Exomorphic and Endomorphic features of *Swertia chirayita*

A.B.D. Selvam

Pharmacognosy Section, Botanical Survey of India, Howrah – 711103, India

ABSTRACT

The trade name '*Chirata*' refers to both *Swertia chirayita* (Roxb. *ex* Flem.) Karsten. (Gentianaceae) and *Andrographis paniculata* (Burm.*f*) Nees (Acanthaceae). This ambiguous trade name gives room for fraudulent activities. The plant *Swertia chirayita* has already been studied by many authors. However, the basic vascular structures of Stem have been misinterpreted in Pharmacognosy books and also in the Indian Herbal Pharmacopoeia. In the present investigation, exomorphic and endomorphic features of *Swertia chirayita* have been critically studied and the correct usage of the scientific terms and distinguishing structural features have also been highlighted, which would be useful in identification and authentication of this plant.

Key words: *Swertia chirayita*, chirata, exomorphic, endomorphic, identification, authentication

INTRODUCTION

The genus *Swertia* belongs to the family Gentianaceae, which comprises of about 250 species throughout the world and about 32 species occur in India, including *Swertia chirayita* (Roxb. *ex* Flem.) Karsten. In early days, the European practitioners in British India had appreciated the value of Chirata. Later on, this plant was recognised in the British and United States Pharmacopoeiae.[4,6,10] This plant species is ranked 'Endangered' among threatened plants of India.[11]

The whole plant is used in Folk, Ayurveda, Siddha, Unani, Homoeopathy and in Modern medicine. It is credited with tonic, febrifuge, alterative, carminative, expectorant, laxative, stomachic, anthelmintic and anti-diarrhoeal properties. It is employed in drug formulations prescribed for the treatment of toxic fever, malarial fever, urinary disorders, bronchial asthma, bilious affections, burning of the body, constipation, diarrhoea, dyspepsia, flatulence and skin diseases.[1,2,11]

Address for correspondence:
A.B.D. Selvam, Pharmacognosy Section,
Botanical Survey of India,
Howrah – 711103, India.
E-mail: abd_selvam@yahoo.co.in

DOI: 10.5530/pj.2011.19.1

The market sample of this raw drug is generally mixed with other species of *Swertia*, of which *Swertia angustifolia* Buch.-Ham ex D. Don and *Swertia alata* Royle are most common. The whole herb of *Andrographis paniculata* (Burm.*f*) Nees (Acanthaceae) is often sold in the name of Chirayita. *Andrographis paniculata* is often substituted for or confused with *Swertia chirayita*.[5,11]

MATERIALS AND METHODS

Proper identification and authentication of plants, particularly medicinal plants at species level is a most important prerequisite, as they are predominantly used in the manufacturing of life saving medicines. Medicinal plants and their plant parts can be identified primarily using Exomorphic (external or macro-morphological) features such as size, shape, surface features (external markings) etc.[9] In addition to the exomorphic features, organoleptic characters are also equally valued in the identification of drug plants/plant parts.[14] In the present study, the exomorphic or macroscopic features of Swertia chirayita was studied initially. Later on, the organoleptic characters (sensory characters) were noted down and are furnished in a tabular form **(Table–1).**

Taxonomists often value endomorphic (anatomical and histological) features for solving taxonomic problems. Anatomical characters are of great value in establishing

botanical identity of crude drugs. Further, it is simple, time saving, cost effective and most reliable. It also plays an important role in checking adulteration, fraud and substitution. The anatomical characters that are either restricted or constant in distribution in plants/plant parts are mostly relied upon for diagnosis viz. laticiferous elements, included phloem (interxylary phloem), crystals, starch grains (size, shape and appearance), pericyclic sclerenchyma, stomata, palisade ratio, vein-islet numbers, among others.[8]

Customary procedure of Microtomy was employed for studying the micro-morphological or endomorphic (anatomical and histological) features of Swertia chirayita. [7,12] Light microscope was used to study anatomical features. For normal structural observations, visible light or bright field was used. For the study of lignified cells, polarized light microscope was employed. The microscopic descriptions of the tissues/cells and the cell inclusions have been supplemented with photomicrographs wherever necessary.

OBSERVATIONS

Several authors have studied the macroscopic, microscopic and organoleptic features of the whole plant (root, stem and leaves) of *Swertia chirayita*.[5,10,11,14] However, the data available on the organoleptic characters of this drug plant is insufficient and it requires a detailed study. Further, some of the anatomical features have been misinterpreted in the published work. Therefore, in the present study, all the above-mentioned parameters have been worked out with a view to bring out a comprehensive structural data on this drug plant, which can be used to distinguish this genuine drug plant from its allied species in its crude form.

EXOMORPHIC FEATURES AND ORGANOLEPTIC CHARACTERS

The data available on the macroscopic features and organoleptic characters of the whole plant of *S. chirayita* were collected and reviewed.[5,10,11,14] They are furnished in a tabular form along with the personal observations (Table–1; Plate–1, figure D).

ENDOMORPHIC FEATURES

In cross sectional view, the whole plant (root, stem and petiole) of *S. chirayita* shows following structural features.

Root: Root studied measures about 4.5 mm in diameter. It has thin, less prominent periderm. The cortical zone is wide, homogeneous and parenchymatous. The cortical cells are polyhederal, thin walled and compact. No specific cell inclusions are evident in the cortex. The cortical zone is gradually transformed in to secondary phloem. The secondary phloem consists of radial files of polyhedral elements that include sieve elements, phloem parenchyma

Table 1: Exomorphic features and Organoleptic characters of the whole plant of *Swertia chirayita*

	Exomorphic features
Occurrence	The dried raw drug sold in the market consists of bundles of long, more often leafless stems topped with flowering or fruiting branches, with a short tapering root.
Size	**Root:** 5–10 cm long, up to 1.25 cm in diameter; **Stem:** 60–125 cm long, about 6 mm in diameter; **Leaves:** 3.5–10.0 x 1.5–4.0 cm.
Shape	**Root** – oblique, twisted and gradually tapering; **Stem** – cylindrical/sub-cylindrical at base and 4-angled upwards; **Leaves** – opposite decussate, broadly ovate or lanceolate, glabrous, obtuse or cordate at base, acuminate at apex, margin entire, usually with 3–7 prominent lateral veins.
Surface	**Root:** bears few rootlets or their remnants, which are pointed and hard; **Stem:** smooth; **Leaves:** glabrous.
	Organoleptic characters
Colour	**External surface of root:** light brown; **Stem:** rusty brown or purplish brown at base, greenish yellow or dark green in the upper region; **Leaves:** fresh dark green; dried leaves upper surface reddish brown, lower surface pale greenish brown. **Internal surface of root:** Yellowish grey or brownish with a hollow or spongy centre; **Stem:** grey or grayish white with a central pith; **Leaves:** grayish brown.
Odour	The drug is odourless.
Taste	All parts of the plant (from root to tip) are very bitter.
Fracture	**Root:** short, complete and splintery; **Stem:** short, incomplete, breaks with a tick sound; **Leaves:** short, complete, breaks with tick sound.
Texture	**Root:** uneven due to irregularly broken surface and rough; **Stem:** uneven and rough; **Leaves:** smooth.
Peculiarities, if any.	The stem forms the major portion of the drug. It has smooth outer surface with shining rusty brown or purplish green colour, a large continuous pith and intensely bitter taste.

Plate - 1. Figures illustrating the morphological features of *Swertia chirayita*.
A - herbs in cultivtion, *B* - herb in its natural habitat,
C - dried plant samples (whole plant) occur in trade.

and phloem rays. There is no clear demarcation in the transition zone of the cortex into phloem.

Secondary xylem is centrally located, solid and wide. The central core consists of narrow mass of irregularly lobed xylem tissue. Outside the central core of xylem tissue occurs wide parenchymatous tissue, in which small nests of xylem elements are scattered. Enclosing the parenchymatous tissue with scattered xylem masses and central core lobed xylem strand occurs fairly thick, closed cylinder of xylem cylinder. Within this cylinder, the xylem elements occur in small clusters or in radial multiples and sometimes they are solitary. Xylem includes fairly wide vessels and thick walled lignified fibres. The vessel elements are abundant, especially in the peripheral zone. The vessels are 35–42.5 μm in diameter **(Plate–2).**

Stem: The young stem has thin epidermal layer followed by a wide heterogeneous cortex. In some of the epidermal cells, the outer tangential walls developed into prominent spherical papillae. The outer part of the cortex consists of polyhedral collenchyma cells with wide air chambers. The inner part of the cortex consists of radially aligned rectangular cells. The vascular cylinder is thick and hollow. Outer phloem is fairly wide and occurs in continuous cylinder of uneven thickness. The secondary xylem elements occur in short, radial parallel lines. Primary xylem cylinder is less prominently seen. Under polarized light, an irregularly shaped discontinuous cylinder of pith fibres is seen. Pith is wide, homogeneous and parenchymatous. At certain places, the pith region is hollow due to disintegration of cells.

Matured stem is 4.25–4.5 mm in diameter. There is a distinct layer of epidermis followed by heterogeneous cortex. The outer cortex has collenchymas cells with wide, rectangular air chambers; the inner cortex is narrow, comprising of 4 or 5 layers of parenchyma cells with wide rectangular air chambers. Inner to the cortex, there is a thin and continuous

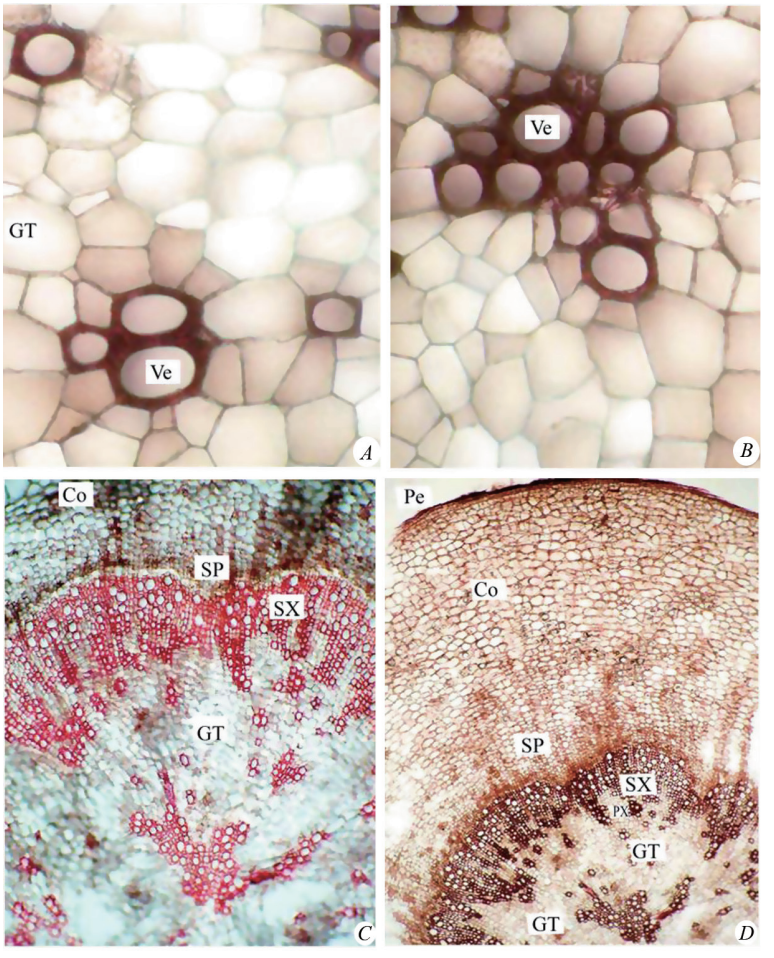


Plate - 2. Transverse section of Root of *Swertia chirayita*.
A - root in cross sectional view - a sector,
B - portion of the root showing vascular and ground tissues,
C - central portion of the root showing ground tissue and xylem elements.

zone of secondary phloem (outer phloem or external phloem) with radial files of phloem elements.

It exhibits initial stage of secondary growth. Secondary xylem cylinder is thick and continuous. It has angular, wide, thick walled vessels which are either in clusters or in short radial multiples. The diameter of the vessels ranges from 20 to 40 µm. The primary xylem occurs along the inner circumference of the secondary xylem cylinder comprising less distinct short radial rows of xylem elements. Inner to the primary xylem cylinder, occurs a wide cylinder of inner phloem or medullary phloem with uneven thickness. The inner phloem is divided into thick masses by short partition segments of thick walled cells. These partition segments are radial extensions of thick walled pith cells. These thick walled cells are fibres since they have lignified walls with wide lumen; the lignification is confirmed by examining the cells under polarized light. Major portion of the pith is homogenous and parenchymatous. The pith cells are angular, thin walled and compact **(Plate - 3).**

Petiole: In cross sectional view, the decurrent petiole (winged petiole) is broadly V-shaped with wide adaxial concavity and three wide prominent abaxial ridges. The midrib/median ridge is 1075–1750 µm horizontally and 1000–1725 µm vertically. The midrib comprises of thin epidermal layer with small, semicircular, thin walled cells. The ground tissue is parenchymatous and homogeneous; the cells are angular, thin walled and compact. The vascular system is multistranded. A wide, arc or bowl shaped median vascular strand and three slightly smaller arc shaped lateral vascular strands either side of the petiole and a smallest, less prominent marginal vascular strand on either side of the petiole are seen. The vascular strands are bicollateral;

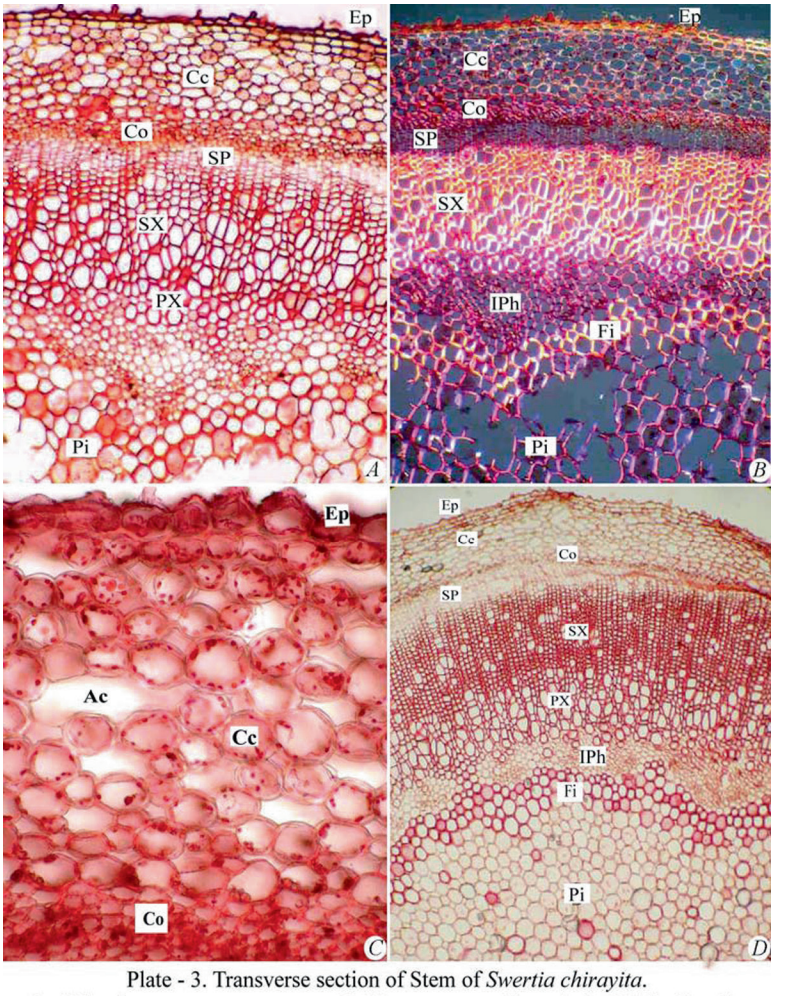


Plate - 3. Transverse section of Stem of *Swertia chirayita*.
A - T.S. of young stem - a sector, *B* - Young stem under polarised light showing vascular and ground tissues, *C* - young stem showing collenchyma cells and cortex, *D* - matured stem showing primary and secondary structures.

they have several closely arranged short parallel rows of xylem elements and thick arcs of phloem elements located all along the outer and inner parts of the xylem strand **(Plate–4).**

SUMMARY AND CONCLUSION

In *Swertia chirayita*, a wide, solid secondary xylem located in the centre of the root. In addition, the xylem elements are scattered in the ground tissue of the root. In the stem, primary and secondary structures are seen. The petiole has thick, wide, arc or bowl-shaped vascular strand in the midrib region and also in the lateral veins, which is bicollateral, having phloem both on the outer and inner sides of the xylem.

In most of the Pharmacognosy text books and Indian Herbal Pharmacopoeia, the vascular cylinder of stem of *Swertia chirayita* has been described as Amphiphloic siphonostele. This term is misnomer for two reasons. The vascular cylinder of matured stem is not a stele according to the original definition of stele.[3] Secondly, the two phloem zones are to be referred to as outer normal secondary phloem and inner medullary or intraxylary phloem.

ACKNOWLEDGEMENTS

The author is thankful to the Director, Botanical Survey of India, Kolkata for providing facilities. He is also grateful to Prof. P. Jayaraman, Plant Anatomy Research Centre, Chennai for his critical comments, and help during the study.

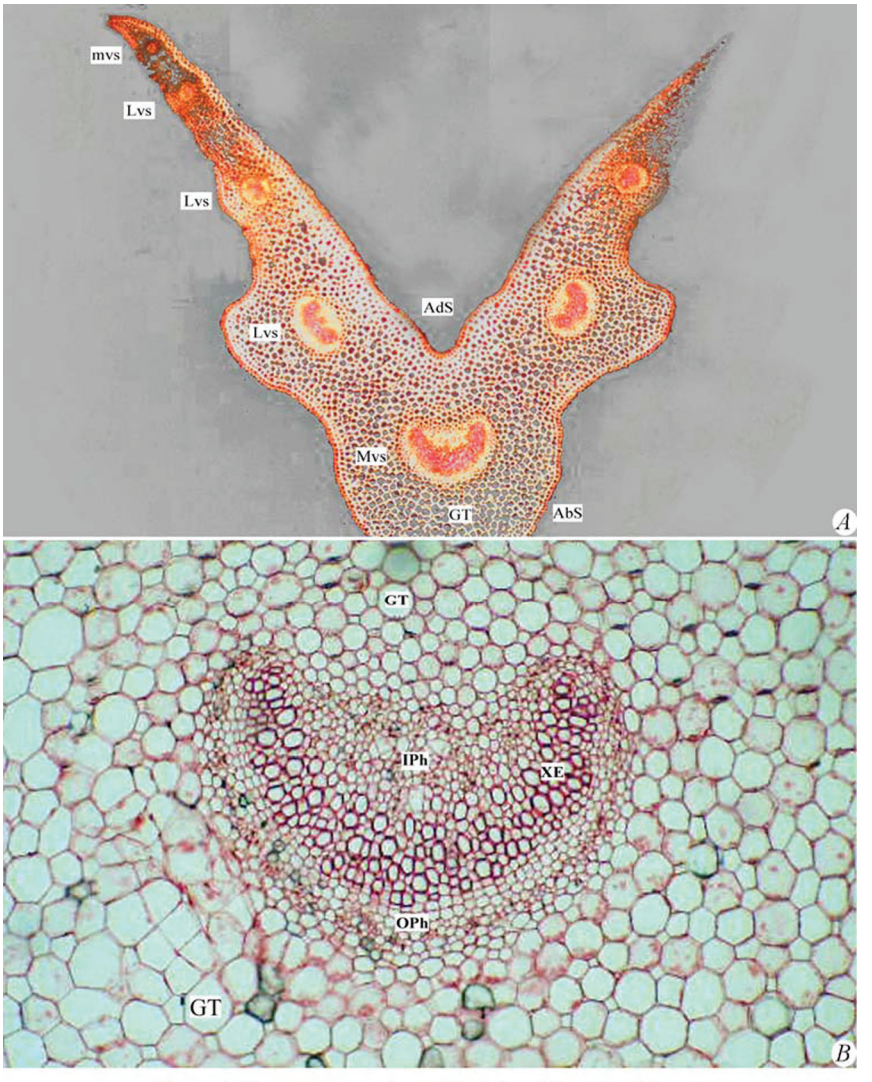


Plate - 4. Transverse section of Petiole of *Swertia chirayita*.
A - petiole in cross sectional view seen under polarised light,
B - central portion of the petiole showing median vascular tissue and ground tissue - a close up view.

REFERENCES

1. Chauhan NS. Medicinal and Aromatic plants of Himachal Pradesh. New Delhi: Indus publishing Company; 2006.
2. Dey AC. Indian Medicinal plants used in Ayurvedic preparations. Dehra Dun: Bishen Singh Mahendra Pal Singh; 1980.
3. Esau K. Plant Anatomy. New York: John Wiley & Sons, Inc.; 1953.
4. Garg S. Gentianaceae of the North West Himalaya (A revision). New Delhi: Today & Tomorrow's Printers & Publishers; 1987.
5. Handa SS, Mundkinajeddu D, Mangal AK. Indian Herbal Pharmacopoeia. Mumbai: RRL, Jammu & IDMA; 1998. I:147-155.
6. Jain SK. Medicinal plants. New Delhi: National Book trust; 2001.
7. Johansen DA. Plant Microtechnique. New York: Mc Graw Hill Book Co.; 1940.
8. Metcalf CR, Chalk L. Anatomy of the Dicotyledons - Leaves, stem and wood in relation to taxonomy with notes on Economic uses. London: Oxford University Press; 1957.
9. Naik VN. Identification of common Indian medicinal plants. Jodhpur: Scientific Publishers (India); 2004.
10. Prasad S, Nayar KC, Bhattacharya IC. Pharmacognostical studies on Chirata and its substitutes and adulterants: Part I – *Swertia chirata* Ham. J Sci Industr Res; 1960. 19C(5):119-125.
11. Sarin YK. Principal crude herbal drugs of India. Dehra Dun: Bishen Singh Mahendra Pal Singh; 2008.
12. Sass JE. Elements of Botanical Microtechnique. New York: Mc Graw Hill Book Co.; 1940.
13. Tyler VE, Brady LR, Robbers JE. Pharmacognosy. Philadelphia: Lea & Febiger; 1976.
14. Wallis TE. Textbook of Pharmacognosy. 5th ed. New Delhi: CBS Publishers & Distributors; 2005.

Comparative Pharmacognostic Evaluation of Three species of *Swertia* L. (Gentianaceae)

Deepa Bisht, Mansi Gupta, Sharad Srivastava, Bhaskar Datt[1] and AKS Rawat*

**Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute (CSIR), Lucknow-226003*
[1]Taxonomy Division, National Botanical Research Institute (CSIR), Lucknow-226003

ABSTRACT

Swertia spp. (Gentianaceae) commonly known as *Chiraita* in herbal drug markets of India is used to protect liver and a wide range of diseases. The official species *Swertia chirayita* (Roxb.) Karsten is known for its potent activity against malaria, liver-disorder, fever, diabetes and also as appetite stimulant. However, several other species of *Swertia* viz. *S. alata* (Royle ex D. Don) Clarke and *S. paniculata* Wall. are being used as substitutes /adulterants for *S. chiraita* in India, Japan, China, Pakistan and other Asian Countries.

In the present communication a comparative botanical, phytochemical and qualitative analysis of biomarker Oleanolic acid in *S. chirayita* along with two allied species of *Swertia* has been done.

