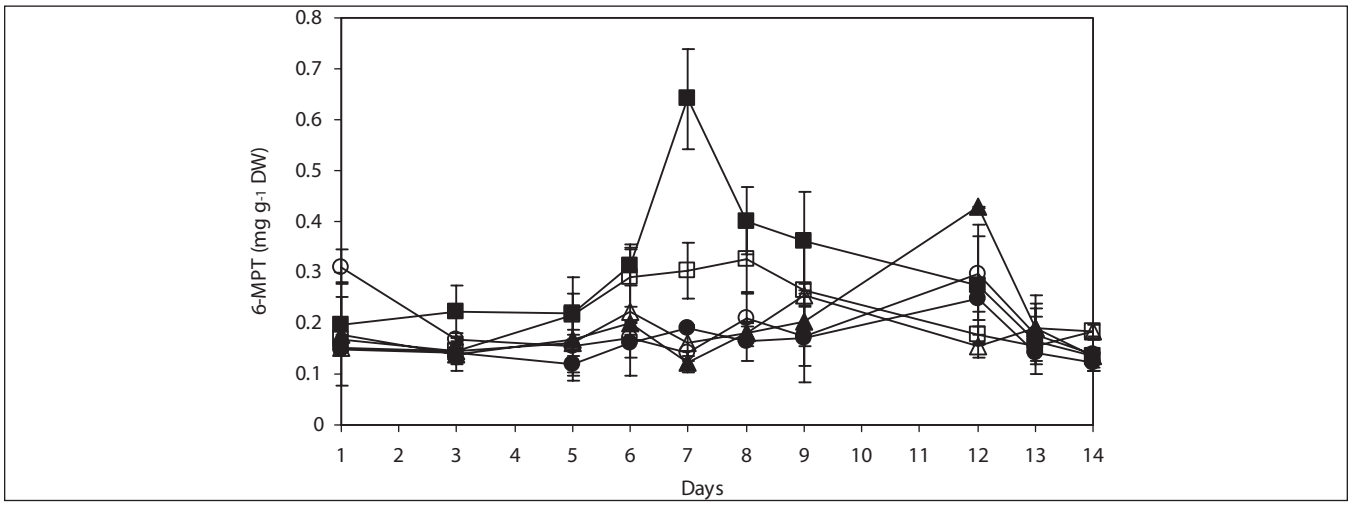

Figure 4: 6-MPT production in *L. flavum* cell suspension culture after treatment with Na_2EDTA 5 mM (■), 2 mM (□), 1 mM (●), 0.5 mM (○), 0.1 mM (▲) and without Na_2EDTA as a control (△). Individual values expressed in mg g^{-1} of dry weight are averages of three independent experiments as means ± standard deviation

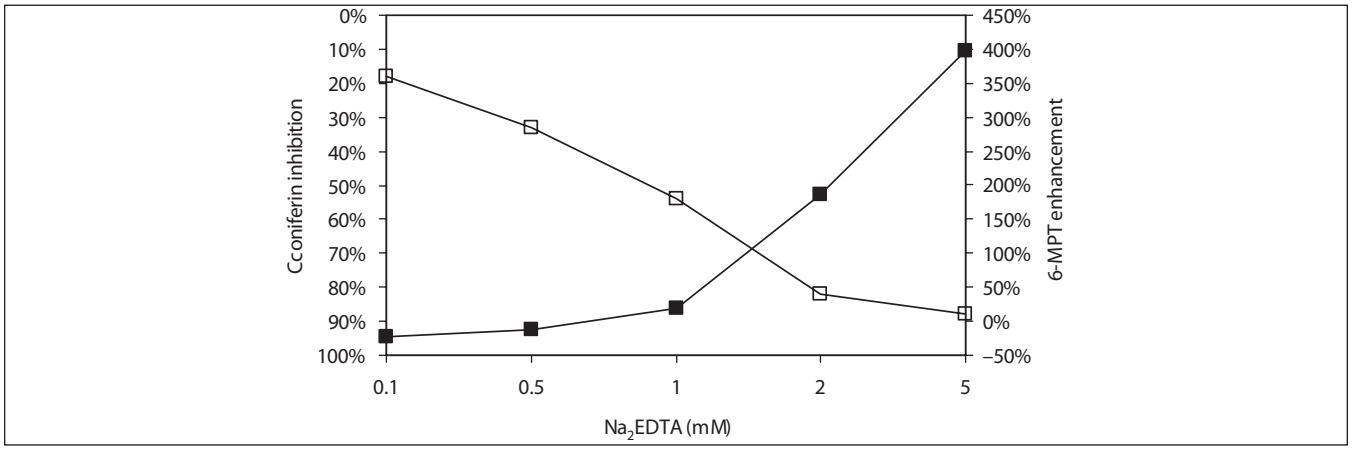

Figure 5: Inhibition of the coniferin production on day 14 (control = 120.7 mg g^{-1} dry weight) (□) and enhancement of the 6-MPT production on the day 7 (control = 0.16 mg g^{-1} dry weight) (■) in *L. flavum* cell suspension culture after treatment with Na_2EDTA