Keywords: *Swertia*; Pharmacognosy; *Chiraita*; Oleanolic acid; HPTLC

INTRODUCTION

Swertia L. (Family: Gentianaceae) is widely distributed in the Temperate regions of Asia, Africa, Europe, North America and Madagascar with *ca.* 150 species. The genus *Swertia* is represented in India by *ca.* 40 species, chiefly distributed in the Himalaya between 1500-2400 m of which *S. chirayita* (Roxb.) Karsten is well-known for numerous medicinal applications[1]. Several species of *Swertia* are being used as substitutes or adulterants of *S. chirayita* and are known for their therapeutic potential against fever, hepatitis, cholecystitis, pneumonia, osteomyelitis, dysentery, scabies, spasm, pain, and malaria, and used as antidepressant, mutagenic, antipsychotic, tuberculostatic, choleretic, antioxidant, anti-inflammatory and antidiabetic drug[2-7]

The genus *Swertia* is known as a rich source of xanthones, some species also containing terpenoids and seco-iridoids[2,3,7]. Previous chemical investigations of the plants have revealed the presence of the Lupeol[8], Mangiferin[9,10], β-sitosterol, 3-β-D-glucoside[11] and ursolic acid[12]. In an earlier study, 1-hydroxy-8-glucosyloxy-3, 5-dimethoxyxanthone, 1, 8-dihydroxy-3, 7-dimethoxyxanthone, 3-methoxy-1, 5, 8-trihydroxyxanthone and ursolic acid have been reported from its aerial parts[7] with potent antioxidant activity[13], but there has been no report on the qualitative analysis of Oleanolic acid in these species, which is an important triterpenoid responsible for hepato-protection, anti-inflammatory and anti-hyperlipidemic properties.

Several species of *Swertia* are highly exploited as raw material for different traditional medicines. Among them, *S. chirayita* is considered to be superior in medicine and trade. One of the main issues in its trade is adulteration with other species which are considered to be inferior in medicinal quality. Hence, there is need for selection of distinct macro- as well as micro-morphological characteristics for correct identification of raw material. The present paper aims to provide HPTLC, physico-chemical, morphological and histological profiles to establish the authenticity of *S. chirayita* which may help to a great extent for differentiating the genuine drug from its other allied species.

Address for correspondence:
AKS Rawat, Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute (CSIR), Lucknow-226003.
Email: pharmacognosy1@rediffmail.com

DOI: 10.5530/pj.2011.19.2

EXPERIMENTAL

Material and methods

Plant material of *S. paniculata* Wall. and *S. alata* (Royle ex D. Don) Clarke were collected from Almora district of

Kumaon region (Uttarakhand) and *S. chirayita* from Darjeeling (West Bengal). It was authenticated on the basis of taxonomical characters and matched with herbarium specimens available at the NBRI herbarium, Lucknow. The voucher specimen of the plant material (*S. chirayita*-285496, *S. alata*-251916, *S. paniculata*–251915) have been deposited at the NBRI, herbarium. Whole plants were preserved in 70% ethyl alcohol for histological studies. Hand sections were cut and stained with safranin and fast green and photographed with Olympus CX 31 camera[14].

Physico-chemical and phytochemical studies like total ash, acid insoluble ash, extractive values, sugar and starch were calculated from the shade dried and powdered (60 mesh) plant material[15-17].

HPTLC studies

Extraction of plant material

Air-dried (45-55^{0}c) powdered sample of three *Swertia* species (2.0g each) was extracted with methanol. Extracts were concentrated under vacuum, re-dissolved in methanol, and finally made concentration of 10mg/ml prior to HPTLC analysis.

Chromatographic studies

HPTLC was performed on 10X10 cm silica gel 60GF$_{254}$ plates (Merck). Methanolic solutions and standard compound Oleanolic acid of known concentration of 1mg/ml were applied, using Camag Linomat 5 automated TLC applicator with the nitrogen flow providing a delivery speed of 150nl/sec from the application syringe. The plates were developed to a distance of 80 mm with toluene: ethyl acetate (8:2) as mobile phase in a Camag twin-trough glass chamber previously saturated with mobile phase vapour. After removal of plates from chamber it was completely dried in air at room temperature and peak areas for samples and standard were recorded by densitometry in absorbance/reflection mode at λ max = 570nm, by means of a Camag TLC Scanner 3 with WINCATS version 3.2.1 software.

RESULT AND DISCUSSION

Macro and microscopic studies

Macroscopically stem of *S. chirayita* is cylindrical below, *tetragonous* upwards, hollow, while it is quadrangular with narrowly winged angles in *S. alata* and cylindrical below and *4 lineolate upwards in S. paniculata*. The leaves are elliptic or ovate -lanceolate in *S. chirayita*, linear or linear-lanceolate in *S. paniculata* and ovate-oblong in *S. alata.*

The main distinguishing features among these species are in the flowers i.e. pentamerous in *S. paniculata* and tetramerous in *S. chirayita* and *S. alata*. Besides, each petal in *S. chirayita* has two basal glands, whereas there is single basal gland on each petal in both *S. alata* and *S. paniculata*.

Microscopically *S. chirayita* contains cavities at pre-medullary zones, while it is absent in *S. alata* and *S. paniculata*. Mucilage and acicular crystals of calcium oxalate are present in *S. chirayita* and absent in *S. alata*. Further, *S. alata* consists of wider zone of interxylary phloem than that of *S. chirayita*. (Fig. 1&2 and Table 1)

Physicochemical studies:

Studies shows that *S. chirayita* has almost maximum percentage of all the physicochemical parameters (total ash 5.30%, ethanol soluble extractive 41.60%, water soluble extractive 31.60%) except starch and sugar which were highest in *S. alata*. (Fig. 3)

HPTLC Studies:

Qualitative HPTLC analysis (Fig.4) of all the three *Swertia* species was also performed for the development of characteristic fingerprint profile, which may be used as marker fingerprints for quality evaluation and standardization of this drug.

S. alata and *S. paniculata* have more or less similar bands at same R_f as in *S. chirayita*. Moreover the concentration of oleanolic acid is maximum in *S. chirayita* than in *S. paniculata,* however, minimum percentage was found in *S. alata*. (Table 2)

CONCLUSION

The detailed morphological and anatomical studies revealed that there are marked differences in morphological characters such as shape of leaves (elliptic or ovate -lanceolate in *S. chirayita*, linear or linear-lanceolate in *S. paniculata* and ovate -oblong in *S. alata*) and stem (narrowly winged in S. alata, while cylindrical to obtusely 4- angled in *S. chirayita* and *S. paniculata*). However, the main distinguishing features among these species are: flowers pentamerous in *S. paniculata* and tetramerous in *S. chirayita* and *S. alata,* and each petal in *S. chirayita* have 2 glands, whereas in other two species there is single gland on each petal.

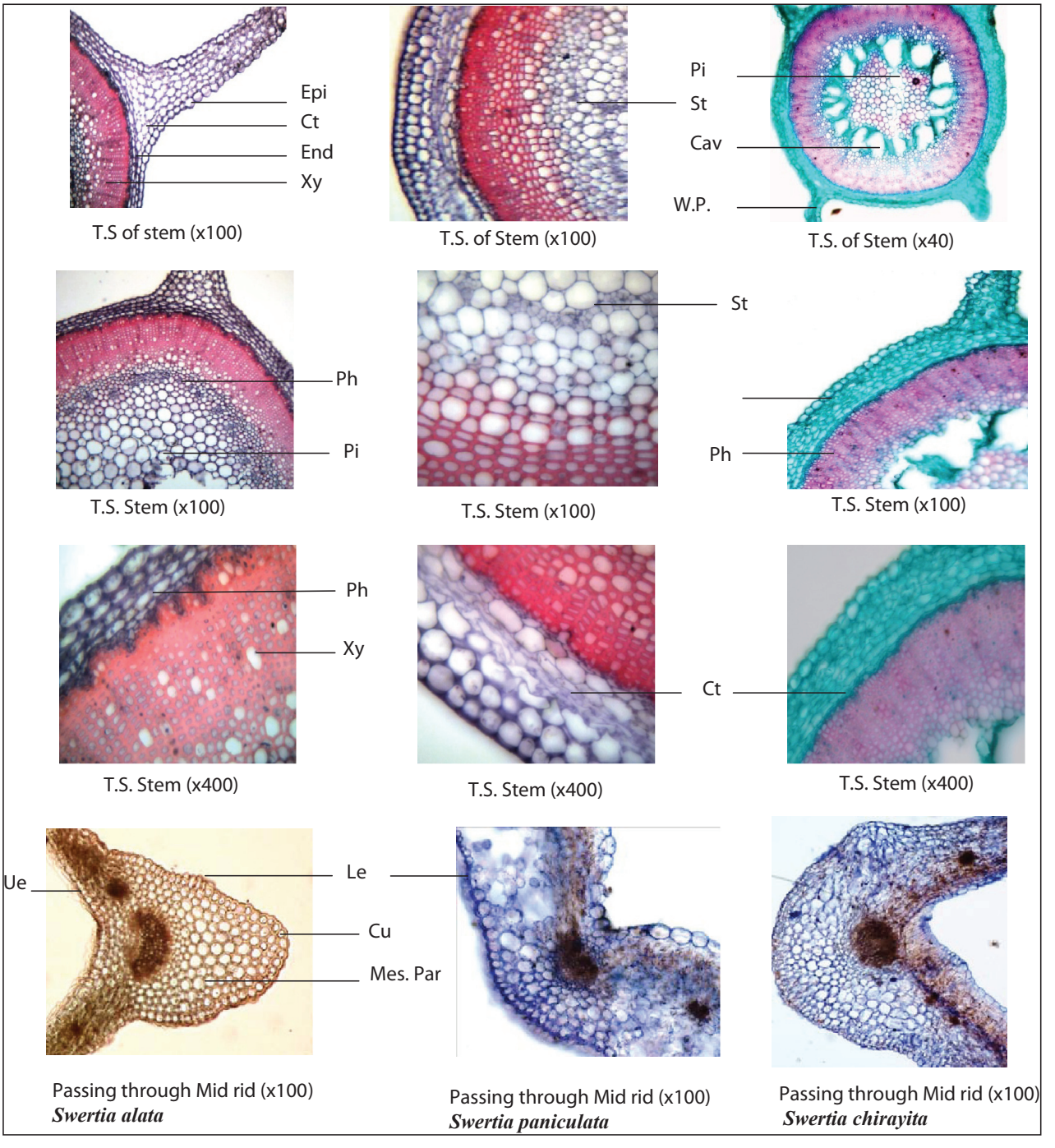


Figure 1: Comparative Microscopic descriptors of three Swertia species
Abbreviation:
Stem - ct-cortex, cu- cuticle, end- endodermis, epi-epidermis, pi-pith, wi-winged extension, xy-xylem,Ph-Phloem, St-Seive tube, Cav-Cavity
Leaf-Le-Lower epidermis, Ue-Upper epidermis,Cu-Cuticle, Mes. Par- Merophyll parenchymatous

There are some minor variations in the microscopic characters as calcium oxalate crystals are absent in *S. alata*. No appearance of cavity in the pith region of stem in *S. alata* and *S. paniculata* while cavities present in premedullary region of *S. chirayita*. Besides, there is also variation in physicochemical parameters and Oleanolic acid concentration among these species.

ACKNOWLEDGMENT

The authors are grateful to the Director, CSIR: NBRI, Lucknow for providing all the facilities to conduct this research work and are also thankful to National Medicinal Plant Board, India for financial support.

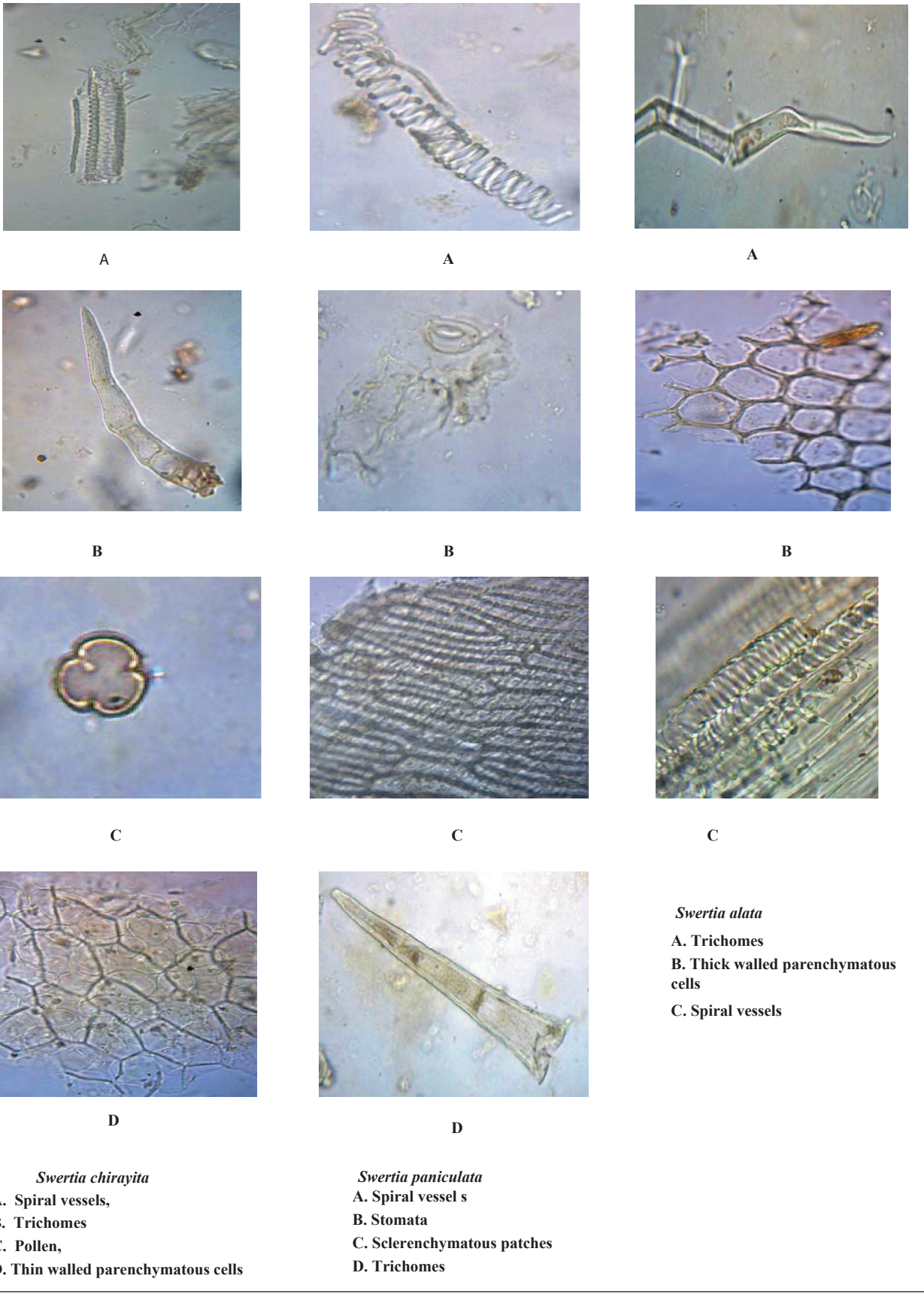


Figure 2: Comparative Powdered Microscopic details of three *Swertia* species

Table 1: Comparative macroscopic and microscopic characters of three *Swertia* species

Characters	*Swertia chirayita*	*Swertia alata*	*Swertia paniculata*
Macroscopic	Annual or biennial, up to 110 cm high; stem cylindrical below, 4-gonous upwards, yellowish brown; leaves 3-7-nerved; flowers tetramerous; petals yellowish green outside, purple inside, glands 2 at the base of each petal; anthers purple.	Annual, up to 90 cm high; stem 4-angular with winged angles, pale green; leaves 3-5-nerved; flowers tetramerous ; petals lurid green, dotted and veined with purple, gland 1 at the base of each petal; anthers yellow.	Annual, up to 50 cm high ; stem cylindrical below, 4 lineolate upwards, greenish purple; leaves 1-3-nerved, flowers pentamerous; petals white with purple blotches at base; gland 1, scaly, at the base of each petal; anthers violet.
Microscopic	T.S. of stem shows single layered epidermis with striated cuticle and 4-winged projections at each corner; cortex 2-6-layered , mucilage and acicular crystals present in cortical cells; stellar system amphiphloic siphonostele; external phloem with usual elements; xylem with tangentially elongated cells, sieve tube, tracheids and fibres. Pith consists of isodiametric cells.	Cubical epidermal cells with striated cuticle; cortex 5-10- layered composed of collenchyma cells; stellar system amphiphloic siphonostele; xylem composed of vessels, tracheids and xylem fibres; inner phloem is developed more than outer, contains wider zone and strands of sieve tube intermixed with phloem parenchyma. Pith consists of isodiametric cells.	Epidermis covered with cuticle; cortex 4-8- layered with rounded to cubical cells; stellar system amphiphloic siphonostele; outer phloem consists of narrow zone; xylem region wide, sieve tube present in more quantity with perenchymatous cells.
	T.S of leaf shows single layered epidermis covered with cuticle. Mesophyll cells spongy, loosely arranged.	Epidermis single layer with cells cubical in shape, covered with cuticle. Mesophyll cells closely arranged.	Epidermis single layer with cells cubical in shape, covered with cuticle. Mesophyll cells loosely arranged.

Table 2: Comparative Qualitative HPTLC details (R_f values) of three *Swertia* species

	Colour of Band		
R_f	*S. chirayita*	*S. alata*	*S. paniculata*
0.10	Light purple	Light purple	Light purple
0.14	Light purple	Light purple	Light purple
0.19	Light Green	Light Green	Light Green
0.40 (Oleanolic acid)	Purple (0.714%)	Purple (0.328%)	Purple (0. 644%)
0.52	Purple	Purple	Purple
0.65	Light Green	Light Green	Light Green
0.76	Light Green	Light Green	Light Green
0.85	Light purple	Light purple	Light purple
0.92	Light pink	Light pink	Light pink

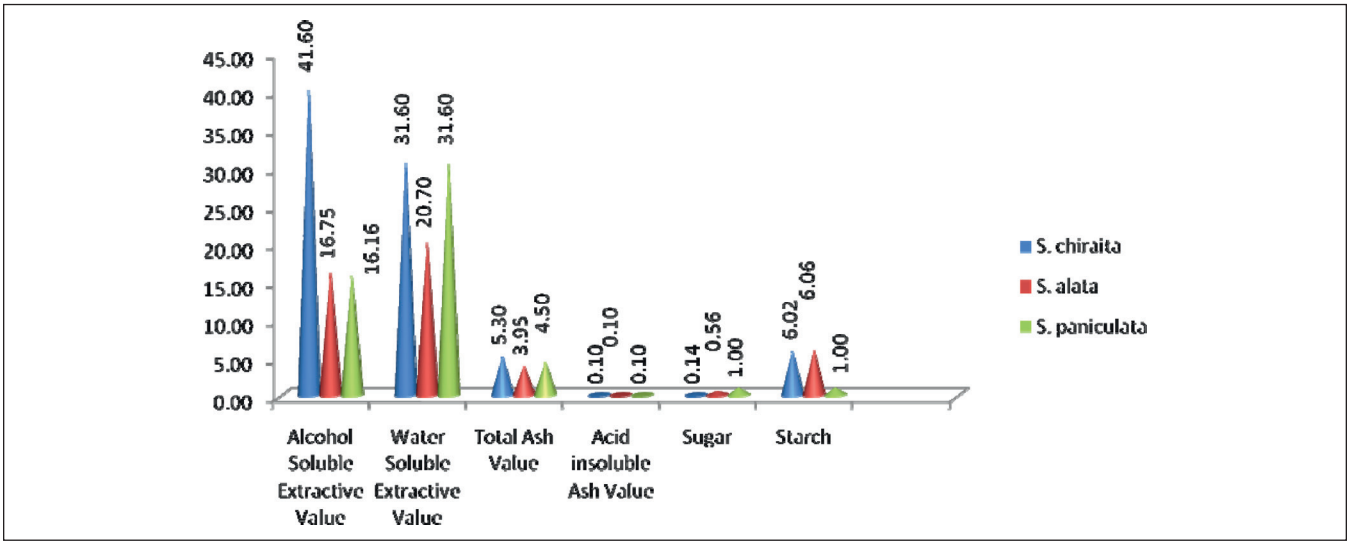


Figure 3: Comparative Physico chemical values of Three Swertia species

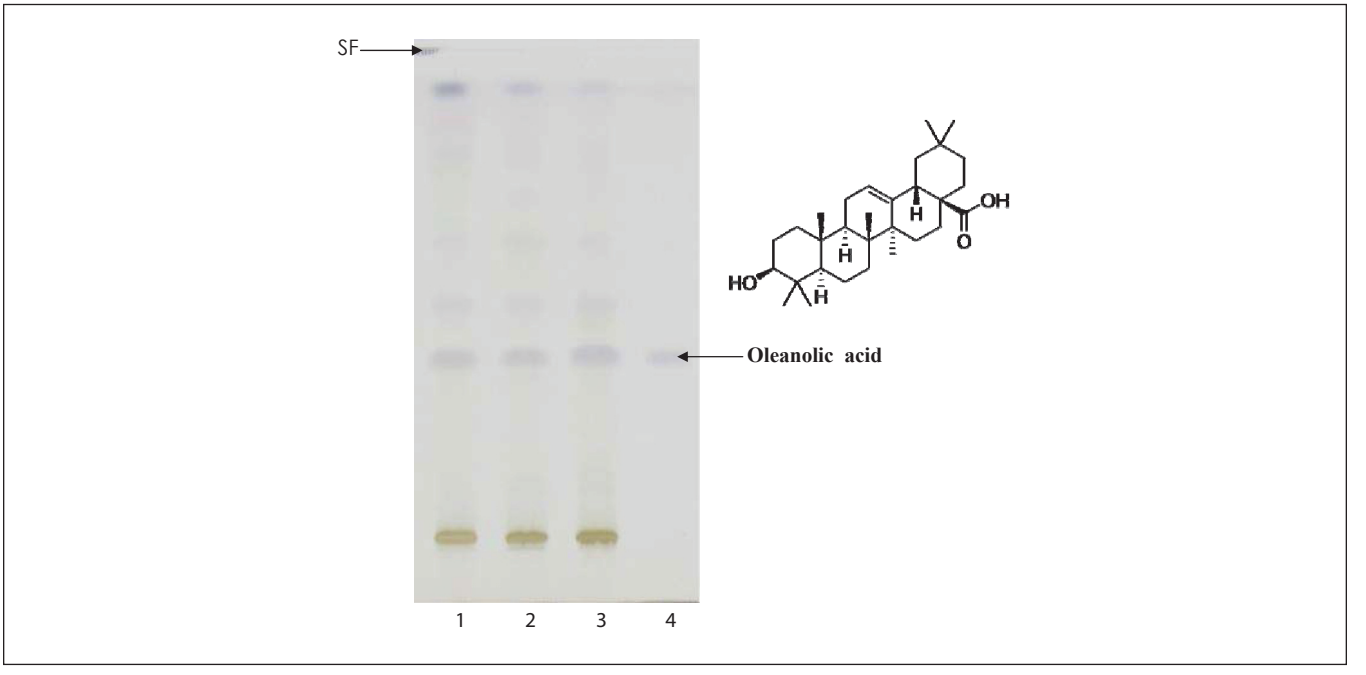


Figure 4: Comparative HPTLC profiles of three *Swertia* species along with Oleanolic acid (Standard) under visible light
1 = *Swertia chirayita*, 2 = *Swertia alata*, 3 = *Swertia paniculata*, 4 = Standard (Oleanolic acid)

REFERENCES

1. Garg, S. (1987). *International Bioscience Monograph*, 17.
2. Anonymous. (1976). *The Wealth of India*, A Dictionary of Indian Raw Materials and Industrial Products, Council of Scientific and Industrial Research, New Delhi, India, **10**, 77-81.
3. Chopra, R.N., Nayar, S.L. and Chopra, I.C. (1956). *Glossary of Indian Medicinal Plants*, CSIR, New Delhi, 237.
4. Naithani, B.D. (1985). *Flora of Chamoli*, Botanical Survey of India, Calcutta, **2**, 433.
5. Dhar, U. and Kachroo, P. (1983). Alpine Flora of Kashmir Himalaya, Scientific Publishers, Jodhpur, India, 132.
6. Ambasta, S.P. (1986). *The Useful Plants of India*, Publication & Information Directorate, CSIR, New Delhi, 608-609.
7. Khetwal, K.S. and Bisht, R.S. (1988). A xanthone glycoside from *Sweria speciosa*. *Phytochemistry*, **27**, 1910.
8. Chakravarty, A.K., Mukhophadhyay, S., Masuda, K., Ageta, H. (1992). More of Swertane Triterpenoids from *S. chirayita* Buch-Ham. *Indian J Chem* B, **31**, 70-71.
9. Ghosal, S., Sharma, P.V., Chaudhuri, R.K. and Bhodtacharya, S.K. (1973). Chemical constituents of the gentianaceae-V: tetraoxygenated xanthones of *Sweria chirayita* Buch-Ham. *J Pharm Sci,* **62**, 926.
10. Chakravarty, A.K., Mukhopadhyay, S., Moitra, S.K. and Das, B. (1994). Syrungareinol, a hepatoprotective agent and other constituents from *Sweria chirayita*. *Indian J Chem* B, **33**, 405-408.
11. Chaudhuri, P.K. and Daniewshi, W.M. (1996). Unambigous assignments of the 1H and 13C chemical shifts of the major bitter principles of *Sweria chiraita* by 2D-NMR study and characterization of other constituents. *Pol J Chem*, **69**, 1514-1519.
12. Chakravarty, A.K., Mkhopadhyay, S. and Das, B. (1991). Swertane terpenoids from *Swertia chirayita*. *Phytochemistry*, **30**, 4092-4087.
13. Rana, V.S. and Rawat, S.M. (2005). A new xantone glycoside and antioxidant constituents from the rhizomes of *Sweria speciosa*. *Chemistry and Biodiversity,* **2**, 1310-1315.
14. Johansen, D.A. (1940). *Plant Microtechnique*. 1st edn. MC Graw Hill Book Co. Inc., New York and London, 15-93.
15. Peach, K. and Tracy, M.V. (1955). *Modern Methods of Plant Analysis*. Springer, Heidelberg, **3**, 258-261
16. Anonymous. (2007). *Indian Pharmacopoeia*. Govt. of India, Ministry of Health and Welfare, Controller of Publications, New Delhi, 191.
17. Anonymous. (1984). *Official methods of Analysis* (AOAC). 4th ed. Association of Official Chemists, Inc. U.S.A., 187-188

Pharmacognostical Evaluation of *Amaranthus spinosus* L

Sangameswaran Balakrishnan*[1], Balakrishnan Natarajan[1], Balasubramaniam, Arumugam[1], Bhaskar Vaidun Hariram[2]

[1]Technocrates Institute of Technology-Pharmacy, Anandnagar, Bhopal - 462021, M.P, India, [2]M.P. Patel College of Pharmacy, Kapadwanj-387620, Gujarat, India

ABSTRACT

Introduction: In increasing demand in the field of herbal medicines and cosmetics, it has become necessary and pertinent to probe into the area of systematic knowledge about herbal drugs. There is a need for the application of this knowledge in authentification, detailed study and practical utilization of crude drugs. The present paper deals with the taxonomy, anatomy, powder study pertaining to organoleptic, microscopic, and physical constant evaluations of *Amaranthus spinosus* Linn (Amaranthaceae). It is a glabrous herb found in tropical and sub-tropical regions of India. The decoction of the leaves is diuretic and febrifuge. Leaves and stems are also used for treating eye diseases and diabetes. **Methods**: In the present study macroscopic, microscopic, ash values and extractive values were carried out to develop the diagnostic parameters for quality control of leaf and stem. The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin – 5 ml + acetic acid – 5ml + 70% Ethyl alcohol – 90ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary – butyl alcohol. The specimens were castled into paraffin blocks. **Results**: Powder showed epidermal cell, anamocytic stomata, calcium oxalate crystals and cross section of leaf and stem showed the bundle sheath cells, median bundle, phloem fibers, vessels, periderm, secondary xylem and pith. Phytochemically, the methanol and aqueous extracts of *A. spinosus* showed maximum phytochemicals like alkaloids, glycosides, steroids, flavonoids, saponin, tannin and phenolic compounds, terpenoids, carbohydrates, etc. The average extractive values of leaf and stem can also be used for determine the quality of raw material of *A. spinosus*. **Conclusion:** The results of this study should provide a standard for identification and preparation of monograph of this drug.

Key words: *Amaranthus spinosus,* extractive and ash values, microscopical characters.

INTRODUCTION

The World Health Organization states that approx 85-90% of the world's population consumes traditional herbal medicines, while the herbal drug industry has been in a high growth in the late 90s due to the growing demand in developing and developed countries.[1] The plant biodiversity in India has served as the foundation for the development of many traditional system of medicine, including Ayurveda, Unani, Siddha and Tibetan.[2] In recent years there has been a rapid increase in the standardization of selected medicinal plants of potential therapeutic significance.[3,4] Standardization is an essential requirement for the whole plant, plant parts or extracts in order to assess the quality of drugs.

A. spinosus (Amaranrheaceae) is a glabrous herb found in tropical and sub tropical region of India. The root of this plant is used as diuretic and febrifuge.[5] Previous report of this plant showed that its extract was used as an anti-malarial[6], anti-diarrheic[7], anti-diabetic, anti-hyperlipidemic and spermatogenic[8], and anti-inflammatory activity[9], stimulates proliferation of β-lymphocytes[10] and haematology.[11] Extract of this plant showed the presence of flavonoid.[12] The present study is focused to Pharmacognostical studies of *A. spinosus*. In folk/tribal medical practice many plants are used to treat many diseases in South India.

Address for correspondence:
Sangameswaran Balakrishnan,
Technocrates Institute of Technology-Pharmacy,
Anandnagar, Bhopal - 462021, M.P, India.
Email: sangameswaran03@gmail.com

DOI: 10.5530/pj.2011.19.3

MATERIALS AND METHODS

Plant material

The plant parts were collected from the foot hills of Yercaud, Salem, in the month of September 2005. The plant was identified and authenticated by the experts in the department of Botany Govt. Arts College, Salem, Tamil Nadu, and India. A voucher specimen (CHL-03) has been kept in our museum for future reference. The plant material was collected and shade dried at room temperature for 10 d and coarsely powdered with the help of a hand-grinding mill and the powder was passed through sieve No.60.

Preparation of the extract

The powdered material of *A. spinosus* was extracted separately by using soxhlet apparatus with different solvents.[13] After extraction, the extracts were concentrated under reduced pressure.

Instruments used

Photographs of different magnifications were taken with Nikon Labphot2 Microscopic Unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background.[14]

MATERIALS AND METHODS FOR ANATOMICAL STUDIES

Collection of specimens

The plant specimens were collected from foothill of Yercaud, Salem Tamil Nadu India, Care was taken to select healthy plants and for normal organs. The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin – 5 ml + acetic acid – 5ml + 70% Ethyl alcohol – 90ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary – butyl alcohol as per the schedule given by Sass, 1940 [15]. The specimens were castled into paraffin blocks.

Botanical description

Amaranthus spinosus is armed, erect herb. Spines axillary, clustered subtending the inflorescences. Leaves are elliptic-obavate, acute base, apex are acutely emarginated. Fascicle axiallary into terminal panicles, bracts and bracteoles narrowly ovate-lanceolate, midribs are green and short. Male flowers: Sepals are in five, unequal with prominent midrib, outer part two lobes which are curved to outwards and inner three lobes which are oblong. Stamens are in five and anthers are oblong. Female flowers: Five sepals which are equal, oblong, flat.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10-12 µm. De washing of the sections were done by customary procedure. The sections were stained with Toluidine blue as per the method published by O'Brien et al[16]. For studying the venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jefferey's maceration fluid was prepared. Glycerin mounted temporary preparations were made for macerated/ cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerin medium after staining. Different cell component were studied and measured.

Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Lab hot 2 Microscopic Unit. For the study of crystals, starch grains and lignified cells, polarized light was employed. Under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale – bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books.

Physicochemical Analysis

The dried powdered material of root was subjected to physicochemical analysis including fluorescence analysis[17], moisture content, total ash, water soluble ash, acid insoluble ash, alcohol soluble extractive and water soluble extractive[18] to determine the quality and purity of the plant material.

Preliminary phytochemical screening

The leaves were dried under shade, powdered with a mechanical grinder and pass through sieve no 45. The sieved powder was stored in airtight container and kept in room temperature until further study .The dried powdered material (250 g) was extracted with methanol by hot extraction method by using soxhlet apparatus and aqueous extraction by cold maceration. The solvents were completely removed under reduced pressure by using vacuum evaporator.

RESULTS

Determination of physicochemical parameters of selected plants

Fresh materials of *A. spinosus* (stem and leaf), were collected and subjected to various physicochemical parameters such as moisture content were observed and recorded.

Ash values are helpful in determining the quality and purity of crude drug, especially in the powder form. Total ash reflects the care taken in its preparation as all traces of organic matters were removed during ash formation and usually consists of carbonates, phosphates and silicates of sodium, potassium, calcium and magnesium. A higher limit of acid insoluble ash reflects the cases where silica may be present or when the calcium oxalate content of the drug is very high (Table 1)

Extractive values of selected medicinal plants like *A. spinosus* (leaf and stem) were observed and tabulated no 1.

The total percentage of ash values, acid insoluble ash, water soluble ash and percentage yield of extractives in different solvents are constant features of a part of the plant which may constitute individual drug. These reports would be of much significance in finding out the genuineness of the drug sample. Medicinal plants are valuable natural sources and regarded as potential and safe drugs. They have been playing an important role as natural drugs to alleviate human sufferings by contribution herbal medicines to the primary health care systems of rural and remote areas where more than 70% of population in India depend on folklore and traditional systems of medicines.

Table 1: Showing physicochemical parameters of selected plants

Parameters (w/w%)	*A. spinosus*	
	Stem	*Leaf*
Moisture content	17.8-14.2	16.26-17.8
Foreign matters	0.2-0.4	0.6-0.8
Total ash	18.4-20.42	14.0-18.62
Acid insoluble ash	12.4-14.64	11.5-13.22
Water soluble ash	11.2-13.64	9.6-10.32
Sulphated ash	16.2-18.84	12.2-14.65
Extractive values		
Pet. Ether	6.10-12.26	6.32-10.86
Chloroform	5.21-10.24	4.42-8.26
Ethyl acetate	4.43-8.56	5.53-9.65
Methanol	9.45-16.8	6.81-11.26
Aqueous	9.52-10.24	10.52-12.26

Preliminary phytochemical studies

The qualitative chemical investigation of all the extracts of selected plant was carried out to check the presence of various phytoconstituents. It revealed the presence of alkaloids, flavonoids, phenolic compounds, phytosterol, tannins, glycosides and carbohydrates.

Botanical description

A. spinosus is armed, erect herb, spines axillary, clustered subtending the inflorescences. Leaves are elliptic-obovate, acute base, apex are acutely emarginated. Fascicle axillary into terminal panicles, bracts and bracteoles narrowly ovate-lanceolate, midribs are green and short. Male flowers: Sepals are in five, unequal with prominent midrib, outer part two lobes which are curved to outwards and inner three lobes which are oblong. Stamens are in five and anthers are oblong. Female flowers: Five sepals which are equal, oblong, flat.

Anatomy of *A. spinosus* (Fig 1.a, b)

Leaf

The leaf has prominent abaxial midrib and thin lamina. The midrib is deeply hallowed on the adaxial side and prominently hemi spherical on the abaxial side. The midrib is 400 μm in vertical plane and 450 μm in horizontal plane. The abaxial part of the midrib is wavy in outline. It has thin epidermal layers of small cells. The ground tissues are homogeneous, paranchymatous, thin walled and compact. There is a wide prominent bowl shaped vascular bundles. The vascular bundles are collateral with wide circular thick walled vessels and parenchymatous xylem elements. Phloem fibers have a thick abaxial sheath. The vessel elements are 30-40 μm in diameters.

Calcium oxalate crystals are abundant in the midrib. Some of them are quite large measuring upto 50 μm in diameter and some of them are smaller measuring upto 20 μm in diameter. They occur in the ground parenchyma in the midrib and they are also localized within the vessel wall (Fig 1 a, b)

Lamina

The lamina is thin glabrous and has thin delegate epidermal layers. The mesophyll tissue has narrow zone of adaxial palisade cells and three or four layers of small lobed spongy parenchyma cells. The median part of the lamina, there are several circular small vascular bundles surrounded by bundle sheath cells called "Kranz-tissues" (Fig 1 c)

Venation Pattern

The lamina shows very characteristic venation pattern. The veins are thin and reticulate with distinct or indistinct vein-islet. The vein-terminations are distinct, shortly and thick.

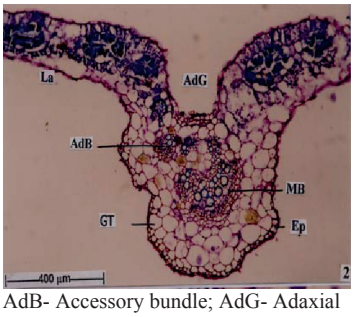
The veins are surrounded by dilated bundle sheath cells (Kranz-tissue) which contain dark prominent chloroplast.

Stem

The young stem measuring about 4 mm diameter. The stem is circular in outer line and smooth, the places where leuticle are present. The epidermis is thin and has started dividing transversely giving rise to initial periderm (Fig 1 h). The periderm is fairly 300 µm wide, it is homogeneous and parenchymatous. The sclernchyma zone has thick walled and lignified in outer part and thin walled in inner part. Cellulose is present in the inner zone.

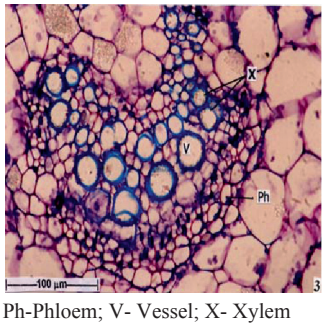
The leuticle has several layers of filling tissue. The sieve tubes are wide, angular thick walled having the companion cells along the corners. Phloem rays are thin and narrow. Secondary xylem is a dense hollow cylinder comprising of their rays and vessels. The vessels are sparse, wide, thick walled and mostly solitary. The widest vessel is 80µm in diameter and

1. a: T.S of Leaf



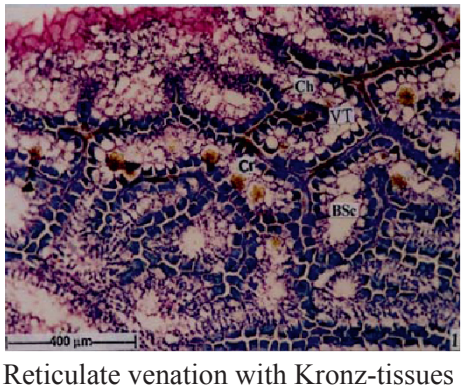
AdB- Accessory bundle; AdG- Adaxial groocle; Ep- Epidermis.

1. b: Mid rib



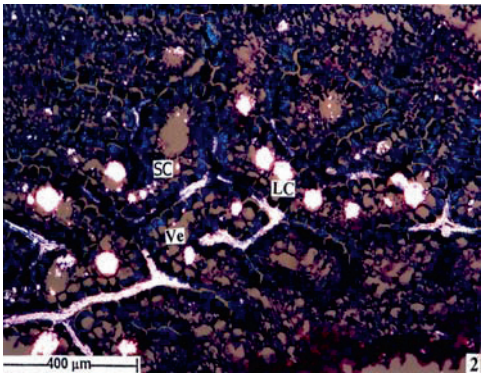
Ph-Phloem; V- Vessel; X- Xylem
GT- Ground tissue; La- Lamina; MB- Median bundle.

1. c: Lamina



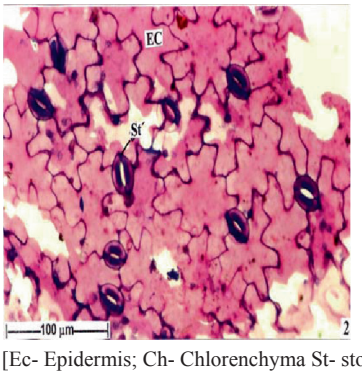
Reticulate venation with Kronz-tissues
Lc- Larger crystal; Sc- Smaller crystal; Ve- vein

1. d: Powder microscopy



BSc- Bundle sheath cells;
VT- Vein-termination

1. f: Powder microscopy (Stomata)



[Ec- Epidermis; Ch- Chlorenchyma St- stomata;]

1. g: Calcium oxalate crystals

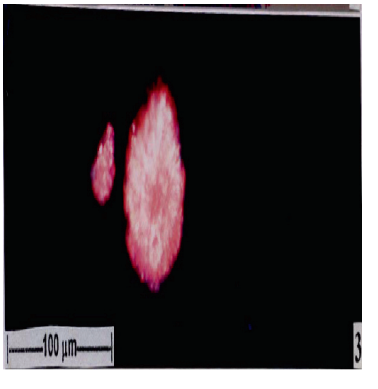
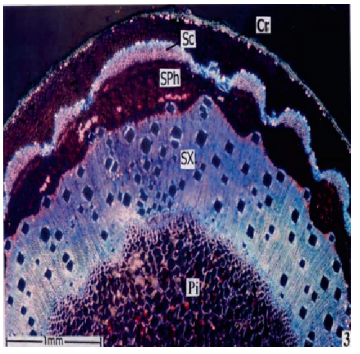


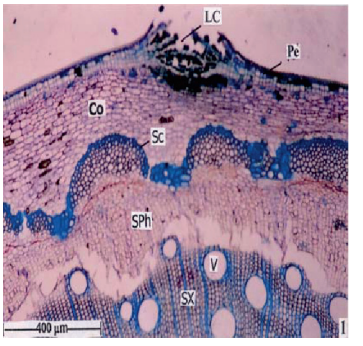
Figure 1: Microscopical Characters of *Amaranthus spinosus*

1. j : T.S of old stem entire view



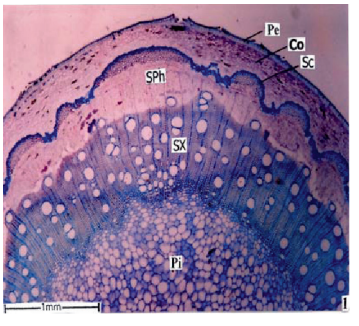
Co- Cortex, Ep- Epidermis, Pe- periderm, Sclerenchymatous, Sph- Secondary phloem, Lenticells, Cr-Crystals.

1. k : T.S of old stem a sector enlargement



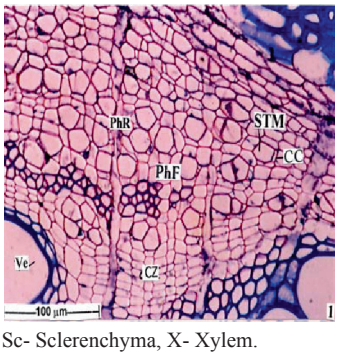
Pi- Pith, Px- Primary xylem, Sc- Sx- Secondary xylem, V- Vessel, Lc-

1. h: T. S of Young Stem- ground and round plane



Co- cortex, Ep- Epidermis, Fi- Fibers, Ph- phloem, Pi- Pith,

1. i: T.S of young stem- Sector enlargement



Sc- Sclerenchyma, X- Xylem.

Figure 1: *continued*

Table 2: Phytochemical screening of extracts of *Amaranthus spinosus*

Name of the constituents	Pet. ether	Chloroform	Ethyl acetate	methanol	aqueous
Alkaloid	-	-	+	+	+
Glycoside	-	-	+	+	+
Steroid	-	-	++	++	++
Flavonoid	-	-	++	++	++
Tanin & Phenolic compound	-	-	+	+	+
Terphenoid	-	-	+	+	+
Carbohydrates	-	-	+	+	++
Fixed oils	-	-	-	-	-
Proteins	-	-	-	-	-
gums & mucilage	-	-	+	-	+
Saponin	-	-	+	-	+

narrow vessels are less than 50µm (Fig 1 a, b). The xylem fibers are thick walled, lignified and random in arrangement. The pith is wide, homogenous and parenchymatous.

When the section is viewed under the polarized microscope, phloem parenchyma tissues are seen. The lignified tissues appear bright against dark background.

Powder microscopy of *A. spinosus* (Fig 1 d)

The powder of the leaf shows small fragments of lamina. These fragments show rerticulate venation with Kronz-tissue.

Some of the fragments show epidermal morphology. The epidermal cells are large and amoeboid in shape. There walls are thin and highly lobed. The stomata are large and elliptical. They are mostly anomocytic type, because no distinct subsidiary cells are seen around the stomata. Crystal distribution is also seen in the powders. Calcium oxalate crystals are abundant in the mesophyll tissue as well as along the vein. The crystals are either very large or very small and these two type of crystals are seen inter mixed.

DISCUSSIONS

Bioprospecting of medicinal plants entails the search for pharmacologically and economically valuable biochemical resources. India has great potential to utilize these resources and knowledge base in traditional medicines as population as well as for economical gain. There is an urgent need for the documentation of folk knowledge, systematic, phytochemical and pharmacognostical studies of medicinal plants and their natural products.

REFERENCES

1. Dinesh Kumar C: Pharmacognosy can help minimize accidental misuse of herbal medicine. Current Sci, 2007; 93:1356-1358.
2. Jadhav RB, Patil CR, Ganbote AJ. Plant biodiversity and its conservation. Indian J Pharm Edu, 2003; 37:162-165.
3. Reddy YSR, Venkatesh S, Ravichandran T,Suburaju T, Surseh B.: Pharmacognostical studies of Wrightia tinctoria bark. Pharm Biol. 1999; 37:291-295.
4. Venkatesh S, Madhava RB, Surseh B, Swamy MM, Ramesh M. Pharmacognostical identification of Rumex nepallensis Spreng (Polygonaceae) – an adulterant for Indian rhubarb. Nat Prod Sci, 2004:10; 43-47.
5. Kirtikar KR. Basu BD. Indian Medicinal Plants Vol III International Book publisher Dehradun 2057-2058; 1993.
6. Hilou A, Nacoulma OG, Guiguemde TR. In vivo anti-malarial activity of extracts from *Amaranthus spinosus* L and *Boerhaavia erecta* L in mice. J Ethnopharmacol.2006; 103:236-40.
7. Sawangjaroen N, Sawangjaroen K. The effects of extract from anti-diarrheic, Thai medicinal plants on the in vitro growth of the intestinal protozoa parasite Blastocystis hominis, J Ethnopharmacol. 2005; 98:67-72.
8. Sangameswaran B, and Jayakar B. Anti-diabetic, anti-hyperlipedemic and spermatogenic effects of *Amaranthus spinosus* L. on streptozotocin induced diabetic rats. J Nat Med 2008; 62:79-82
9. Sangameswaran B, Balakrishnana BR, Balakrishnan N, and Jayakar B. Anti-inflammatory activity of stem of *Amaranthus spinosus*. Int. J Pharmacol Biol Sci, 2008; 2(2):59-62.
10. Bi-Fong Lin, Bor-Luen Chiang, Jin-Yuarn Lin, *Amaranthus spinosus* water extract directly stimulates proliferation of B-lymphocytes in Vitro. Int. J Immunopharmacol. 2005; 4:711-722.
11 Olufemi BE, Assiak IE, Ayoade GO, Onigemo MA. Studies on the effects of *Amaranthus spinosus* Leaf extract on the haematology of growing pigs. African J Biomed Res. 2003; 6:149-150.
12. Azhar-ul-Haq., Malik, A., Khan, A.U., Shah, M.R., Muhammad, P. Spinoside new coumaroyl flavone glycoside from *Amaranthus spinosus*. Arch Pharm Res. 2004; 27:1216-19.
13. Kokate CK. Practical pharmacognosy 3rd ed Vallabh Prakashan New Delhi. 107-109; 1994.
14. Esau K. Anatomy of Seed Plants, second edition. New York, John Wiley, pp. 550; 1997.
15. Sass JE. Elements of Botanical Micro technique. McGraw Hill Book Co, New York , 222; 1940.
16. O'Brien TP, Feder N and Mc cull ME. Polychromatic Staining of Plant cell walls by Toluidine blue – O. Protoplasma. 1964; 59:364-373.
17. Kokashi CJ, Kokashi RJ, Sharma M. Fluorescence of powdered vegetable drugs in ultra-violet radiation. J Am Pharm Assoc, 1958; 47:715-717.
18. Khandelwal KR. Practical Pharmacognosy. 11th Ed. Pune, India, Nirali Prakashan, 2004; 149-153 and 157-159.