Table 1: CAGT activity in *L. flavum* cell suspension cultures on various days and the percentage inhibition after treatment with Na_2EDTA. Each percentage is calculated on its respective control. Individual values expressed in mkat g^{-1} of protein are averages of three independent experiments as means ± standard deviation. [a]P<0.05, [b]P<0.01 (compared to control values, Students t-test)

Na2EDTA (mM)	CAGT activity (mkat g^{-1}) ± (SD) and % inhibition							
	Day 1		Day 6		Day 13		Day 14	
	Activity	% inhibition	Activity	% inhibition	Activity	% inhibition	Activity	% inhibition
0	13.7 (±1.3)	0	2.5 (±0.3)	0	6.6 (±1.0)	0	8.8 (±0.5)	0
0.l	9.6 (±2.4)	30	2.2 (±0.4)	12	6.0 (±2.5)	4	8.5 (±0.4)	14
0.5	10.8 (±2.5)	22	1.9 (±0.5)	24	2.5 (±0.4)	62[a]	4.8 (±1.8)	46[a]
1	7.5 (±1.6)	46[a]	1.0 (±0.3)	60[b]	1.9 (±0.3)	72[b]	3.9 (±0.3)	56[b]
2	5.2 (±1.3)	62[a]	1.1 (±0.1)	56[b]	1.3 (±0.1)	80[b]	3.1 (±0.2)	65[b]
5	5.9 (±1.0)	57[a]	1.2 (±0.4)	52[a]	1.4 (±0.0)	79[b]	1.0 (±0.1)	89[b]

For CAGT purification, the enzyme was extracted from 1 day-old cell suspension cultures (highest CAGT activity). The purification procedure, summarized in Table 2, ultimately resulted in 41.2-fold enhancement of the CAGT activity, 13.1 % recovery of total activity and a product with a specific activity of 256 μkat g^{-1} of protein. The amount of protein obtained however, was insufficient to carry out the incubation experiments with Na_2EDTA. Therefore the previous

Table 2: Preparation steps of the partially purified CAGT preparation

Fraction	Protein (mg)	Total activity (µkat)	Specific activity (µkat g–1 protein)	Purification (fold)	Yield (%)
Crude extract	302.4	1.52	5	1	100
Ammonium sulphate precipitation	28	1.12	40	8	73
Desalting	4.4	0.25	56.8	11.4	16.4
HiTrap DEAE FF	0.8	0.20	256	41.2	13.1