Pharmacognostical studies on *Ficus racemosa* stem bark

Faiyaz Ahmed, Asna Urooj

Department of Studies in Food Science and Nutrition, University of Mysore, Mysore - 570 006, India

ABSTRACT

Ficus racemosa (Moraceae) is a popular medicinal plant in India, which has long been used in Ayurveda, the ancient system of Indian medicine for various diseases/disorders including diabetes, liver disorders, diarrhoea, inflammatory conditions, hemorrhoids, respiratory and urinary diseases. The present paper highlights the macroscopic and microscopic characters of the *Ficus racemosa* bark along with physicochemical evaluation and phytochemical characteristics. These observations would be of immense value in the botanical identification and standardization of the drug in crude form. This study would help distinguish the drug from its other species.

Keywords: *Ficus racemosa*, Pharmacognostic, Extractive, Histology, Histochemical reactions

INTRODUCTION

Ficus racemosa Linn. (Moraceae) is an evergreen, moderate to large sized spreading, lactiferous, deciduous tree 15-18 m high, without much prominent aerial roots.[1] It belongs to the genus *Ficus* which is an exceptionally large pantropical genus with over 700 species[2] and belongs to the family Moraceae which is distributed widely throughout the warmer parts of Asia, Africa, America and Australia. It is retained as a single, large genus because it is well defined by its unique reproductive system, involving synconia fig and specialized pollinator wasps.[3] *F. racemosa* is commonly known as 'Gular', and all parts of this plant are regarded medicinally important in Ayurveda and has been used extensively in the treatment of biliary disorders, jaundice, dysentery, diabetes, diarrhoea and inflammatory conditions.[4-6]

Ficus racemosa bark is a rich source of polyphenolic compounds and has shown to possess excellent antioxidant properties *in vitro*, *ex vivo*[7] and *in vivo* in streptozotocin-induced diabetic rats.[8] We have also reported *F. racemosa* stem bark to exhibit various biological effects such as *in vitro* antidiabetic,[9] *in vivo* antihyperglycemic,[10] hepatoprotective effect against CCl_4 induced hepatotoxicity[11] and carbohydrate hydrolyzing enzyme inhibitory activity.[12] Various reports also indicate *F. racemosa* bark to exhibit chemopreventive,[13] antidiabetic,[14] anti inflammatory,[15] antipyretic,[16] antitussive[17] and antidiuretic effects.[18] In spite of its abundant uses, the pharmacopoeial standards of *F. racemosa* bark have not been reported. Hence, the present pharmacognostical study was undertaken to supplement useful data in regard to its correct identity, as this plant is broadly used in indigenous system of medicine.

Address for correspondence:
Dr. Faiyaz Ahmed,
Department of Studies in Food Science and Nutrition,
University of Mysore, Manasagangotri, Mysore - 570 006, India.
E-mail: fayaz_ahmed09@yahoo.co.in

DOI: 10.5530/pj.2011.19.4

MATERIALS AND METHODS

Plant material and chemicals

Ficus racemosa stem bark was collected from Mukkadahally, Chamarajanagar district of Karnataka, India during September 2007, subsequently identified by Dr. Shivprasad Huded and the voucher specimen (BOT-001/2008) was deposited at the herbarium of Department of Studies in Botany, University of Mysore, Mysore, India. The bark was cut into small pieces, dried (50°C) and powdered, passed through 60 mesh sieve (BS) and stored in an air tight container at 4°C till further use. All the chemicals and reagents used in the study were of extra pure analytical grade.

Organoleptic properties

The freshly peeled and the dried stem barks were spread on a clean dry plastic sheet and investigated different organoleptic features such as condition, thickness, color, odour, taste and fracture by repeated observations using a magnifying glass (where required) and recorded.[19]

Histology and histochemical color reactions

Small fragments of the fresh bark were embedded in paraffin and transverse sections (4-5 µm) were obtained. Good sections were collected and placed on a grease free microscopic slide along with a drop of glycerin and water (1:1). The sections were covered with clean cover slip and observed under the compound microscope at 10x magnification and the sections were suitably traced out.[19] Histochemical color reactions were also carried out on the transverse sections, wherein the sections were treated with various chemical reagents and observed under microscope to detect the presence of various phytoconstituents.[20]

Color reactions

Behavior of bark powder with different chemical reagents was studied to detect the presence of different phytoconstituents with color changes under daylight. [21]

Fluorescence characteristics

A small quantity of the bark powder was placed on a grease free clean microscopic slide and added 1-2 drops of the freshly prepared reagent solution, mixed by gentle tilting the slide and waited for 1-2 minutes. Then the slide was placed inside the UV viewer chamber and viewed in day light, short (254 nm) and long (365 nm) ultraviolet radiations. The colors observed by application of different reagents in different radiations were recorded.[19]

Extractive values

The bark powder (100 g) was extracted sequentially with petroleum ether, chloroform, acetone, methanol and water in a soxhlet extractor by continuous hot percolation to yield sequential petroleum ether extract (FRSPE), sequential chloroform extract (FRSCE), sequential acetone extract (FRSACE), sequential methanol extract (FRSME) and sequential aqueous extract (FRSAE). Each time before extracting with the next solvent of higher polarity, the powdered drug was dried in a hot air oven below 50°C for 10 min. solvents were evaporated in a rotary vacuum evaporator and the dried extracts were weighed. Their percentages were calculated in terms of initial air dried plant material. The colors of extracts were also observed.[19]

RESULTS AND DISCUSSION

The present study reports the pharmacognostical characteristics of *Ficus racemosa* bark. To ensure reproducible quality of herbal products, proper control of starting material is utmost essential. Thus in recent years there has been an emphasis in standardization of medicinal plants of therapeutic potential. Despite the modern techniques, identification and evaluation of plant drugs by pharmacognostical studies is still more reliable, accurate and inexpensive means. According to World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken.[22]

The various organoleptic characteristics recorded are presented in Table 1. Organoleptic evaluation is a technique of qualitative evaluation based on the study of morphological and sensory profiles of whole drugs.[23] The organoleptic or macroscopic studies yielded important characteristics, such as the fractured surfaces of fresh and dried bark, typical tongue sensitizing aromatic taste and aromatic and characteristic odour of the bark; which are useful diagnostic

Table 1: Organoleptic characteristics of fresh and dried bark

	Fresh bark	Dry bark
Condition	Moist, soft, sticky, thick, fissured and warty or corky, becoming rough. When wounded, the bark exudes a gum which is initially white in color but changes to reddish brown or brownish black on exposure	Hard and contracted
Shape of the pieces	Semicircle but not contracted	Recurved and channeled quills
Thickness	Varies, 0.8-1.4 cm	Varies, 0.6-1.0 cm
Color	Outer surface pale greenish with white flakes Inner surface light brown	Outer surface: blackish brown Inner surface: brownish buff colored
Odour	Odourless	Slight woody odour
Taste	Bland and woody	Same as fresh bark
Fracture	Fibrous; outer surface of the bark had scattered lenticels and small and large scars left by the prickles and branches. Outer surface was marked by wavy longitudinal striations; inner surface also had longitudinal striations. The cork was found frequently exfoliated	Splintery

characters. Similarly the microscopic or histological features, e.g. presence of tannins, fiber cells and absence of sclereids, etc may be useful for this purpose.

Microscopic analyses and qualitative parameters were carried out on plant samples in order to establish appropriate data that can be used in identifying crude drugs particularly those supplied in powder form. They are standard pharmacognostic parameters that can be used to differentiate closely related plant species or varieties with similar constituents or pharmacological activities.[24]

The transverse section (T.S.) of young stem comprising of bark was taken as the mature bark was brittle and contained large amounts of secondary dead cells. The T.S. showed a periderm consisting of the following three layers (Figure 1A and 1B).

1. Outer phellum (cork): consisted two layers of thickly suberised cells.
2. Middle phellogen (cork cambium): forms a continuous layer of tangentially elongated and thin walled cells.
3. Inner phelloderm (secondary cortex): consisted a few layers of parenchymatous cells, some of the cells contained numerous chloroplasts, while a few others showed thick walled fibers.

The cortex region showed tannin filled cells and calcium oxalate crystals [rhomboidal (Figure 1C) and prismatic (Figure 1D)]. Next to cortex, secondary phloem was present followed by secondary xylem. In between few layers of conjunctive tissues were seen. Next to secondary xylem, primary xylem followed by central pith were seen.

Histochemical color reactions were carried out on the transverse sections of mature bark and the results are shown in Table 2. The results revealed the presence of lignin as indicated by the development of yellow and pink colors with aniline SO_4-H_2SO_4 and phloroglucinol-HCl reagents, respectively. The lignin was found mainly in the periderm. The presence of starch granules was indicated by the development of blue color with iodine solution, while the absence of cellulose was evidenced by the reaction with concentrated sulphuric acid. Histochemical reactions of the bark sections showed the presence of cardiac glycosides with development of blue with Kedde reagent and absence of anthraquinone glycosides as no change in color was observed with 5% aqueous KOH.

With antimony chloride the sections developed reddish pink color indicating the presence of steroids and triterpenoids. Similarly, red color was developed with Millon's reagent indicating the presence of proteins containing

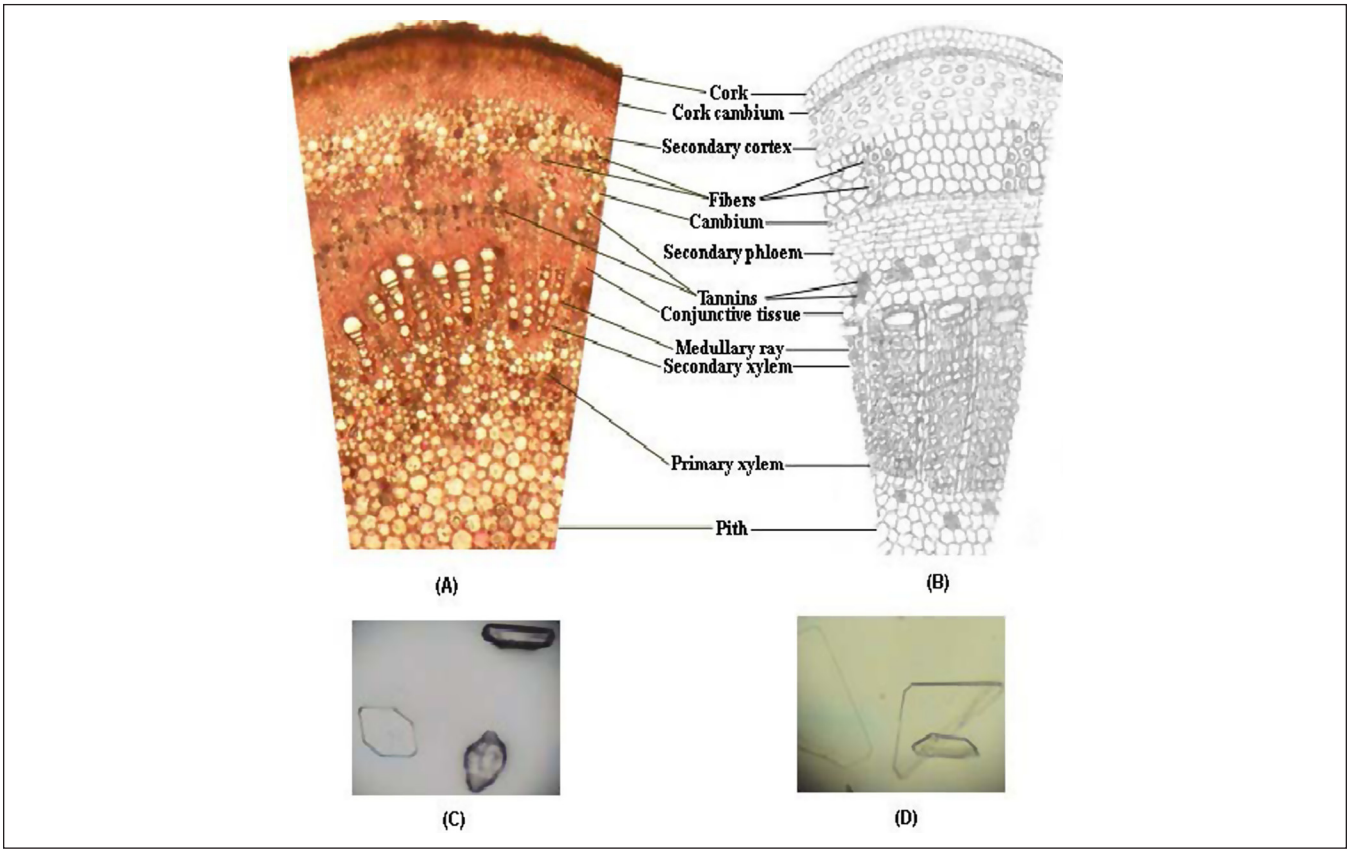


Figure 1: (A) T.S. of the young stem containing the bark. (B) Schematic diagram of the T.S. (C) Rhomboidal crystals. (D) Prismatic crystals.

Table 2: Histochemical color reactions of the *Ficus racemosa* bark

Reagent	Constituent	Color	Degree of intensity
Aniline SO_4 + H_2SO_4	Lignin	Yellow	++
Phloroglucinol + HCl	Lignin	Pink	++
Conc. H_2SO_4	Cellulose	Brown	-
Weak Iodine solution	Starch	Blue	++
Millon's reagent	Proteins	Red	+
Dragendorff's reagent	Alkaloids	No change	-
Caustic alkali + HCl	Calcium oxalate	Precipitate	++
Kedde reagent	Cardiac glycosides	Blue	+
$SbCl_3$	Steroids/triterpenoids	Reddish pink	+++
5% Aq. KOH	Anthraquinone glycosides	No change	-

**+++: high, ++: moderate, +: slight, -: negative*

Table 3: Behavior of the *Ficus racemosa* bark powder with different chemical reagents

Reagent	Color/precipitate	Inference
Picric acid	No precipitation	Alkaloids absent
Conc. H_2SO_4	Reddish brown	Steroids/triterpenoids present
Aq. $FeCl_3$	Greenish black	Tannins, flavonoids present
Iodine solution	Blue	Starch present
Ammonia solution	No change	Anthraquinone glycosides absent
5% Aq. KOH	No change	Anthraquinone glycosides absent
Mayer's reagent	No precipitation	Alkaloids absent
Spot test	Stain observed	Fixed oils present
Aq. $AgNO_3$	Precipitation observed	Proteins present
Aq. NaOH	Reddish brown	Flavonoids present
Mg-HCl	Magenta	Flavonoids present
Dragendorff's reagent	No precipitation	Alkaloids absent
Aq. Lead acetate	White precipitate	Tannins present
Liebermann Burchard test	Reddish green	Steroids/triterpenoids present

**NaOH: sodium hydroxide, KOH: potassium hydroxide, Mg-HCl: magnesium metal-hydrochloric acid, $FeCl_3$: ferric chloride, H_2SO_4: sulphuric acid, $AgNO_3$: silver nitrate*

tyrosine resides. The presence of calcium oxalate crystals observed in the transverse sections was confirmed by their precipitation in the presence of caustic alkali and hydrochloric acid. The observations also revealed that the bark does not contain alkaloids as changes were observed by the addition of Dragendorff's reagent.

Behavior of bark powder with different chemical reagents was studied to detect the presence of major phytoconstituents with color changes under daylight and the results are shown in Table 3. The bark powder did not produce any precipitation with picric acid, Mayer's reagent and Dragendorff's reagent implied the absence of alkaloids, while absence of Anthraquinone glycosides was confirmed as no change in color was observed with either of ammonia or potassium hydroxide solutions. The presence of steroids/triterpenoids and triterpenoids was confirmed as the bark powder reddish brown and reddish green color with H_2SO_4 and Liebermann Burchard reagents respectively. Further, spot test indicated the presence of fixed oils, while lead acetate and ferric chloride tests confirmed the presence of tannins. Presence of flavonoids was confirmed by the reaction of the bark powder with ferric chloride, sodium hydroxide and Mg-HCl. The powder produced a dark blue color with iodine solution indicating the presence of starch.

The fluorescence characteristics of the bark powder with different chemical reagents are summarized in Table 4. Although, a change in color was observed by the addition of various reagents under day light, none of the reagents

Table 4: Fluorescence characteristics of *Ficus racemosa* bark powder

	Visible/Day light	UV 254 nm (short)	UV 365 nm (long)
Powder as such	Light brown	Black	Blackish brown
Powder + 1M NaOH	Dark brown	Black	Black
Powder +1% Picric acid	Yellowish brown	Black	Black
Powder + Acetic acid	Brown	Blue	Black
Powder + 1M HCl	Brown	Blue	Black
Powder + Dil HNO_3	Brown	Black	Dark brown
Powder + 5% Iodine	Yellowish brown	Black	Black
Powder + 5% $FeCl_3$	Greenish brown	Black	Black
Powder + HNO_3 + 25 % NH_3	Light brown	Black	Dark brown
Powder + Methanol	Brown	Dark brown	Brown
Powder + 50% HNO_3	Yellowish brown	Black	Black
Powder + 1M H_2SO_4	Reddish brown	Dark brown	Dark brown
Powder + Dil. NH_3	Dark brown	Dark black	Dark brown
Powder + Conc. HNO_3	Yellowish brown	Black	Black
Powder + 10% Potassium dichromate solution	Yellowish green	Black	Black
Powder + 25% Liquid NH_3	Dark brown	Dark black	Dark brown

**NaOH: sodium hydroxide, HCl: hydrochloric acid, HNO_3: nitric acid, $FeCl_3$: ferric chloride, H_2SO_4: sulphuric acid, NH_3: ammonia*

Table 5: Extractive values of *Ficus racemosa* bark

Extract	Extractive value (% w/w)
Sequential petroleum ether extract (FRSPE)	2.5 ± 0.31
Sequential chloroform extract (FRSCE)	0.35 ± 0.07
Sequential acetone extract (FRSACE)	0.83 ± 0.05
Sequential methanol extract (FRSME)	5.8 ± 0.21
Sequential water extract (FRSAE)	4.0 ± 0.17

**Values are mean ± SD of triplicate determinations.*

induced any fluorescence to the bark powder under both short and long UV radiations. Under UV light, dark brown and black colors were prominent.

Fluorescence is the phenomenon exhibited by various chemical constituents present in the plant material. Some constituents show fluorescence in the visible range in daylight. The ultra violet light produces fluorescence in many natural products (e.g. alkaloids like berberine), which do not visibly fluoresce in daylight. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives or decomposition products by applying different reagents. Hence, some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostical evaluation.[25-28]

The extractive values of *F. racemosa* by various solvents are summarized in Table 5. Among sequential extractions, extractive value of methanol was highest followed by water and petroleum ether. The extractive value of chloroform was the least.

CONCLUSION

In conclusion, the present study on pharmacognostical characters of *Ficus racemosa* Linn. bark provides useful information in regard to its correct identity and help to differentiate from the closely related other species of *Ficus*.

ACKNOWLEDGEMENTS

The authors acknowledge University Grants Commission, New Delhi, India, for the financial assistance (F.31-278/2005).

REFERENCES

1. Varier FS. Indian Medicinal Plants. Vol. 3, Hydrabad: Orient longman, p. 34-37;1995.
2. Berg CC. Classification and distribution of *Ficus*. Experimentia. 1989;45:605-11.
3. Novotny V, Basset Y, Miller SE, Drozd P, Cizek L. Host specialization of leaf chewing insects in New Guinea rainforest. J Animal Ecology. 2002;71:400-12.
4. Kirtikar KR, Basu BD. Indian Medicinal Plants. 2nd edn, Dehra Dun, p. 2327-28;1975.
5. Nadkarni KM. *Indian materia medica*. Vol 1, Bombay: Popular Prakashan, p. 1031;1976.
6. Chopra RN, Chopra IC, Handa KL, Kapur LD. Indigenous Drugs of India. 2nd edn, Calcutta: Academic Publisher, p. 508-674;1958.
7. Ahmed F, Urooj A. Antioxidant activity of various extracts of *Ficus racemosa* stem bark. Nat J Life Sci. 2009;6:69-74.
8. Ahmed F, Urooj A. Glucose lowering, hepatoprotective and hypolipidemic activity of stem bark of *Ficus racemosa* in streptozotocin-induced diabetic rats. J Young Pharm. 2009;1:160-4.

9. Ahmed F, Urooj A. *In vitro* studies on the hypoglycemic potential of *Ficus racemosa* stem bark. J Sci Food Agric. 2010;90:397-401.
10. Ahmed F, Urooj A. Antihyperglycemic activity of *Ficus glomerata* stem bark in streptozotocin-induced diabetic rats. Global J Pharmacol. 2008;2:41-45.
11. Ahmed F, Urooj A. Hepatoprotective effects of *Ficus racemosa* stem bark against carbon tetrachloride-induced hepatic damage in albino rats. Pharm Biol. 2010;48:210-16.
12. Ahmed F, Urooj A. Effect of *Ficus racemosa* stem bark on the activities of carbohydrate hydrolyzing enzymes-An *in vitro* study. Pharm Biol. 2010;48:518-23.
13. Khan N, Sultana S. Chemomodulatory effect of *Ficus racemosa* extract against chemically induced renal carcinogenesis and oxidative damage response in Wistar rats. Life Sci. 2005;29:1194-1210.
14. Rao BR, Murugesan T, Sinha S, Saha BP, Pal M, Mandal SC. Glucose lowering efficacy of *Ficus racemosa* bark extract in normal and alloxan diabetic rats. Phytother Res. 2002;16:590-2.
15. Li RW, Leach DN, Myers SP, Lin GD, Leach GJ, Waterman PG. A new anti-inflammatory glucoside from *Ficus racemosa* L. Planta Med. 2004;70:421-6.
16. Rao BR, Anipama K, Swaroop A, Murugesan T, Pal M, Mandal SC. Evaluation of anti-pyretic potential of *Ficus racemosa* bark. Phytomed. 2002;9:731-3.
17. Rao BR, Murugesan T, Pal M, Saha BP, Mandal SC. Antitussive potential of methanol extract of stem bark of *Ficus racemosa* Linn. Phytother Res. 2003;17:1117-8.
18. Ratnasooriya WD, Jayakody JRAC, Nadarajah T . Antidiuretic activity of aqueous bark extract of Sri Lankan *Ficus racemosa* in rats. Acta Biol Hung. 2003;54:357-64.
19. Bhattacharya S, Zaman MK. Pharmacognostical evaluation of *Zanthoxylum nitidum* bark. Intl J Pharm Tech Res. 2009;1:292-8.
20. Trease GE, Evans WC. Pharmacognosy. 12th ed, East Bourne: Bailliere Tindal, p. 136-204;1986.
21. Chase CR, Pratt RJ. Fluorescence of powdered vegetable drugs with particular reference to development of a system of dentification. J Am Pharmacol Assoc. 1949;38:32-34.
22. Anonymous. Macroscopic and microscopic Examination: Quality Control Methods for Medicinal Plant Materials, WHO, Geneva; 1998.
23. Kokate CK. Practical Pharmacognosy. 4th ed., New Delhi: Vallabh Prakashan, p. 112-20;1994.
24. Ayinde BA, Omogbai EKI, Amaechina FC. Pharmacognosy and hypotensive evaluation of *Ficus exasperata* Vahl *(moraceae)* leaf. Acta Poloniae Pharmaceutica ñ Drug Res. 2007;64:543-6.
25. Kokashi C J, Kokashi RJ, Sharma M. Fluorescence of powdered vegetable drugs in ultra-violet radiation. J Am Pharm Assoc. 1958;47:715-7.
26. Wallis TE. Textbook of Pharmacognosy. 5th edn, New Delhi: CBS Publications, p. 111-7;1985.
27. Mukherjee PK. Quality Control of Herbal Drugs: An Approach to Evaluation of Botanicals. Business Horizones;2002.
28. Ansari SH. Essentials of Pharmacognosy. 1st edn. New Delhi: Birla Publications Pvt. Ltd;2006

Isolation of Antibacterial Flavonoids from the aerial parts of *Indigofera secundiflora*

A. A. Ahmadu[1*], A. Onanuga[2] and B.U.Ebeshi[1]

[1]*Department of Pharmaceutical and Medicinal Chemistry, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria*

[2]*Department of Pharmaceutical Microbiology and Biotechnology, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria*

ABSTRACT

Background: The genus *Indigofera* belongs to the family leguminasae consisting of about 700 species. which are distributed throughout the tropical regions. Previous phytochemical studies on other species of Indigofera led to the isolation of Flavonoids,Dihydrostilbenes,Triterpenoids. **Method:** The aerial parts of *Indigofera secundiflora* was collected in July 2008 in Samaru, Zaria, Nigeria and authenticated at the Herbarium section, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. The air-dried plant (250 g) was extracted with acetone (2.5 l) at room temperature to exhaustion and then 70%methanol at room temperature.The combined acetone extract (5.2 g), a portion was separated for antibacterial activity, while the rest portion (3 g) was chromatographed on silica gel column using a polarity gradient of N-hexane and N-hexane :ethyl acetate mixtures and then ethyl acetate:methanol mixtures and finally pure methanol,Gel filteration over sephadex LH-20 eluting with methanol and preparative TLC afforded compounds Quercetin(I),Quercetin 3-O-methylether (II) and Quecretin 3,3^1,4^1-trimethylether(III). Agar well diffusion assay method was employed in the antibacterial studies with the acetone extract at concentration of 5 and 10 mg/ml respectively, while for the isolated flavonoids, concentrations of 100,200 and 300 µg/ml were used for studies. The standard antibiotics were Gentamicin 10 µg/ml and Ciprofloxacin 5 µg/ml respectively. **Results:** Phytochemical studies of the acetone extract by a combination of Flash column chromatography, gel filteration over sephadex and preparative thin-layer chromatography afforded Quercetin,Quercetin 3-O-methylether and Quercetin 3,3^1,4^1-trimethylether, the flavonoids were identified by comaparison of their ^{1}H and ^{13}C-NMR spectra with literature data. Antibacterial studies of the extract and isolated flavonoids revealed that the extract and the isolated flavonoids showed activity, however Quercetin [I] showed the least activity, Quercetin-3-O-methylether (II) showed significant activity against Staphylococcus aureus, while Quercetin 3,3′,4′-trimethylether (III) showed activity against all the tested pathogens. **Conclusion:** Chemical investigation of the acetone extract of the aerial parts of I.secundiflora led to the isolation of three flavonoids for the first time from this plant,anti-bacterial studies of the extract and the isolated compounds showed activity against the pathogens used with Quercetin being the least active.

Key words: *Indigofera secundiflora*, Flavonoids, antibacterial activity

INTRODUCTION

The genus *Indigofera* belongs to the family leguminasae consisting of about 700 species [1] which are distributed throughout the tropical regions including: Nigeria, Burkina faso, India and Pakistan[2]. Various species of *indigofera* have been used in ethnomedicinal practice of Northern Nigeria in particular, *Indigofera secundiflora* find use in ethnomedicinal practice of Northern Nigeria to treat diarrhoea, malaria and microbial infections [3]. Flavonoids, triterpenoids, steroids and stilbenoids have been isolated in other species of *indigofera* [4,5,6]; and to our knowledge there have been no documented report on the chemical and biological investigation of *indigofera secundiflora*. As part of our continuing research into the chemical constituents of *indigofera* species of Nigeria, we report here the isolation and anti-bacterial activity of Flavonoids from the aerial parts of *indigofera secundiflora*.

Address for correspondence:
A. A. Ahmadu,
Department of Pharmaceutical and Medicinal Chemistry,
Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.
E-mail: ahmadu2001@yahoo.com(A.A.Ahmadu)

DOI: 10.5530/pj.2011.19.5

MATERIALS AND METHODS

Flash Column chromatography was carried on silica gel G(40-63µm)Merck, Thin layer chromatography(TLC) and Preparative thin layer chromatography were carried on

Pre-coated silica gel on aluminium sheets and glass plate of thickness 0.2 mm and 0.5 mm (Merck) respectively, while gel filteration was carried out over sephadex LH-20(Sigma), IR spectroscopy was recorded on a Pye-Unicam FT-IR,NMR spectroscopy was carried out on a Bruker DRX 500MHz for ^{1}H and 125MHz for ^{13}C with TMS as internal standard using CD_3OD.

Plant material

The aerial parts of *Indigofera secundiflora* was collected in July 2008 in Samaru, Zaria, Nigeria and authenticated at the Herbarium section, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, where a voucher specimen (1096) was kept.

Extraction and isolation

The air-dried plant (250 g) was extracted with acetone (2.5 L) at room temperature to exhaustion and then 70%methanol(2.5L) at room temperature. The combined acetone extract (5.2 g,0.21%w/w), a portion was separated for antibacterial activity, while the rest portion (3 g) was chromatographed on silica gel column using a polarity gradient of N-hexane and N-hexane :ethyl acetate mixtures and then ethyl acetate:methanol mixtures and finally pure methanol,Gel filteration of the N-Hexane :Ethyl acetate(1:1) pooled fractions over sephadex LH-20 eluting with methanol and preparative TLC using N-Hexane:Ethylacetate(2:1) afforded compounds I, II and III.

Anti-bacterial activity

Agar well diffusion assay method describe by[7] was employed in the antibacterial studies with the acetone extract at concentration of 5 and 10 mg/ml respectively, while the isolated flavonoids concentrations of 100,200 and 300 µg/ml were used for studies. The standard antibiotics were Gentamicin 10 µg/ml and Ciprofloxacin 5 µg/ml respectively. Nutrient agar media are prepared and sterilized in an autoclave and 10ml transferred to previously sterilized plates. After solidification, the plates were inoculated with *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* aseptically. Gentamicin (10 µg/ml), Ciprofloxacin (5 µg/ml) were used as standard. Using a sterile cork borer, four wells were made and 0.1 ml each of the extract and isolated flavonoids were poured aseptically into the wells while the standard antibiotic discs were placed on the agar in the same plate. They were incubated at $37^{0}C$ for 24hrs. The zones of inhibition were measured to the nearest mm.

RESULTS AND DISCUSSION

Compound I a yellow powder(12 mg) UV_{MEOH} 228,246,354nm, IR(Nujol v_{max} cm^{-1}): 3440,1655,1604,^{1}H-NMR(CD_3OD,δ): 6.12(1H,d,J=2Hz,H-6),6.34(1H,d,J=2Hz,H-8),6.85(1H,d,J=8Hz,H-5^{1}),7.61(1H,d,d,J=2,8Hz,H-6^{1}),7.6991H,d,J=2Hz,H-2^{1}) was identified as Quercetin by direct comparison of the spectral data with literature[8-9]

Compound II a pale yellow amorphous powder(10 mg) UV_{MEOH} 256,358nm,IR(Nujol v_{max} cm^{-1}): 3446,1653,1605,805, ^{1}H-NMR(CD_3OD,δ): 6.22(1H,d,J=2Hz,H-6),6.36(1H,d,J=2Hz,H-8) ,6.8(1H,d,J=8Hz,H-5^{1}), 7.54(1H,d,d,J=2,8Hz,H-6^{1}),7.61(1H,s,H-2^{1}),3.78(3H,s,OCH_3)

13C-NMR(Table1),the compound was identified as Quercetin 3-methyl ether by comparison of the spectral data with literature [10-11].

Compound III a yellow solid (5 mg),IR(Nujol v_{max},cm^{-1}): 3447,1650,1604,814

^{1}H-NMR(CD_3OD,δ): 6.09(1H,d,J=2Hz,H-6),6.29(1H,d,J=2Hz),6.82(1H,d,J=8Hz,H-5^{1})

7.54(1H,d,d,J=2,8Hz,H-6^{1}),7.61(1H,d,J=2Hz,H-2^{1}),3.69(3H,s,OCH_3),3.77(3H,s,OCH_3)

3.84(3H,s,OCH_3),^{13}C-NMR(Table 1),was identified as Quercetin 3, 3^{1}, 4^{1} -trimethyl ether by direct comparison of the spectral data with literature [12-13].

The acetone extract at conc. of 10 mg/ml inhibit the growth of the test pathogens: Clinical isolates; *S. aureus, B. subtilis,*

Table 1: ^{13}C-NMR Spectra of Compounds II and III in CD3OD,δ-values in ppm

	Compound II	Compound III
2	146.0	146.2
3	135.6	135.7
4	179.5	174.8
5	158.4	158.6
6	99.8	100.1
7	163.1	163.9
8	94.7	95.1
9	158.4	151.4
10	104.6	104.8
1^{1}	123.0	123.7
2^{1}	116.2	112.8
3^{1}	145.9	146.1
4^{1}	149.9	149.9
5^{1}	117.5	117.5
6^{1}	123.9	123.7
3-OCH3	56.7	61.1
31-OCH3	-	60.8
5-OCH3	-	56.8

E.coli and *Ps. aeruginosa* (Table 2) with the highest zone of inhibition shown against *S. aureus* and *B. subtilis* both gram positive bacteria (Table 2).The activity of the extract on *S. aureus* was higher than the standard antibiotics; Gentamicin and Ciprofloxacin which both have zone of inhibition of 15 and 20 mm respectively. The isolated flavonoids showed the same trend against *S. aureus,* with compound III at concentration of 200 µg/ml showing activity against all the test pathogens, while compound II showed the highest zone of inhibition against *S. aureus* at 20 mm as compared to the standard antibiotics, it is interesting to note that Quercetin(compound I) which lacks the methoxyl group showed the least antibacterial activity contrary to previous reports that Quercetin has antibacterial activity[14]. The observed antibacterial activity of the extracts can be related to the methoxylated flavonoids as these classes of flavonoids have been known to exhibit antibacterial activity [15].The observed activity could justify the folkloric uses of this plant.

ACKNOWLEDGEMENT

The authors wish to thank Prof. Simon Gibbons of the University of London for the NMR spectra of the isolated Flavonoids.

REFERENCES

1. Bakasso S,Lamienda A,Lamien CE,Kiendrebeogo M,Milogo J,Ouedrago AG et al. Antioxidant activities of five Indigofera species from Burkina faso. Pakistan. Journ. of Biol. Sci 2008; 11 suppl 11:1429-1435
2. Aziz-UR-Rehamn, Abdul Malik, Naheed Riaz, Habib Ahmad,Sarfraz A and Muhammed IC. Lipoxygenase inhibitory constituents from *Indigofera hethranta* Chem Pharm Bull 2005; 53:suppl 3 263-266
3. Thusoo A,Raina N ,Ahmad SR. J. Indian Chem. Soc 1982; 59:1007-1008
4. Merogelman KM,Mckee TC,Boyd MR. Anti HIV prenylated flavonoids from *Monotes africanus* . J Nat Prod 2001; 64:546-548
5. Rehaman,A;Malik,A;Mehmood,S;Jahan,E;and Ahmad,A. Phytochemical studies of *Indigofera hetrantha*. Journ of Pak Chem.Soc 2005; 27 :440-442
6. Aliyu M,Haruna AK,Ilyas M,Ahmadu A, Rahman MM, and Gibbons S .Dihydrostilbenoids from *Indigofera pulchra*. Natural Prod. Commun 2008; 3 suppl 5:805-808
7. Mendoza L,Wilkens M and Urzua A. Antibacterial studies of resinous diterpenoids and Flavonoids of some Chilean pseudographalium(Asteralae). Journ. of Ethnopharmacol 1997; 58 suppl 2:246-252
8. Zuhal G and Damirez,LO . Flavonoid glycosides from *Asperula arvensis* . Turk Jour. of Chem 2005; 29 :163-169
9. Harbone,J.B(1994). The Flavonoids Advances in research since 1986: Chapman and Hall
10. Roitman J and James,LF. Highly oxygenated flavonol methylethers from *Gubierrezia microcephala*. Phytochem 1985;24:835-848
11. Eunhalea,H;Yun,SS;Changbae,J;Kyung-Tae lee et al. Constituents of stem and fruits of *Opuntia-ficus indica var saboten*. Arch.Pharm. Res 2003; 26 suppl12:1018-1023
12. Agrawal PK. ^{13}C-NMR of Flavonoids. Elsevier Publication 1989; 158-163
13. Mabry TJ,Markham KR and Thomas MB .The Systematic identification of Flavonoids A Spring- Verlag Publication 1970; 292-300

Table 2: Antibacterial activity of *I.secundiflora* acetone extract and isolated flavonoids #*

Extract/Compound	Concentration(µg/ml)	*S.aureus*	*B.subtilis*	*E.coli*	*Ps.aeruginosa*
Acetone extract	10000	21.0	23.0	16.0	16.0
Quercetin	200	-	-	-	-
	300	-	16.0	-	-
	200	20.0	18.0	-	-
	200	15.0	13.0	12.0	12.0
	300	nt	nt	nt	15.0
	5	10.5	26.5	31.0	31.0
	10	13.0	21.5	20.0	17.5

-: No inhibition
nt: not tested
#* Values are zone of inhibition diameter in millimetre and mean of triplicates

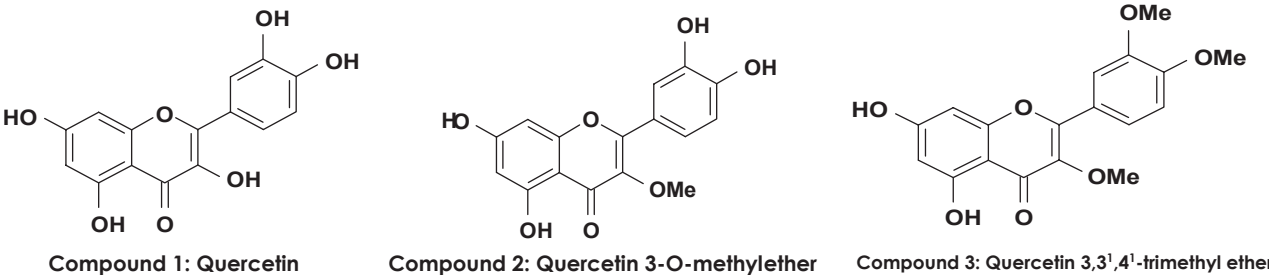


Figure1: Structures of isolated flavonoids.

14. Mori A,Nishino C,Emoki N and Tawata S.Anti bacterial activity and mode of plant flavonoids against *Proteus vulgaris* and *Staphyloccus aureus*. Phytochem 1987;26:223-224

15. VanPuyvelde L,De Kimpe N,Costa J,Munyjabo V,Nyirankulaza A,et al. Isolation of flavonoids and chalcone from *Helichrysum odoratissimum*. J Nat Prod. 1989;52:629-633

Pharmacognostical and Preliminary Phytochemical Investigation of *Blepharis molluginifolia*, Pers. – A Threatened Medicinal Herb.

Pramod V. Pattar.[1]*, Jayaraj M.[1], Arunkumar B.S.[1], Ananth B.[2]

[1]P. G. Department of Botany, Karnatak University, Dharwad, Karnataka, India. [2]Department of Pharmacognosy, KLES's College of Pharmacy, Hubli, Karnataka, India.

ABSTRACT

Plants are the great sources of medicines, especially in traditional system of medicine, which are useful in the treatment of various diseases. *Blepharis molluginifolia* Pers. is threatened medicinal herb belongs to the family Acanthaceae. The plant selected for the present study is used traditionally to treat bone fractures, skin diseases, urinary discharges, and allergies. Further, it has not yet been studied pharmacognostically. Hence, the present study is aimed to know the pharmacognostic parameters to determine the quality of the plant *Blepharis molluginifolia*. This study comprises the detailed macroscopic, microscopic and preliminary phytochemical investigations of the herb were studied.

Key words: *Acanthaceae, Blepharis molluginifolia, Pharmacognosy, Phytochemical screening.*

INTRODUCTION

Blepharis molluginifolia Pers. is a threatened medicinal herb,[1] commonly known as Haridachchu in Kannada.The leaf of this plant is mainly used for bone fractures, skin diseases, urinary discharges, and allergies.[2] According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicines for their primary health care needs. The medicinal value of plants due to the presence of chemical substances that produce a definite physiologic action on the human body. Plants are found along sandy places and rocky regions. In nature (*in vivo*) the plants are seasonal and appear for few months. The roots and few basal stem stocks retaining in the soil and are regenerating during rainy season and soon after the rainy season the aerial part dries up and the plants disappear. Propagation of the species by seeds or any other conventional methods are not reliable because the plants are seasonal and available for short duration in nature. Moreover, the seeds are viable for short period and loose their viability within few weeks. The species is under threat due to their exploitation from their natural habitat by traditional healers, over grazing, seasonal habitat and short seed dormancy. At the same time, no data are available concerning its biological activities.[3] The qualitative chemical tests of the alcohol extracts of plant material revealed the presence of alkaloids, triterpenoids, flavonoids and carbohydrates and saponinglycosides. The study scientifically validates the use of plant in traditional medicine and it contributes to the development and standardization of parameters of herbal drugs used in Indian system of medicine. Therefore, the aim of the study presented here is to elaborate a protocol for pharmacognistical works including macroscopic, microscopic and preliminary phytochemical investigations.

Address for correspondence:
Pramod V. Pattar, P. G. Department of Botany, Karnatak University, Dharwad, Karnataka, India.
E-mail: pams238@gmail.com

DOI: 10.5530/pj.2011.19.6

MATERIALS AND METHODS

Macroscopic studies of Blepharis molluginifolia, Pers.

Blepharis molluginifolia, Pers. is a prostrate herb, leaves narrow, oblong to oblanceolate or ovate, and flowers violet to purple in axillary cymes, seeds four, flattened, orbicular and echinate on retinacula. Procumbent, slender, hairy. Leaves apparently 4-nate in distant whorls, up to 1 by 1/4 in., from narrow-elliptic to linear-oblong, narrowed at both ends, sessile. One-flowered branches (reduced spikes) sessile, axillary,

solitary (rarely 2–3 approximately); bract 1/4–1/3 in. long, quadrate-obovate, strongly 3-nerved, with 7–15 spines, each 1/12–1/10 in. long, round its triangular-obtuse head; bracteoles 1/3 in. long, lanceolate, bristle-tipped. Posticous calyx-segment 1/2–2/3 in. long; 2 innermost calyx-segments linear-lanceolate, nearly as long. Corolla 1/2 in. long. Capsule 1/4 in. long. Flowering and fruiting time is between September and January.

Collection of plant material and authentication

Fresh whole plants of *Blepharis molluginifolia*, Pers. were collected from Karnatak University campus Dharwad and were authenticated by one of the authors Dr. M. Jayaraj. A voucher specimen has been deposited in the P. G. Department of Botany, Karnatak University, Dharwad for future reference.

Drying of plant material

The whole plant material of *Blepharis molluginifolia*, Pers. was subjected to shade drying for about 10 weeks. The shade dried plant material was further crushed to powder and the powder was passed through the mesh 22 and stored in air tight container for further analysis.

Macroscopic and microscopic analysis

The macroscopy and microscopy of the plant studied according to the method.[4,5,6] Transverse sections of Leaf, Stem, Roots and Stomatal index were prepared and stained with Safranin and Fast green as per the procedure.[7] Powder microscopy is performed according to the prescribed procedure.[8,9] The microphotographs were taken by Bright field microscope with digital camera Canon Photo Shot G2 for detailed studies.

Determination of behaviour of plant powder

Behaviour of powdered plant material with different chemical reagents were determined under natural light.

Extraction of powdered plant material

The plant material collected from their natural habitat was cleaned, shade dried at room temperature, coarsely powdered and stored in an air tight glass container. 100gm of coarse powder was successively extracted with Alcohol (40–60) in Soxhlet extractor for 18 hours. The extracts were filtered and concentrated using rotary flash evaporator and residues were dried in desiccators over sodium sulfite below 60°C. Freshly prepared extract was subjected to phytochemical evaluation for the detection of various constituents using conventional protocol.[10]

RESULTS AND DISCUSSION

Pharmacognistical investigations

The detailed and systematic pharmacognistical evaluation would give valuable information for the future studies. The detailed morphology of *Blepharis molluginifolia*, Pers. was carried out to support proper identification of drug.

Stomata and Stomatal index

Blepharis molluginifolia showed various kinds of stomata are predominantly diacytic, anisocytic and rarely paracytic. In *Blepharis repens* diacytic and anisocytic,[11] *Blepharis boerhaaviafolia* anamocytic[12] and *Blepharis maderaspatensis* diacytic[13] stomata were present. The epidermal cells are larger than subsidiary cells. Stomatal index, the percentage of stomata found in unit area of leaf exhibited marked variation in the adaxial and abaxial surface of the leaf. Abaxial surface has an increased stomatal frequency than adaxial surface (Fig. 1. D). The values are represented in the Table 1.

MICROSCOPY

Microscopy of Root (T.S)

In T.S of root (Fig.1.A), epidermis was made up of one layer of cork cells covered by cuticle, unicellular covering trichomes were seen. Cortex layer was composed of 8-9 layers of parenchymatous cells, which were irregularly arranged some times, these cells are tangentially elongated and compressed.[12] Pericycle was made up of 1-2 layers. Phloem was seen composed of 1-2 layers. Xylem vessels are present, varying in size and accompanied by inter xylary parenchymatous cells, which are lignified. Major portion of root occupied by secondary xylem which consist of vessels, fibres, tracheids and xylem parenchyma, xylem vessels are reticulate and pitted type.[11] Pith is made up of somewhat rounded parenchymatous cells with intercellular space.

Miroscopy of Stem (T.S)

In T.S of stem (Fig.1.B), outer epidermis consisted of single layer of tangentially elongated parenchymatous cells. It was covered by a thin cuticle. Multicellular uniseriate covering trichomes were present, whereas in other species of *Blepharis* trichomes are unbranched, thin walled pointed and curved.[12] Cortex below the epidermal cells, three layers of collenchymatous, four layers of parenchymatous cells and endodermis were seen. Pericycle made up of 4-5 layers of cells followed by phloem. Phloem is composed group of small sized polyhedral cells closely arranged with parenchymatous cells. Xylem consists of annular and reticulated thick walled vessels with tracheid fibers but in

other species these are large and arranged in radial rows with lignified xylem parenchyma.[11] It consisted of metaxylem and protoxylem. Pith was made up of somewhat rounded parenchymatous cells with large intercellular space.

Microscopy of Leaf (T.S)

In T.S of leaf (Fig.1.C), upper epidermis is composed of tangentially arranged single layer of subrectangular cells with smooth cuticle. Trichomes are multicellular, uniseriate. Palisade parenchyma is composed of elongated and more or less cylindrical cells, which are close together with long axes of the cells perpendicular to the epidermis and spongy parenchyma was made up of loosely arranged parenchymatous cells. The mesophyll containing rarely calcium oxalate crystals some times fairly abundant in the leaf.[12] Lignified pericyclic sclerenchymatous cells of 1-2 layers present above and below the xylem vessel. It consists of single layer of tangentially arranged subrectangular cells with smooth cuticle. Just below the lower epidermis, two layers of collenchymatous cells are present.

PRELIMINARY PHYTOCHEMICAL ANALYSIS

Preliminary phytochemical screening of the *Blepharis molluginifolia*, Pers. plant powder is done following standard methods [10] and results are presented in the Table 2. Alcohol extract shows the presence of mainly alkaloids, triterpenoids, flavonoids and carbohydrates and saponinglycosides.

Table 1: Stomatal index on adaxial and abaxial leaf surfaces of *Blepharis molluginifolia*, Pers.

Trials	Adaxial surface of leaf		Abaxial surface of leaf	
	Margin	Middle	Margin	Middle
1.	143	135	152	210
2.	160	156	164	236
3.	156	132	155	210
4.	158	154	170	220
5.	165	160	172	238
Average	156.40	147.40	162.59	222.80
	151.90		192.69	

Table 2: Phytochemical analysis of whole plant extract of *Blepharis molluginifolia*, Pers.