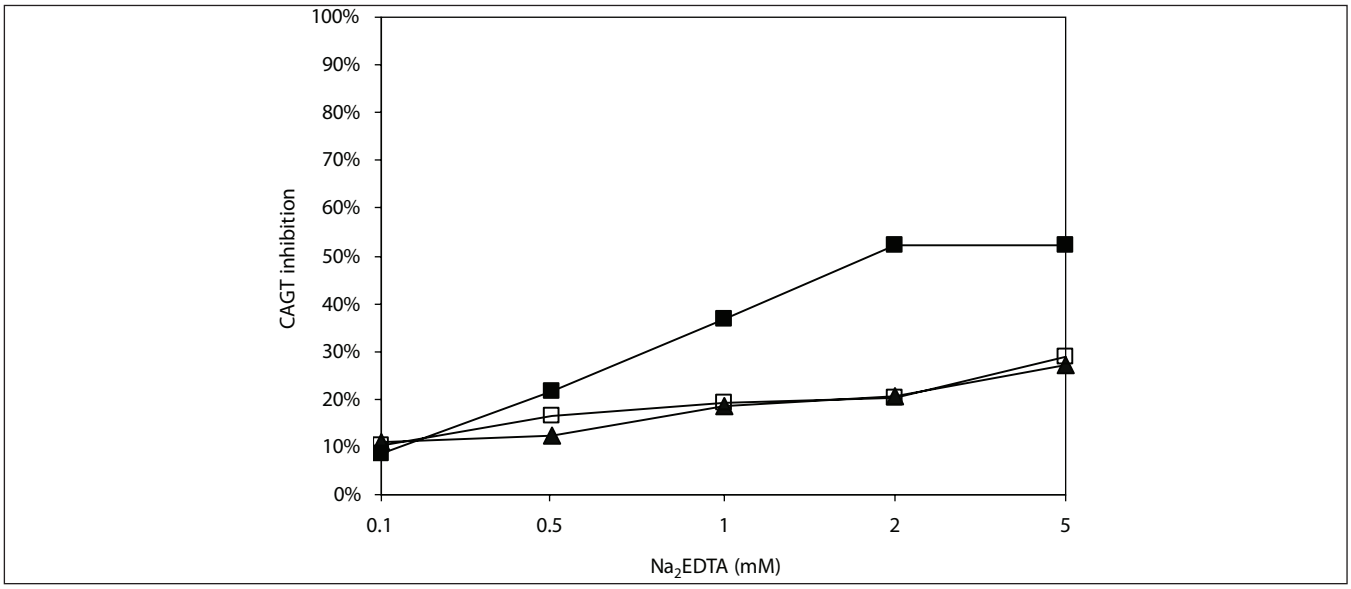

Figure 6: Inhibition of CAGT activity in the partially purified CAGT preparation by Na_2EDTA after incubation for 1 day (▲), 6 days (□) and 14 days (■)

fraction, originating from the desalting step (see Table 2; 11.4-fold purified) was used. Na_2EDTA inhibited the CAGT activity in this partially purified enzyme preparation, up to 53% after 14 days incubation (Figure 6).

DISCUSSION

CAGT is a glucosyltransferase that converts coniferyl alcohol into coniferin. In order to enhance the 6-MPT production in *L. flavum* cell suspension cultures, the formation of coniferin was blocked by inhibition of CAGT using several potential glucosyltransferase inhibitors. Na_2EDTA inhibited the production of coniferin in the suspension cell of *L. flavum* in a concentration-dependent way, in a range of 0.1-5 mM. The results were confirmed by hydrolysis of the coniferin-containing water layer of the cell extract by enzymatic hydrolysis using β-glucosidase. Coniferin was completely converted into coniferyl alcohol and the concentration of the formed coniferyl alcohol related to the original coniferin concentration.

There was a correlation between the coniferin and the 6-MPT production in *L. flavum* cell suspension cultures. Higher coniferin contents corresponded with lower 6-MPT levels. This supports our hypothesis that blocking the branch leading to the formation of lignins by inhibition of glucosyltransferase may result in an enhanced production of lignans. By inhibiting the coniferin production, coniferyl alcohol accumulates and is available as a substrate to produce of 6-MPT and other lignans.

The high 6-MPT content on day 7 of the growth cycle (Fig. 3), correlates with the low CAGT content as measured on day 6 (Table 1). This is in agreement with earlier observations,[13] showing that a maximal coniferyl alcohol content in *L. flavum* cell suspension cultures was preceeded by a maximal activity of the enzyme β-glucosidase.

At the beginning of the growth cycle of the control cell suspension cultures no clear relationship existed between CAGT activity and coniferin content. However, after day 6 it appeared that a low activity of CAGT related to a low coniferin content. CAGT activity then increased until day 14, with a simultaneous increase of the coniferin accumulation.

The highest CAGT activity in cell suspensions was found on day 1. Then it declined to a lower level on day 6 and re-increased on day 13 and 14. This is probably affected by β-glucosidase that converts coniferin into coniferyl alcohol. The reaction catalysed by β-glucosidase is opposite to that of CAGT. In *L. flavum* cell suspension cultures, β-glucosidase activity increased to a maximal value on day 4 of the growth cycle and declined to lowest activity on day 14.[13] A high CAGT activity apparently relates to a low β-glucosidase activity.

Reduction of CAGT activity correlated with a reduction of coniferin production in the cell suspensions. The coniferin production and CAGT activity were reduced to >88% at the end of a growth cycle after treatment with 5 mM Na_2EDTA, while control values were at their maximum at this time point. These results strongly suggest that Na_2EDTA inhibits CAGT activity thereby inhibiting the conversion of coniferyl alcohol into coniferin in *L. flavum* cell suspension cultures. Our hypothesis that Na_2EDTA is an inhibitor of CAGT activity is further supported by the inhibitory effect on CAGT activity in a 11.4-fold purified enzyme preparation. The effect, in terms of % inhibition, however, is less pronounced than in the cell suspensions. This different may be due to a toxic effect of Na_2EDTA on the cell suspension cultures.

CONCLUSION

In conclusion, Na_2EDTA appears to be an inhibitor of CAGT activity both *in vivo* and *in situ*. Because of a lack of information about the structure and the function of the CAGT it is not yet clear which mechanism underlies the inhibition by EDTA. If CAGT needs a metal ion as a co-factor for its activity, it can be understood that the Na_2EDTA complexes with the metal ion, thereby reducing the enzyme activity. Further studies directed to the purification, structure and function determination of the enzyme are in progress.

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