Phytochemicals	Observation
Steroids	-
Tritepenoids	+
Alkaloids	+
Flavanoids	+
Tannins	-
Saponinglycosides	+
Aanthraquinone glycoside	-
Cardiac glycoside	-
Carbohydrates	+
Test for Proteins	-

+ = Present, - = Absent.

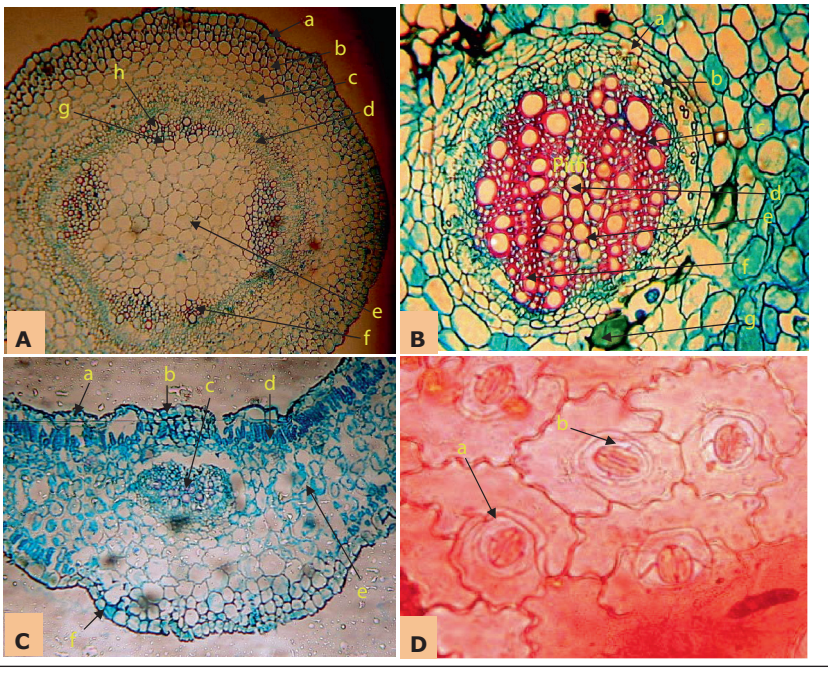


Figure 1: A-T.S. of young root, a-epidermis, b-cortex, c-endodermis, d-pericycle, e-medulla, f-phloem, g-protoxylem, h-metaxylem. **B**-T.S.of stem, a-epidermis, b-hypodermis, c-conjuctive tissue, d-meta xylem, e- proto xylem, f-phloem, g-vascular cambium. **C**-T.S. of leaf, a-upper epidermis, b-cuticle, c-vascular bundles, d-palisade parenchyma, e-spongy parenchyma, f-lower epidermis. **D**-Surface view of leaf epidermis showing a-anisocytic stomata and b- diacytic stomata.

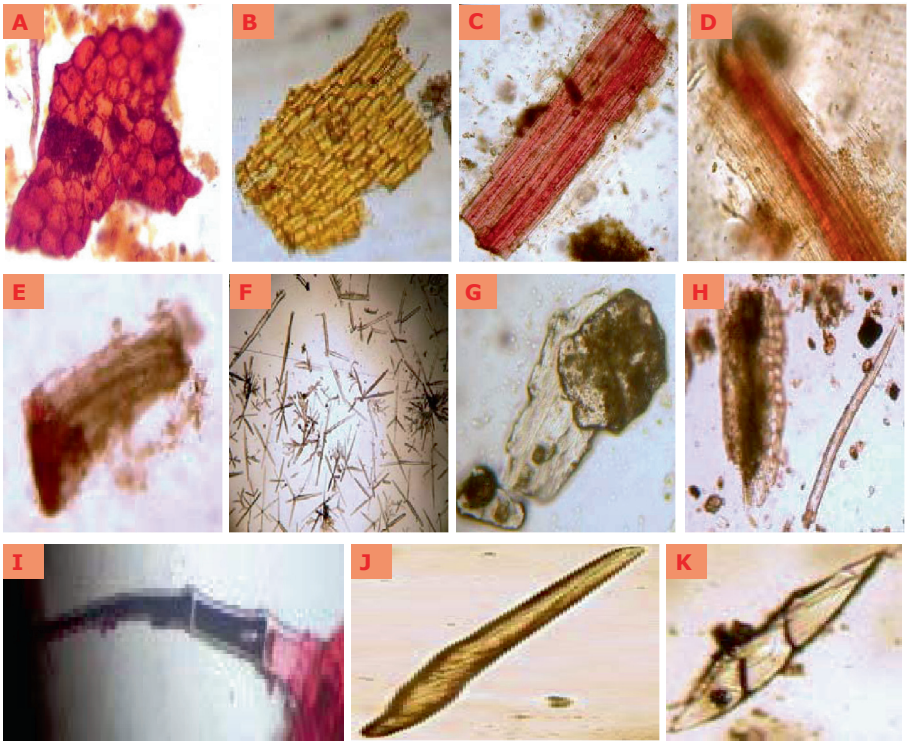


Figure 2: A-Cork cells, **B**-Fragment of parenchyma , **C**-Tracheid fibers, **D**-Annular & Reticulated xylem vessel, **E**-Phloem fibres, **F**-Vessel elements and fibres, **G**-Oxalate crystals, **H**-Warty trichome, **I**- Multicellular, uniseriate trichome, **J**-Narrow fibre, **K**-Xylem vessel.

BEHAVIOUR OF WHOLE PLANT POWDER WITH DIFFERENT CHEMICAL REAGENTS

Behaviour of *Blepharis molluginifolia*, Pers. with different chemical reagents is to detect the colour changes under ordinary daylight by standard method. [14] Table 3.

Table 3: Behaviour analysis of whole plant powder of *Blepharis molluginifolia*, Pers. with different chemical reagents.

Treatment	Colour of powder
Powder as such	Light brown
Powder + Picric acid	Yellowish green
Powder + HNO_3	Faint brown
Powder + HCL	Light green
Powder + $H2SO_4$	Faint brown
Powder + $FeCl_3$	Dark brown
Powder + NaOH	Light green
Powder + Glacial acetic acid	Blackish green
Powder + Iodine solution	Faint black
Powder + Aqueous solution	Light green
Powder + Aq. Mercuric chloride	Light green
Powder + HNO_3 + Ammonia solution	Light brown

CONCLUSION

In present investigation, various standardized parameters such as macroscopy, microscopy, phytochemical screening was carried out and which could be helpful in authentification of ***Blepharis molluginifolia*, Pers.** The result of present study will also serve as reference material in preparation of monograph. It is present need to conserve the plant for medicinal usage. Tissue culture techniques may be more useful in the conservation point of view and to make the drug available throughout the year.

Table 4: Organoleptic evaluation of various parts of *Blepharis molluginifolia*, Pers.

	Flower	Fruit	Seed	Leaves	Stem	Root
Colour	Violet/Purple	Dark green	Brown	Dark green	Light brown	Yellowish brown
Odour	Odourless	Odourless	Odourless	Odourless	Odourless	Odourless
Taste	Bitter	Bitter	Pungent	Bitter	Slight bitter	Slight bitter

ACKNOWLEDGEMENTS

Authors acknowledge the financial assistance from UGC New Delhi, under RFSMS for one of the author, Chairman, P.G. Department of Botany, Karnatak University, Dharwad. for extending facilities.

REFERENCES

1. Balakrishna Gouda, M.M. Nissar, Y.N.Seetharam and S.R.Ramesh. The Threatened Plants of Peninsular India. My Forest. 1997; Vol.33 (1):327-334.
2. Khare C. P. Indian Medicinal Plants: An Illustrated Dictionary, Springer-Verlag Berlin/Heidelberg. 2007; 94.
3. Saxena H. O. A survey of the plants of Orissa (India) for tannins, saponins, flavanoids and alkaloids, Llyoda. 1975; 38:346-374.
4. Pullaiah T.,V. Ramakrishnaiah, S.Sandhya Rani and P.N.Rao. Flora of Guntur District, Andhra Pradesh, India. 2000; 265.
5. Evans W.C., Trease and Evans Pharmacognosy, WB Saunders Ltd. London. 2002; 32-33 & 95-99,512, 547.
6. Wallis T.E., Textbook of Pharmacognosy, Published by SK Jain. 1985; 572-575.
7. Dwivedi J.N., R. B. Singh., Essentials of Plant Techniques 2nd Ed., Scientific Publishers.1990; 26-28.
8. Khandelwal K.R., Practical Pharmacognosy, 18th Ed. Nirali Prakashan, Pune. 2007; 31-40 & 100-101.
9. Khasim S.M., Botanical Microtechnique: Principles & Practice. Capital Publishing Company, New Delhi. 2002; 41-52 & 85-88.
10. Kokate C.K. Preliminary phytochemical screening. In 4th Ed. Practical Pharmacognosy. Nirali Prakashan, Pune, India. 2000; 107–11.
11. Sayed S.V and B.R.Zate. Pharmacognostic study of *Blepharis repens*. Proceedings of the 33rd Conference of Indian Botanical Society and International Symposium On The New Horizons of Botany. 2010; Nov 10th-12th:137.
12. Devi P. and Meera R. Pharmacognostic and Preliminary Physio - Phyto chemical investigations on the leaves of *Blepharis boerhaaviafolia*. Journal of Pharmaceutical. Science & Research. 2010; Vol.2 (2):99-106.
13. Mohan V.R., Amish Abragam D., Kalidass C., Maruthupandian A. Pharmacognostical and phytochemical investigation of whole plant of *Blepharis maderaspatensis* (L.) Heyne ex Roth. Pharmacognosy Journal. 2010; Vol 2:1-5.
14. Chase C.R. and Pratt R.J., Fluoresence of powder vegetable drugs with particular reference to development system of identification. Jr. Amer. Pharm. Assoc.1949; 38:324-331.

Acid-Base Indicator Properties of Dyes from Local Flowers: *Cassia aungostifolia* Linn., *Thevetia peruviana* (Pers.) K. Schum and *Thevetia thvetiodes* (Kunth) K. Schum

Sudarshan Singh[1] *, S. B. Bothara[2], Sangeeta Singh [3]

Shree H.N.S.IP.E.R, Rajkot, Gujarat, India. Shri GM BILAKHIA College of Pharmacy ROFEL, Vapi, Gujarat, India. Innovative Groups of college, Delhi, India.

ABSTRACT

Indicators used in titration show well-marked changes of color in certain intervals of pH. Most of these indicators are organic dyes and are of synthetic origin. The environmental pollution caused by chemical industries in the synthesis of organic dyes had made the scientist in the developing country to enter in to an era, in which plant product serve as an alternative to synthetic products. The advantages of the plant products are local and easy accessibility, environmental friendly nature, and lower price compare to the synthetic products. Herbs are non-polluting renewable supplies of chipper products for the worlds growing population. Natural pigments in plants are highly colored substances and may show color changes with variation of pH. Curcumin isolated from *Curcuma longa* is as example of natural indicator used in analytical chemistry. Hence the work was carried out to study the indicator property of flowers *Cassia aungostifolia* Linn., *Thevetia peruviana* (Pers.) K. Schum and *Thevetia thvetiodes* (Kunth) K. Schum from ethanolic extract. It was found that the extract changes the color at different pH and can be used successfully as a compound indicator.

Key words: Acid-base indicator, *Cassia aungostifolia* Linn., *Thevetia peruviana, Thevetia thvetiodes*, Anthocynins.

INTRODUCTION

In spite of the numerous instrumental techniques currently available for the chemical analyses of various samples, conventional methods of analyses are still relevant and find application in many situations. Some conventional analytical techniques that are still popular include gravimetry and titrimetry. In titrimetry, the equivalence point is usually determined by the end point in the titration. The end point in traditional titrimetry is usually indicated by some substances added into the analyte solution, which change color immediately after the equivalence point has been attained. These substances are generally referred to as indicators.

Address for correspondence:
Mr. Sudarshan singh, M. Pharm., Lecturer,
Dept. of Pharmaceutics,
Shree H.N.S.I.P.E.R., C/o BM Kiayda Campus,
Nr. Lal Pari Lake, B/H Marketing Yard, Amargardh - Bichari,
Rajkot, 360002 (Gujarat.) India.
Mobile Number: +919978819269
E-mail: sudarshansingh83@hotmail.com

DOI: 10.5530/pj.2011.19.7

Several types of indicators are available for different types of titrimetric analyses. For acid-base titrations, organic dyes, which are either weak acids or bases, serve excellently as indicators. A large number of dyes are obtainable as natural products. In Nigeria, several workers have extracted a number of dyes from a variety of local plants. According to Akpuaka and Osabohien *et al.*, the local plants-Camwood, Redwood, Henna, Annato, Rothmania, Terminalia, Indiqovine, Kola, Banana, Tumeric, Roselle and Ginger all contain different types of dyes which are used for various purposes.[1,2] The suitability of some of these dyes for dyeing purposes has been investigated on different types of fabrics. An evaluation of other properties of a number of dyes including synthetic dyes have also been reported by a number of workers.[3,4] Ekandem and Eze *et al.* have also reported their findings on the use of some natural dye extracts as indicators in acid-base titrimetry.[5,6] Other than these few reported cases, very little attention has been paid to the use of local dye extracts as indicators in acid-base titrimetry.

Cassia aungostifolia linn. commonly known Tinnevelly Senna or Tanner's cassia, is a well known source of sennosides as belonging to family Caesalpiniaceous. This herb contains

anthraquinones, flavonoids and flavan-3-ol derivatives.[7] *Cassia aungostifolia* has been used as natural medicine for the treatment of Anti-viral, anti-cancer and hypoglycemic. The whole plant possesses medicinal properties useful in the treatment of skin diseases, inflammatory diseases, rheumatism, anorexia and jaundice.[8] *Thevetia peruviana* (Pers.) K. Schum commonly known as yellow oleander belonging to family Apocynaceae it contain a milky sap containing a compound called thevetin that is used as a heart stimulant but in natural from is extremely poisonous as are all part of plant.[9] *Thevetia thvetiodes* (Kunth) K. Schum commonly known giant thevetia as belonging to family Apocynaceae; The leaves and bark are used as an emetic and purgative. The flowers of the tree are cultivated for their seeds that contain a strong heart drug. All parts of the tree are poisonous and the latex is an irritant that causes blisters upon skin contact.[10] It seems that no work has been done on the suitability of *Cassia aungostifolia, Thevetia peruviana* and *Thevetia thvetiodes* as indicator in acid-base titration as compared to the relatively common Methyl Red and Phenolphthalein using acid-base titration. Hence the present vocation was attempted to appraise the flower as a natural indicator.

MATERIAL AND METHOD

Material

Fresh flower of were collected from the Rajkot region, Gujarat, and they were authenticated from NISCAIR, New Delhi, Ref No: NISCAIR/RHMD/Consult/2010-11/1468/69. All other ingredients were of analytical grade and purchased from Loba chemicals, Mumbai.

Method

The flower were cleaned by distilled water and cut into small pieces and macerated for two hours in 25ml of 90% ethanol. The extract was preserved in tight closed container and stored away from direct sun light.[11]

The experiment was carried by using the same set of glassware's for all types of titrations. As the same aliquots were used for both titrations i.e. titrations by using standard indicators and flower extract, the reagents were not calibrated. The equimolar titrations were performed using 10 ml of titrant with three drops of indicator. All the parameters for experiment are given in Table 1. A set of five experiments each for all the types of acid base titrations were carried out. The mean and standard deviation for each type of acid base titrations were calculated from results obtained. The extract was also analyses for its λmax in UV-Visible range on Systronics single beam spectrophotometer (Shimadzu UV 1800).

RESULT

The extract was found to contain compound anthocynins Table 2 as it gives blue color to aqueous sodium hydroxide solution, yellow orange color to concentrated sulphuric acid while red color which fed out on standing with magnesium-hydrochloric acid solution.

The flower extract of *C. aungostifolia, T. peruviaana* and *T. thvetiodes* showed λmax in both regions i.e. UV and visible (Table 3). Both λmax suggested the presence of anthocynins in the extract.

Table 1: Standard chart for Phytochemical identification

Phytochemicals	*Color with aq. NaOH*	*Color with Conc. H_2SO_4*	*Color with Mg-HCl*
Anthocynins	Blue violet	Yellow to orange	Red (fades to pink)
Flavones	Yellow	Yellow to orange	Yellow to red
Flavonones	Yellow to orange (cold) Red to purple (hot)	Crimson Orange	Red, magenta, violet, blue
Isoflavones	Yellow	Yellow	Yellow
Leucoanthocyanins	Yellow	Crimson	Pink

Table 2: Technological characterization for analysis of chemical test.

Sample	Poly-Phenolic compound		Flavonoid	Anthrocynins		
	Color with $FeCl_3$	*Color with Lead acetate*	*Shinoda test*	*Color with aq. NaOH (Blue violet)*	*Color with Conc. H_2SO_4 (Yellow orange)*	*Color with Mg-HCl (Red)*
CAI	+	+	+	+	+	+
TPI	+	+	+	+	+	+
TTI	+	+	+	+	+	+

+; Presence of compound

The Flower extract was screened for its use as an acid-base indicator in various acid-base titrations, and the results of this screening were compared with the results obtained by standard indicators methyl red, phenolphthalein and mixed indicator [methyl orange: bromocresol green (0.1:0.2) results are presented in Table 4.[12] The titrations of strong acid with strong base (HCl & NaOH), strong acid with weak base (HCl & NH_4OH), weak acid with strong base (CH_3COOH & NaOH), and weak acid with weak base (CH_3COOH and NH_4OH) were carried out using standard indicators and flower extract. The results of these titrations are given in Table 5,6,7,8. It could be due to these flavonoids and anthocyanins, the sharp end point appeared in the above mentioned titrimetric analyses. The flower extract of *C. aungostifolia, T. peruviaana* and *T. thvetiodes* was found to have Poly-Phenolic, flavonoids, anthocyanins and is pH sensitive. The end point determination of acid base titrations by the traditional indicators, compared with flower extract indicator, it was observed that traditional indicators gave incorrect results due to addition of excess of titrant (base) after the neutralization reaction was completed, but flower extract indicator has given sharp end point because solutions give sharp color change at the equivalence points. Thus natural indicator employed in the acid base titrations was found economic, safe and an efficient alternative for traditional indicators. In comparison to this, chemical indicators were found more expensive and hazardous, which proves that flower extract of *C. aungostifolia, T. peruviaana* and *T. thvetiodes*, as a natural indicator is more worthy.

Table 3: Determination of UV Visible absorption

Sample code	UV λmax	Visible λmax
CAI	275	596
TPI	338	555
TTI	291	537

Table 4: Technological characterization for analysis and comparisons of color change.

Titrant	Titrate	Indicator Color Change			
		Standard (pH Range)	CAI (pH Range)	TPI (pH Range)	TTI (pH Range)
HCl	NaOH	Yellow - pink	Yellow- Colorless (11.19-1.04)	Greenish yellow-Colorless (12.72-2.32)	Light Green to Colorless (12.72-5.37)
HCl	NH_4OH	Pink -Colorless (10.98-6.74)	Yellow- Colorless (11.29-1.28)	Greenish yellow-Colorless (11.05-2.0)	Light Green to Colorless (11.0-2.69)
CH_3COOH	NaOH	Yellow- light red	Yellow- Colorless (12.95-6.12)	Greenish yellow-Colorless (12.82-7.22)	Light Green to Colorless (12.76-6.52)
CH_3COOH	NH_4OH	Orange to blue-green (4.73-2.86)	Yellow- Colorless (11.31-5.10)	Greenish yellow-Colorless (11.05-6.7)	Light Green to Colorless (10.95-5.92)

HCl: Hydrochloric acid, CH_3COOH: Acetic Acid, NaOH: Sodium Hydroxide, NH_4OH: Ammonium Hydroxide, CAI: *C. aungostifolia*, TPI: *T. peruviana*, TTI: *T. thetiodes.*

Table 5: Technological characterization of acid-base titration using standard indicator.

Titration (Titrant v/s Titrate)	Strength in moles	Indicator	Mean ± S.D.*	Color	pH
NaOH v/s HCl	0.1	MR	12.3 ± 0.12	Yellow to pink	12.32-5.77
	0.5	MR	11.2 ± 0.16	Yellow to pink	12.55-4.87
	1.0	MR	11.2 ± 0.15	Yellow to pink	12.63-3.30
HCl v/s NH_4OH	0.1	PT	05.9 ± 0.01	Pink to colorless	10.50-6.74
	0.5	PT	06.6 ± 0.08	Pink to colorless	10.61-8.28
	1.0	PT	06.5 ± 0.16	Pink to colorless	10.98-8.29
CH_3COOH v/s NaOH	0.1	MR	12.0 ± 0.11	Yellow to light red	12.33-6.01
	0.5	MR	11.9 ± 0.14	Yellow to light red	12.56-5.96
	1.0	MR	12.0 ± 0.09	Yellow to light red	12.67-5.99
CH_3COOH v/s NH_4OH	0.1	MI	05.0 ± 0.05	Orange to green	03.25-4.52
	0.5	MI	05.6 ± 0.19	Orange to green	02.81-4.68
	1.0	MI	06.1 ± 0.17	Orange to green	02.86-4.73

*All values are mean ± S.D. for n=3
HCl: Hydrochloric acid, CH_3COOH: Acetic Acid, NaOH: Sodium Hydroxide, NH_4OH: Ammonium Hydroxide, MR; Methyl Red, MI: Mixed Indicator, PT: Phenolphthalein.

Table 6: *Cassia aungostifolia* as indicator

Titration (Titrant v/s Titrand)	Strength in moles	Indicator	Mean ± S.D.	Color	pH
	0.1	CAI	13.1 ± 0.11	Yellow to colorless	11.19-3.14
NaOH v/s HCl	0.5	CAI	10.0 ± 0.18	Yellow to colorless	11.62-1.65
	1.0	CAI	10.7 ± 0.12	Yellow to colorless	11.36-1.04
	0.1	CAI	19.0 ± 0.18	Yellow to colorless	10.46-2.01
HCl v/s NH_4OH	0.5	CAI	12.6 ± 0.05	Yellow to colorless	10.13-1.28
	1.0	CAI	6.0 ± 0.03	Yellow to colorless	11.29-1.31
	0.1	CAI	11.5 ± 0.13	Yellow to colorless	12.83-6.12
CH_3COOH v/s NaOH	0.5	CAI	10.6 ± 0.17	Yellow to colorless	12.95-6.32
	1.0	CAI	10.3 ± 0.08	Yellow to colorless	12.18-6.17
	0.1	CAI	4.4 ± 0.16	Yellow to colorless	09.28-7.35
CH_3COOH v/s NH_4OH	0.5	CAI	8.4 ± 0.05	Yellow to colorless	10.18-5.35
	1.0	CAI	8.9 ± 0.06	Yellow to colorless	11.31-5.10

*All values are mean ± S.D. for n=3
HCl: Hydrochloric acid, CH_3COOH: Acetic Acid, NaOH: Sodium Hydroxide, NH_4OH: Ammonium Hydroxide, CAI: *C. aungostifolia*

Table 7: *Thevetia thvetiodes* as indicator

Titration (Titrant v/s Titrand)	Strength in moles	Indicator	Mean ± S.D.	Color	pH
	0.1	TTI	11.6 ± 0.06	Light green to Colorless	12.33-6.48
NaOH v/s HCl	0.5	TTI	10.1 ± 0.05	Light green to Colorless	12.46-6.43
	1.0	TTI	10.0 ± 0.12	Light green to Colorless	12.72-5.37
	0.1	TTI	6.5 ± 0.08	Light green to Colorless	10.38-4.60
HCl v/s NH_4OH	0.5	TTI	7.3. ± 0.18	Light green to Colorless	10.78-2.69
	1.0	TTI	7.0 ± 0.16	Light green to Colorless	11.0-5.42
	0.1	TTI	10.9 ± 0.13	Light green to Colorless	12.36-9.20
CH_3COOH v/s NaOH	0.5	TTI	11.5 ± 0.17	Light green to Colorless	12.73-6.52
	1.0	TTI	11.3 ± 0.18	Light green to Colorless	12.76-6.55
	0.1	TTI	6.6 ± 0.05	Light green to Colorless	10.20-6.62
CH_3COOH v/s NH_4OH	0.5	TTI	7.5 ± 0.03	Light green to Colorless	10.73-5.99
	1.0	TTI	8.0 ± 0.11	Light green to Colorless	10.95-5.92

*All values are mean ± S.D. for n=3
HCl: Hydrochloric acid, CH_3COOH: Acetic Acid, NaOH: Sodium Hydroxide, NH_4OH: Ammonium Hydroxide, TTI: *T. thvetiodes*

Table 8: *Thevetia peruviana* as indicator

Titration (Titrant v/s Titrand)	Strength in moles	Indicator	Mean ± S.D.	Color	pH
	0.1	TPI	11.5 ± 0.06	Greenish yellow to Colorless	12.33-6.97
NaOH v/s HCl	0.5	TPI	10.5 ± 0.05	Greenish yellow to Colorless	12.47-4.81
	1.0	TPI	10.5 ± 0.12	Greenish yellow to Colorless	12.72-2.32
	0.1	TPI	8.3 ± 0.08	Greenish yellow to Colorless	10.29-6.35
HCl v/s NH_4OH	0.5	TPI	7.3. ± 0.18	Greenish yellow to Colorless	10.88-2.0
	1.0	TPI	7.1 ± 0.16	Greenish yellow to Colorless	11.15-2.36
	0.1	TPI	11.4 ± 0.13	Greenish yellow to Colorless	12.55-7.35
CH_3COOH v/s NaOH	0.5	TPI	10.7 ± 0.17	Greenish yellow to Colorless	12.82-8.08
	1.0	TPI	11.5 ± 0.18	Greenish yellow to Colorless	12.78-7.22

Table 8: *continued*

	0.1	TPI	6.6 ± 0.05	Greenish yellow to Colorless	10.48-6.24
CH_3COOH v/s NH_4OH	0.5	TPI	6.4 ± 0.03	Greenish yellow to Colorless	11.05-6.7
	1.0	TPI	5.7 ± 0.11	Greenish yellow to Colorless	11.22-7.99

*All values are mean ± S.D. for n=3
HCl: Hydrochloric acid, CH_3COOH: Acetic Acid, NaOH: Sodium Hydroxide, NH_4OH: Ammonium Hydroxide, TPI: *T. peruviana*

CONCLUSION

The results obtained in all the types of acid-base titrations lead us to conclude that, it was due to the presence of flavonoids and anthocyanins, sharp color changes occurred at end point of the titrations. We can also conclude that, it is always beneficial to use *C. aungostifolia, T. peruviaana* and *T. thvetiodes* flower extract as an indicator in all types of acid base titrations because of its economy, simplicity and wild availability.

REFERENCES

1. Akpuaka MU, Chukwuneke C, Agbo G. The dyeing of textile fabrics with natural dyes from some local trees. J. Chem. Soc. Nig. 1998; 23:47-52.
2. Osabohien E and Ukpumwan DO. Extraction of natural dyes from some local plants. J. Chem. Soc. Nig. 2002; 27(2):139-142.
3. Obanda OD, Dambata BB, Ukpumwan DO. Predicting light fastness of dyes on textiles from spectra of their photo-fading in solutions – A preliminary report. J. Chem. Soc. Nig. 1995; 20:8-17.
4. Dambata BB and Gumel SM. Dye-fiber bond stabilities of some reactive dyes on nylon. J. Chem. Soc. Nig. 1997; 22:121-131.
5. Ekandem JE and Owor AU. Local flower extracts as simple acid-base indicators. J. Chem. Soc. Nig. 1997; 22:98-103.
6. Eze SO and Okereafo A. Industrial and analytic potentials of dye extracts from Telfaira occidentalis (Fluted pumpkin). J. Chem. Soc. Nig. 2002; 27:143-146.
7. Kritikar KR and Basu BD. Indian Medicinal Plants. Vol.-IV, 2nd edtion, International Book Distribution, Dehradun, India; 2006. p. 1225-1226.
8. Anonymous: The wealth of India. Publication and information Directorate (CSIR), New Delhi; Vol-III (Ca-Ci), 1992:354-360.
9. http://en.Zipcodezoo.com/ *Thevetia peruviana* use/. Accessed - Dec 10, 2010.
10. http://en.Zipcodezoo.com/ *Thevetia thvetiodes* use/. Accessed - Dec 10, 2010.
11. Sandeep B Patil et. al., Use of Flower Extracts as an Indicator in Acid-Base Titrations, Research J. Pharm. and Tech, 2009; 2(2):421-422.
12. http://en.wikipedia.org/wiki/PH_indicator. Accessed - May 30, 2010.

HPTLC Fingerprinting Profile of Marker Compound (Berberine) in Roots of *Berberis aristata* DC

Rashmi[1*], Jagdish Pant[2], and A. Rajasekaran[3]

[1] Chemistry Division, Forest Research Institute, Dehradun-248006. [2]AUM Agrotech Ltd., Chennai- 600028. [3] Himalayan Forest Research Institute, Shimla (Himachal Pradesh)

ABSTRACT

A sensitive and reliable densitometric High Performance Thin Layer Chromatography method has been developed for the quantification of berberine, an alkaloid present in roots of *Berberis aristata*. Chromatographic analysis was performed using methanol extract of roots of *Berberis aristata* on silica gel $60F_{254}$ GLP (E.Merck) plates using the solvent system, n-propanol: formic acid: water (9:01:0.9). Detection and quantification of berberine was done by densitometric scanning at 364 nm. The results of linearity range and correlation coefficient show that there was a good correlation between peak area and corresponding concentration of berberine. The proposed HPTLC method provided a good resolution of berberine from other constituents present in methanol extract of roots of *B. aristata* and can be used for the quantification of berberine.

Key words: *Berberis aristata,* Berberidaceae, berberine, HPTLC, Rasaut.

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.[1]

Berberis aristata DC (Berberidaceae; Hindi: Daruharida, Rasaut; English: Indian barberry) is one of the economically important and of high medicinal value species of temperate areas. It is an erect, glabrous spinescent shrub, 3-6 m in height with obovate, subacute and entired leaves. It is distributed in India upto an altitude of 1500-2400 m. Its stem, roots and fruits are used in many ayurvedic preparations. The plant got hepatoprotective, antitumour, sedative and wound healing properties. Rasaut is one of the very important and a useful preparation obtained from this plant and is used in curing many human ailments.[2] The dried berries are edible. The fresh berries are laxative and antiscorbutic and useful in piles, sores and eye diseases particularly conjunctivitis. A decoction is used as a mouthwash for treatment of swollen gums and toothache. Berberine is one of the important marker alkaloidal active principles of this plant.[3, 4] Berberis species are major source of berberine and other alkaloids also namely berbamine, palmatine, isotetrandrine and jatrorrhizine. Palmatine was shown to have anticholinesterase activity. Jatrorrhizine reduces spontaneous activity of mice and prolongs the animal sleep elicited by pentobarbital. It also induces sleep in mice given sub threshold doses of pentobarbital.[5] Berberine, an isoquinoline alkaloid presents in numerous species of genera Berberis. It has a wide range of pharmacological and biological activities, including anti-inflammatory, antimicrobial and anti-tumour.[6-11] A review has also been published by us on berberis genus showing its importance.[12]

Though, *B. aristata* is prescribed in several traditional pharmaceutical preparations, but lack of technological inputs

Address for correspondence:
Rashmi, Chemistry Division, Forest Research Institute,
P. O. New Forest, Dehradun-248006.
Phone no.- 91-135-2752671
Fax no.- 91-135-2756865
E-mail: reshmi233@rediffmail.com, rashmi@icfre.org

DOI: 10.5530/pj.2011.19.8

to identify and define molecular landscape of potentially bioactive compounds and their quantification bearing this medicinal plant is missing. In this paper, standardization of berberine alkaloid by High Performance Thin Layer Liquid Chromatography technique that identifies, defines and quantifies active molecular fingerprints has been attempted. This approach may serve as a simple, genuine and appropriate technique to quantify berberine in *B. aristata* for further therapeutic exploitation.

EXPERIMENTAL

Plant Material

The plant was identified and authenticated by Head, Botany Division, Forest Research Institute, Dehradun, India. Roots of *B .aristata* were collected from six different places of Himachal Pradesh. About 2kg of root samples were collected from each place randomly from 2 or 3 mature plants. The collected root samples were shade dried and powdered.

Preparation of sample solution

Powdered root material was extracted with methanol by using soxhlet apparatus and extract was concentrated to small volume on water bath at 100^{0}C. The solvent was completely removed on a flash evaporator and yield of the extract was determined on the moisture free basis of root weight. Yield of methanol extracts of roots was found 9.60%. On Co-TLC with berberine standard, the methanol extract of *B .aristata* gave similar spots.

Reagents and standard

n-Propanol and formic acid used were of analytical grade. Standard berberine was procured from Sigma Aldrich, Germany.

Preparation of standard

240 mg of standard berberine was dissolved in 100 ml of methanol (AR grade). 500 mg of the dried methanol extracts of powdered root samples were dissolved in 5 ml of methanol and 5 µl of each sample were used.

HPTLC method for the estimation of berberine

The methanol extracts of roots of *B .aristata* was subjected to HPTLC analysis and a method was developed and standardized to obtain the quantitative yield of marker compound berberine in the extract. The conditions of HPTLC analysis of root methanol extract are as follows:

Instrumentation

A CAMAG HPTLC system comprising LINOMAT5_110922 sample applicator and TLC SCANNER 3 controlled by WIN CATS software v 1.3.4 was used for sample application and quantitative estimation.

Procedure

A number of solvent systems were tried for methanol extract. A good separation was observed in the solvent system: n-Propanol: Formic acid: Water (9: 0.1: 0.9 v/v/v). Samples were applied on precoated silica gel 60F_{254} GLP (E.Merck) (20x10 cm). Along with this varying concentration of berberine standard from 2 µl to 8µl were also applied on TLC plates from about 1 cm edge using a band length of 8 mm. The chromatogram was developed in a twin trough chamber upto a distance of 80 mm and slit dimensions was 6.0x0.45 mm

Detection of Spots

The plate was taken out, air dried and it was viewed in ultra violet radiation to mid day light. The chromatogram was scanned at 350 nm in fluorescence mode (figure 1). The R_f values and fingerprint data were recorded by WIN CATS software. The amount of berberine was determined by plotting a calibration curve between concentration and peak areas of berberine standard (figure 2).

RESULTS AND DISCUSSION

Standard berberine showed single peak in HPTLC chromatogram. The calibration curve of berberine (figure 1) was prepared by plotting the concentration of berberine versus average area of the peak over the ranges of 2 µl to 8 µl/spot. The correlation co-efficient was found to be linear. Amount of berberine in the sample (methanol extract of *B .aristata*) was computed from calibration curve (figure 3 and figure 4). Satisfactory resolution was found in solvent system n-propanol: formic acid: water (9: 0.1: 0.9 v/v/v). The R_f values obtained were calculated through WINCATS HPTLC software supplied with the instrument. The R_f value of marker compound (berberine) was found to be 0.26. On spectral assignment we observed that all the spectra appeared at 0.26 are of same type and it shows the uniformity of compound.

CONCLUSION

The proposed HPTLC method was found to be rapid, simple and accurate for quantitative estimation of berberine in *Berberis aristata* roots extract. The results of linearity range and correlation coefficient show that within the concentration

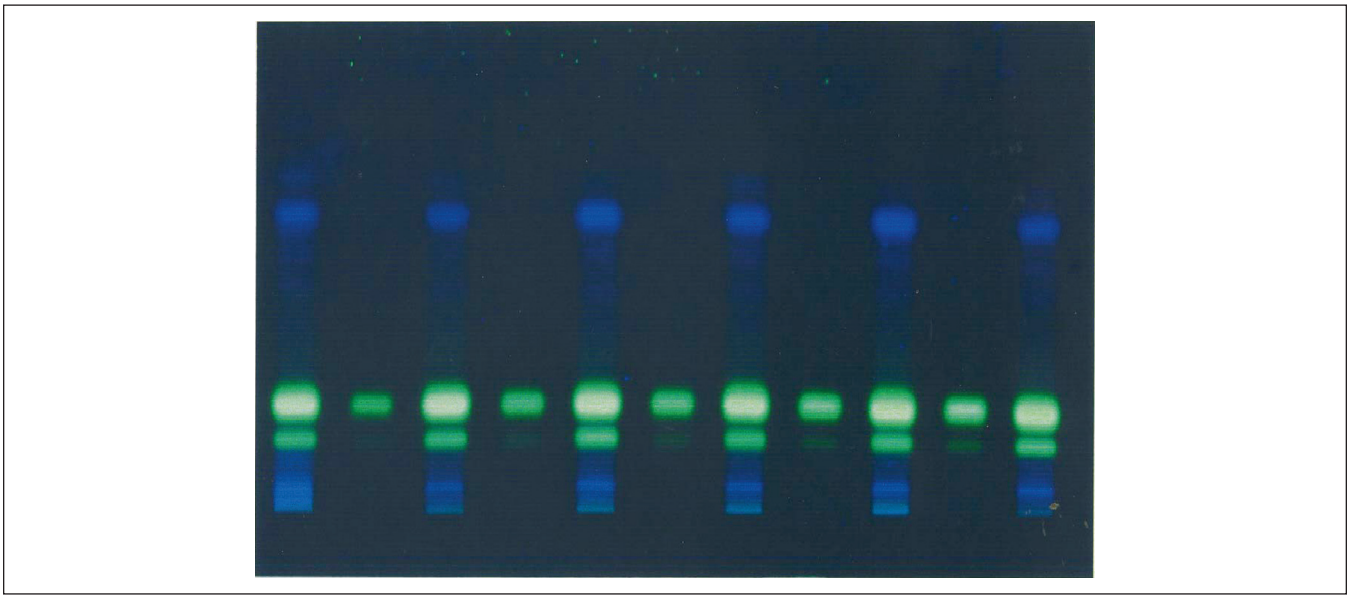


Figure 1: Developed TLC plate

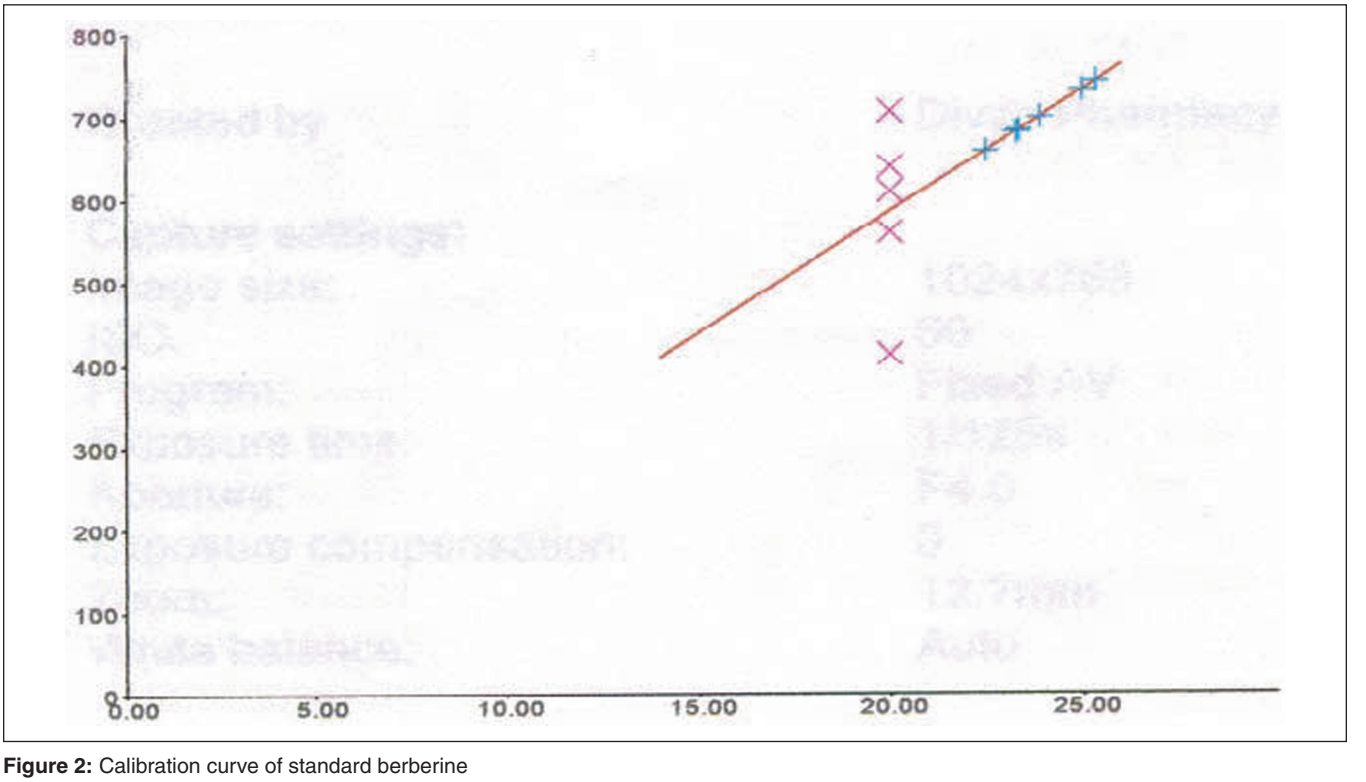


Figure 2: Calibration curve of standard berberine

range, a good correlation between peak area and corresponding concentration of berberine. HPTLC fingerprinting profile is very important parameter of herbal drug standardization for the proper identification of medicinal plants. This parameter can also be a very important tool if some adulteration is suspected in plant material. The present HPTLC fingerprinting profile can be used as a diagnostic tool to identity and to determine the quality and purity of the plant material in future studies. This method allows reliable identification and quantification of marker compound berberine in *Berberis aristata* DC.

ACKNOWLEDGEMENT

The authors are thankful to Department of Biotechnology, New Delhi for providing financial support.

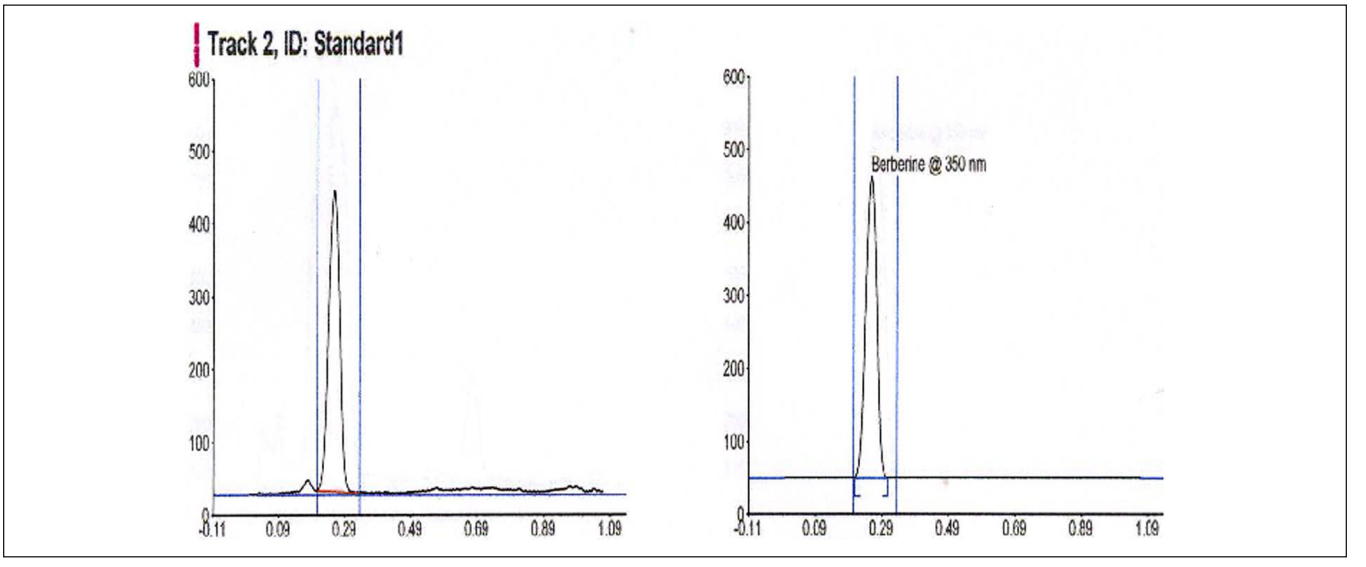


Figure 3: Chromatogram of Standard

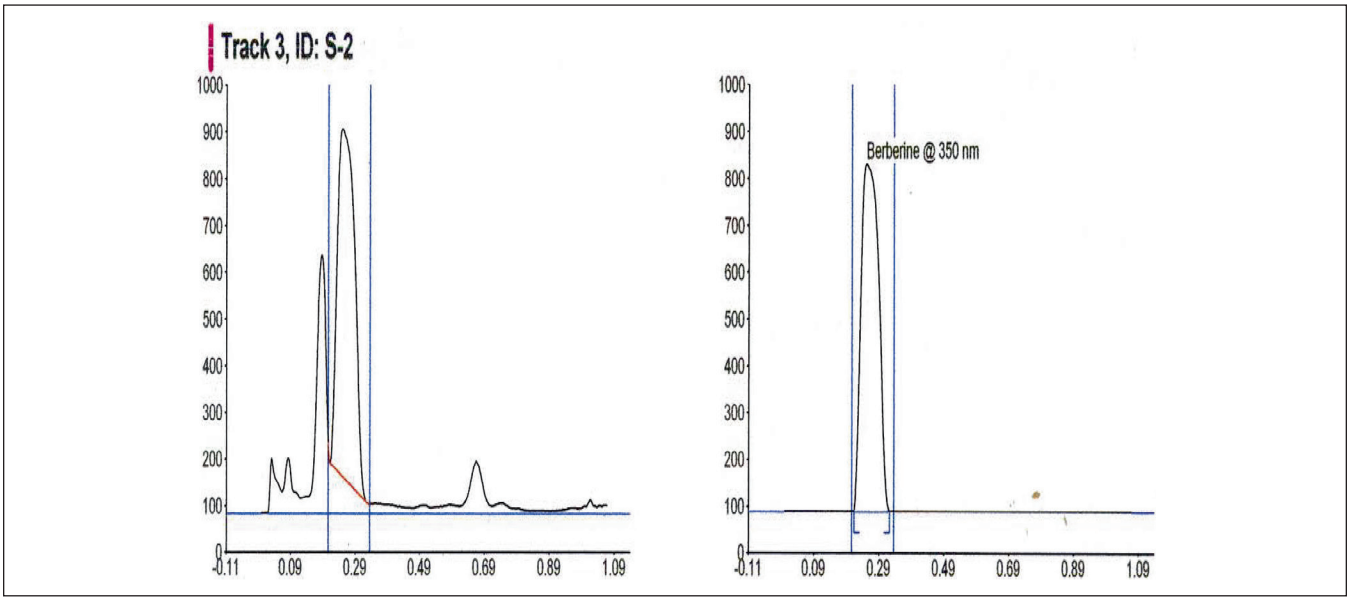


Figure 4: Chromatogram of Sample

REFERENCES

1. Pulok K M. Quality control of herbal drugs. 2nd ed. New Delhi: Business Horizons; 2007.
2. CSIR. *The Wealth of India:* Raw materials. New Delhi: Publications and Information Directorate; 1988; 2 B.
3. Atta-ur-Rahman and Ansari A. A. *Journal of Chemical Society Pakistan* 1983; 5:283.
4. Gilani A.U.H. and Janbaz K.H. *Phytotherapy Research* 1995; 9 (7):489- 494.
5. Chang, H. M. and Butt, P.P.H. *Pharmacology and Applications of Chinese Materia Medica*, (World Scientific, Singapore, USA) 1987; vol. 2.
6. Akhter, M. H., Sabir M. and Bhide, N. K., Anti-inflammatory effect of berberine in rats injected locally with cholera toxin. *Indian J Med Res.* 1977; 65:133-141.
7. Li, S.Y., Ling, H., Teh, B.S., Seow, W. K. and Thong, Y. H., Anti-inflammatory and immunosuppressive properties of the bis-benzylisoquinolines: *In vitro* comparisons of tetrandrine and berbamine. *Int J Immunopharmacol.* 1989; 11:395-441.
8. The, B.S., Seow, W. K., Li, S. and Thong, Y. H., Inhibition of prostaglandin and leukotriene generation by the plant alkaloids tetrandrine and berbamine. *Int J Immunopharmacol.* 1990; 12:321- 326.
9. Kondo,Y. Imai,Y. Hojo, H., Hashimoto Y. and Nozoe, S., Selective inhibition of T-cell-dependent immune responses by bis-benzylisoquinoline alkaloids *in vivo. Int J Immunopharmacol.* 1992; 14:1181-1186.
10. Seow, W.K., Ferrante, A., Summors, A. and Thong, Y. H. Comparative of tetrandrine and berbamine on the production of the inflammatory cytokines interleukin-1 and tumor necrosis factor. *Life Science.* 1992; 50:53-58.
11. Ivanovska, N., Philipov, S., Study on the anti-inflammatory action of *Berberis vulgaris* root extract alkaloid fractions and pure alkaloid. *Int J Immunopharmacol*. 1996; 18:553-561.
12. Rashmi, Rajasekaran, A., Pant, J. The Genus Berberis Linn.: A Review. *Pharmacognosy Reviews* 2008; 2 (4):369-385.

Hypoglycaemic Activity of Seed Extract of *Clitoria ternatea* Linn in Streptozotocin- Induced Diabetic Rats.

Bandaru Venkata Kalyan*, Hariprasath Kothandam, Venkatesh Palaniyappan, Akula Raghu Praveen.

Sir C.R.Reddy college of Pharmaceutical Sciences, Eluru-534007, Andhra Pradesh, India.

ABSTRACT

Introduction: Traditional plant medicines are used throughout the world for a range of diabetic complication. The evaluation of phytochemical is the logical way of searching for the new drugs to treat diabetes. The leaves and flowers of *Clitoria ternatea* have been reported for antidiabetic activity, hence an attempt was made to evaluate the antidiabetic potential of seeds of *Clitoria ternatea.* **Methods:** Ethanol extract of seeds of *Clitoria ternatea* Linn was subjected to preliminary phytochemical investigations. The seed extracts at two dose levels like 200mg and 400mg/kg body weight were screened for hypoglycaemic activity in Streptozotocin induced diabetic rats (60mg/kg, i.p.). **Results:** Ethanol extract showed the presence of various phytoconstituents viz. sterols, alkaloids, glycosides, saponins, tannins, carbohydrates, proteins, phenolic compounds and flavonoids. The ethanol extract at 400mg/kg.b.wt dose showed significant decreased blood glucose ($p < 0.001$), cholesterol ($p < 0.05$), alkaline phosphatase ($p < 0.001$), aspartate amino transferase ($p < 0.001$) and alanine amino transferase ($p < 0.001$), when compared to diabetic control. **Conclusion:** Further study is required to isolate active phytoconstituents from ethanolic extract of seeds of *Clitoria ternatea* Linn.

Keywords: *Clitoria ternatea* Linn, Antidiabetic, Streptozotocin.

INTRODUCTION

Traditional plant medicines are used throughout the world for a range of diabetic complication. The study of such medicine offers a natural key to unlock a diabetologist pharmacy for the future. Plant based medicines have enormous therapeutic potential with simultaneous mitigation of many of the side effects that are often associated with synthetic antidiabetic drugs. So the evaluation of phytochemical is the logical way of searching for the new drugs to treat diabetes[1].

Clitoria ternatea Linn is a plant species belonging to the Fabaceae family and it is native to tropical equatorial Asia. It is a perennial herbaceous plant. Its leaves are elliptic and obtuse. It grows as a vine or creeper, doing well in moist neutral soil. The most striking feature about this plant is its vivid deep blue flowers. They are solitary, with light yellow markings. They are about 4 cm long by 3 cm wide. There are some varieties that yield white flowers. The fruits are 5-7 cm long, flat pods with 6 to 10 seeds in each pod.[2]The roots of *Clitoria ternatea* have been reported for activities like Antidiarrhoeal[3], antipyretic[4], Antihelmintic.[5] The leaves and flowers of *Clitoria ternatea* have been reported for antidiabetic activity[6], Antimicrobial activity[7]. Phytoconstituents Malonylated flavonol glycosides[8], Anthocyanins[9], ternatin[10], Acylated anthocyanins[11], Acylated delphindin glycosides and flavonols[12], Taraxerol[13], Taraxerone[14] were isolated from *Clitoria ternatea* flowers. Oil content and Fatty acid composition[15,16] were studied in seeds of *Clitoria ternatea.*

Hence an attempt was made to evaluate the antidiabetic potential of seeds of *Clitoria ternatea.*

Address for correspondence:
B. Venkata Kalyan,
Sir C. R. Reddy College of Pharmaceutical Sciences,
Eluru-534007, Andhra Pradesh, India.
Phone No: 09704572458
E-mail: b_v_kalyanpharma@yahoo.com

DOI: 10.5530/pj.2011.19.9

MATERIALS AND METHODS

Plant Collection

The seeds of the plant *Clitoria ternatea* were collected during the month of June 2009 from Rajahmundry, Andhra Pradesh, India and was identified and authenticated in Department of Botany, Acharaya Nagarjuna University, Guntur, Andhra Pradesh, India.

Extraction procedure

The seeds of *Clitoria ternatea* was dried at room temperature, the dried seed material was powdered mechanically. Around 500gm of finely powdered seeds were extracted by cold maceration method with aqueous ethanol (30:70) for one week with occasional shaking. After extraction the solvent was distilled off and extract was concentrated on water bath to a dry residue and dried in a dessicator.

Phytochemical Screening

The ethanolic extract was subjected to qualitative phytochemical investigation for the identification of the phytoconstituents viz., sterols, alkaloids, glycosides, saponins, tannins, carbohydrates, proteins, phenolic compounds and flavonoids[17].

Experimental Animals

Healthy adult Wistar rats weighing 150-220 g were used for the antidiabetic activity. The animals were housed in clean polypropylene cages and maintained in a well-ventilated temperature controlled animal house with a constant 12h light/dark schedule.

Acute toxicity studies

The acute toxicity test of the extract was evaluated in Wistar rats (150-200gm), at dose of 2000 mg/kg.b.wt (p.o.). The treated animals were monitored for 14 days, for mortality and general behaviour. No death was observed till the end of the study. The test samples were found to be safe up to the dose of 2000 mg/kg, and, from the results, 200 mg/kg and 400 mg/kg dose were chosen for further experimentation.

Antidiabetic screening

For experiment overnight fasted Wistar rats was induced by a single intraperitoneal administration of Streptozotocin (60 mg/kg.b.wt) in 0.1 M citrate buffer, pH 4.5. Those animals with fasting blood glucose level more than 300 mg/dl after Streptozotocin administration were selected for the study and they were divided into four groups of six animals each.

Group I served as diabetic control and received 0.3% CMC,

Group II served as positive control and received glibenclamide (10 mg/kg.b.wt),

Groups III and IV received the ethanolic extract (200 mg/kg.b.wt and 400 mg/kg.b.wt respectively). The treatment was continued for fourteen days, orally, once daily. After the treatment period the blood glucose level, cholesterol and triglyceride were estimated. Blood samples were collected by orbital sinus puncture under mild ether anaesthesia and blood glucose was estimated by electronic glucometer (Accu-Check active).

For the estimation of cholesterol, triglyceride, alkaline phosphatase (ALP), aspartate amino transferase (AST) and alanine amino transferase (ALT), the blood samples were collected in Eppendroff's tubes (1 ml) containing 50 µl of anticoagulant (10% trisodium citrate) and plasma was separated by centrifuging at 6000 rpm for 15 min and analyzed in Autoanalyzer Microlab 200 using Ecoline-kits (E merck).

Statistical analysis

Values are expressed as mean ± standard error mean (SEM) and analyzed using statistical package for social sciences (SPSS) version 7.5 using ANOVA followed by Dunnett's test. P values < 0.001 were considered significant.

RESULTS AND DISCUSSION

The qualitative phytochemical evaluation of Ethanolic extract showed the presence of sterols, alkaloids, glycosides, saponins, tannins, carbohydrates, proteins, phenolic compounds and flavonoids.

Streptozotocin is a nitrosourea compound produced by *streptomyces achrogenes* which induce DNA strand breakage in β-cells leads to insulin deficiency. Insulin deficiency leads to various metabolic disorders viz. increased blood glucose, cholesterol, alkaline phosphatase, aspartate amino transferase and alanine amino transferase[18]. Oral treatment of 200mg/kg.b.wt and 400 mg/kg.b.wt. showed decreased blood glucose, cholesterol, alkaline phosphatase, aspartate amino transferase and alanine amino transferase. But 400mg/kg.b.wt dose showed significant decreased blood glucose ($p < 0.001$), cholesterol ($p < 0.05$), alkaline phosphatase ($p < 0.001$), aspartate amino transferase ($p < 0.001$) and alanine amino transferase ($p < 0.001$), when compared to diabetic control (Table-1 and 2).

CONCLUSION

From the results it indicates ethanolic seed extract of *Clitoria ternatea* showed potent antidiabetic activity but it is dose dependent. Flavonoids, proteins and saponins have been reported to possess significant anti-diabetic activity and antilipidemic activity.[19,20] *Clitoria ternatea* Linn seed extract showed the presence of flavonoids, proteins and saponins; hence the activity of the plant may be due to this phytoconstituents. However, further study is required to isolate active phytoconstituents from Ethanolic extract.

Table 1: Effect of Ethanolic seed extract of *Clitoria ternatea* on blood glucose level.

		Glucose level		
Group	**Dose**	**Normal**	**After Streptozotocin**	**After Treatment**
Control	0.3% CMC	93.64 ± 4.25	312.16 ± 12.04	338.10 ± 10.12
Glibenclamide	10 mg/kg.b.wt	98.34 ± 6.08	302.05 ± 8.56	102.34 ± 8.34[a]
Ethanolic extract of *Clitoria ternatea*	200mg/kg.b.wt	102.12 ± 10.12	308.46 ± 20.03	198.16 ± 15.08[b]
Ethanolic extract of *Clitoria ternatea*	400mg/kg.b.wt	88.63 ± 1.02	304.24 ± 5.08	103.64 ± 3.14[a]

Values are Mean ± SEM of Six Animals. Statistical Significance: a = p < 0.001 and b = p < 0.05 as compared to control

Table 2: Effect of Ethanolic seed extract of *Clitoria ternatea* on biochemical parameters.

Treatment	**Cholesterol (mg/dl)**	**Triglycerides (mg/dl)**	**Alkaline Phosphatase (U/L)**	**Aspartate Amino Transferase (U/L)**	**Alanine Amino Transferase (U/L)**
Control (0.3% CMC)	184.21 ± 4.08	172.46 ± 12.46	258.13 ± 16.01	134.67 ± 3.92	88.12 ± 5.86
Glibenclamide (10mg/kg.b.wt)	79.08 ± 6.20[a]	94.16 ± 6.24[a]	184.24 ± 8.12[a]	88.13 ± 10.02[a]	48.24 ± 3.93[a]
Ethanolic extract of *Clitoria ternatea* (200mg/kg.b.wt)	134.24 ± 6.42[b]	142.08 ± 12.21[b]	208.12 ± 12.34[b]	102.24 ± 8.12[b]	64.12 ± 8.12[b]
Ethanolic extract of *Clitoria ternatea* (400mg/kg.b.wt)	82.34 ± 3.04[a]	95.23 ± 02.13[b]	189.64 ± 8.32[a]	92.12 ± 5.12[a]	49.46 ± 2.96[a]

Values are Mean ± SEM of Six Animals. Statistical Significance: a = p < 0.001 and b = p < 0.05 as compared to control

REFERENCES

1. Tiwari AK, Rao JM. Diabetes mellitius and multiple therapeutic approaches of phytochemicals: present status and future prospects. Cur Sci 2002;83:30-8.
2. The Ayurvedic Pharmacopoeia of India (Part I Volume II). Ministry of Health and Family Welfare. p. 10–11.
3. Nitinkumar U, Roshan P, Naheed W, Naveen Kumar M. Evaluation of Antidiarrhoeal Activity of the Root of *Clitoria Ternatea* Linn. Int J Pharm Sci Rev Res 2010;5:131-134.
4. Parimaladevi B, Boominathan R, Subhash C. Evaluation of antipyretic potential of *Clitoria ternatea* L. extract in rats. Int J Phytother Phytopharmacol, 2004;1:121-124.
5. Khadatkar SN, Manwar JV, Bhajipale NS. In-*vitro* anthelminitic activity of root of *Clitoria ternatea*. Phcog Mag 2008;4:148-150.
6. Daisy P, Santosh K, Rajathi M. Antihyperglycemic and antihyperlipidemic effects of *Clitoria ternatea* Linn in alloxan- induced diabetic rats. Afr J Microbiol Res 2009;3:287-291
7. Babu U, Kesani P, Sadayappan R. Phytochemical Analysis and Antimicrobial activity of *Clitorea ternatea* Linn against extended spectrum beta lactamase producing enteric and urinary pathogens. Asian J Pharma Clin Res 2009;2:94-96.
8. Kazuma K. Malonylated flavonol glycosides from the petals of *Clitoria ternatea*. Phytochemistry 2003;62:229-37.
9. Terahara, N.; Eight new anthocyanins, ternatins C1-C5 and D3 and preternatins A3 and C4 from young *Clitoria ternatea* flowers. J Nat Prod 1998;61:1361-1367.
10. Terahara, N, Saito N, Honda T, Toki K, Osajima Y. Structure of ternatin A1 the largest ternatin in the major bluce anthocyanin from *Clitoria ternatea* flowers. Tetrahedron Lett 1990;31:2921-2924.
11. Terahara, N, Saito N, Honda T, Toki K, Osajima Y. Acylated anthocyanins of *Clitoria ternatea* flowers and their acyl moieties. Phytochemistry 1990;29:949-953.
12. Saito N, Abe K, Honda T, Timberlake CF, Bridle P. Acylated delphindin glycosides and flavonols from *Clitoria ternatea*. Phytochemistry 1985;4:1583-1586.
13. Banerjee Sk, Chakravarti RN. Taraxerol from *Clitoria ternatea*. Bull Calcutta Sch Trop Med 1963;11:106-107.
14. Banerjee Sk, Chakravarti RN. Taraxerone from *Clitoria ternatea*. Bull Calcutta Sch Trop Med 1964;12:23.
15. Vianni R, Soute SM. Oil content and Fatty acid composition of *Clitoria ternatea* seeds. Arquivos Da Universidade Federal Rural Do Rio De Janeriro 1971;1:47-50.
16. Joshi SS, Shrivastava RK, Shrivastava DK. Chemical examination of *Clitoria ternatea* seeds. J Am Oil Chem Soc 1981;55:714-715.
17. Kokate CK, Purohit AP, Gokhale SB. Text Book of Pharmacognosy. 7th ed. Nirali Prakashan (Publ); 1997. p.105-144.
18. Shanmugasundaram KR, Panneerselvam C, Samudram P, Shanmugasundaram ER. Enzymes changes and glucose utilisation in diabetic rabbits, the *Gymnema sylvestrae*. J Ethnopharmacol 1983;7:205-234.
19. Rajnarayana K, Sripal Reddy M, Chaluvadi MR, Krishna DR. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. Indian J Pharmacol 2001;33:2.
20. J.S.Choi, O.H.Yokozawa; Hypolipidemic effect of *Prunus dadidian*. J Nat Prod 1991;54:218.

Hepatoprotective and cytotoxic activities of *Delonix regia* flower extracts

Aly M. El-Sayed[1], Shahira M. Ezzat *[1], Maha M. Salama [1] and Amany A. Sleem [2]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Kasr el-Aini street, Cairo11562, Egypt. [2]Department of Pharmacology, National Research Centre, Giza 12622, Egypt.

ABSTRACT

Fractionation of the ethanolic extract of the flowers of *Delonix regia* led to the isolation of three sterols, namely, stigmasterol (1.54 %), β-sitosterol and its 3-O-glucoside (6.93 %), a triterpene, namely, ursolic acid (3.61 %) and four flavonoids: quercetin (2.92 %), quercitrin (0.59 %), isoquercitrin (3.87 %) and rutin (5.12 %) in addition to the amino acid L- azeditine-2-carboxylic acid. The structures of the isolated compounds were established on the basis of physicochemical properties and spectral analysis (IR, UV, EI/MS, ^{1}H-NMR and ^{13}C-NMR). The concentration of the isolated compounds was determined by HPLC technique. The ethanolic extract and its non-polar and flavonoid rich fraction as well as the isolated compounds evidenced cytotoxic activities against human liver cancer cell line (HEPG2) which were potent for ursolic acid (IC_{50} 0.55 µg/ml) and L-azeditine-2-carboxylic acid (IC_{50} 2.51 µg/ml). Meanwhile, rutin and isoquercitrin were inactive. Moreover, the ethanolic extract and its two fractions were tested for hepatoprotective activity against CCl_4 induced hepatic cell damage in rats at two dose levels (50 and 100 mg/kg), and the flavonoid rich fraction showed statistically significant hepatoprotection at 100 mg/kg. The presence of the aforementioned flavonoids with their efficient free radical scavenging properties may explain this liver protection ability. This could suggest the use of the ethanolic extract of the flowers of *D. regia* as a chemopreventive agent against the two main causes of liver damage; liver toxicity by chlorinated agents and liver cancer.

Keywords: *Delonix regia*, Hepatoprotective; Anticancer; Azeditine.

INTRODUCTION

Delonix regia (Hook.) Raf. (*Poinciana regia* Boj. ex Hook., Royal Poinciana, Gul mohar, Flame tree or Flamboyant, Fabaceae –Caesalpinioideae) is a large ornamental tree (10-18 m hight) with fern-like bipinnately compound leaves native to Madagascar and carrying the attractive red peacock flowers. It flowers in April and lasts for several months[1,2]. It is widely grown in Egypt lining the streets and gardens with its beauty, especially in Cairo, North coast and Sinai. The ethanolic extract of the flowers inhibited β- lactamase producing methicillin- resistant *Staphyllococcus aureus* (MRSA) and methicillin-sensetive *S. aurues* (MSSA) and evidenced antioxidant property[3,4]. On the other hand, the ethyl acetate extract of the flowers showed a molluscidal activity[5]. Chemically, anthocyanins were reported in the flowers[6-10]. Carotenoids were isolated from the non-polar fraction of the flowers[11], keto and imino acids were detected in the flowers of *Delonix regia*[12]. The flower extract also was reported as a useful natural colour and as an acid-base indicator[7,9].

The aim of this study is to undergo a phytochemical investigation of the flowers in an attempt to isolate the major compounds and evaluate their possible hepatoprotective and cytotoxic activity to justify their use as chemopreventive agents. Accordingly, the ethanolic extract, as well as its different fractions (rich in these compounds) are traced for the same biological activities.

Address for correspondence:
Shahira M. Ezzat, Department of Pharmacognosy,
Faculty of Pharmacy, Cairo University, Kasr el-Aini street,
Cairo11562, Egypt.
tel. 0122336716
E-mail: shahyelkomy@hotmail.com

DOI: 10.5530/pj.2011.19.10

MATERIALS AND METHODS

Plant Material

The flowers of *Delonix regia* (Hook.) Raf. were obtained from the trees growing in the North coast (at the 24th km

during spring 2007/2008. The plant was kindly identified at the Botany Department, Faculty of Science, Cairo University, Giza, Egypt. A voucher specimen (No.: D-3) is kept in the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt.

General

Authentic flavonoids, sterols and triterpenes were obtained from E. Merck, Darmstadt, Germany. Silica gel H (E-Merck, Darmstadt, Germany) for vacuum liquid chromatography (VLC), silica gel 60 (Fluka, 70-230 mesh ASTM, Germany) for column chromatography (CC) and sephadex LH 20 (Pharmacia) were used. Thin-layer chromatography (TLC) was performed on silica gel GF_{254} precoated plates (Fluka, Germany) using solvent systems S_1: *n*-hexane-ethyl acetate (90:10); S_2: *n*-hexane- ethyl acetate (80:20), S_3: chloroform-methanol (95:5) S_4: chloroform-methanol (90:10), S_5: chloroform-methanol (80:20) and S_6 ethyl acetate-methanol-water (100:16.5:13.5). The chromatograms were visualized under UV light (at 254 and 366 nm) before and after exposure to ammonia vapour and spraying with Al Cl_3, as well as after spraying with *p*-anisaldehyde-sulphuric acid, ninhydrin and Dragendorff's spray reagents[13].

Melting points (uncorrected) were determined on a D. Electrothermal 9100 (U.K.). Mass spectra were measured using Shimadzu QP-2010 Plus, 70 ev. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were measured on Varian Mercury-VX-300 NMR instrument. The NMR spectra were recorded in $CHCl_3$-d_6 and DMSO-d_6 and chemical shifts were given in δ (ppm) relative to TMS as internal standard. IR spectra were measured on Jasco FT/IR-460 plus, Japan Infrared Spectrophotometer

Extraction and Isolation

The air-dried powdered flowers of *Delonix regia* (Hook.) Raf. (500 g) were extracted by cold percolation with 95 % ethanol (5 x 3 L) till exhaustion. The ethanolic extract was evaporated under reduced pressure to give 70 g of reddish-brown residue. Forty grams of this residue was chromatographed on a VLC column (220 g silica gel H, 7 x 20 cm) using *n*-hexane, ethyl acetate and methanol. Fractions, were collected and monitored by TLC. The *n*-hexane fraction was evaporated to yield 15 g residue, representing the non-polar fraction **A**. The ethyl acetate fraction was also evaporated to yield 9 g residue representing the flavonoid rich fraction **B**. The methanol fraction **C** was similarly evaporated to yield 980 mg residue. The non polar fraction **A**, was purified over several silica gel columns using *n*-hexane-ethyl acetate mixtures as an eluent to obtain compound **1** (112 mg), compound **2** (42 mg), compound **3** (62 mg) and compound **4** (72 mg). The flavonoid rich fraction **B** was purified over successive sephadex LH-20 columns using methanol and methanol- water mixtures to afford compounds **5** (35 mg), **6** (25 mg)**, 7** (84 mg) and **8** (102 mg). The methanol fraction **C** showed one spot on TLC which reacted positively with ninhydrin and Dragendorff's reagents, and this was compound **9**.

HPLC analysis

A reversed phase column Lichrospher C_{18} (Merck, 250x4.6 mm i.d., 5μm), column temperature: 30°C, the mobile phase used for flavonoid analysis was acetonitrile-water-acetic acid (20:80:1 v/v/v), while a solvent system acetonitrile-methanol(50:50 v/v) containing 3% water (v/v) was used as a mobile phase for sterols. The flow rate was 1 ml/min. UV wavelength was 280 nm for flavonoids and 205 nm for sterols. Calculations for percentages were done considering standards and samples AUC. Sample preparation for HPLC: For sterols: the dried flowers (10 gm) were exhaustively extracted with petroleum ether (60-80°C). Unsaponifiable matters were separated from saponified petroleum ether extract. The unsaponifiable fraction was quantitatively used for sample preparation. For flavonoids: the dried flowers (10 gm) were exhaustively extracted with methanol and quantitatively used for sample preparation.

Material for biological study

Silymarin (Sedico Pharmaceutical Co., 6 October City, Egypt) and carbon tetrachloride (analar, El-Gomhoreya Co., Cairo, Egypt). Transaminase Kits (Bio-Meriéux Co.): biochemical kits for assessment of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase enzymes (ALP). Adult male albino rats of Sprauge Dawely Strain weighing 100-150 g and albino mice (20 - 25g) were used. All animals were kept on standard laboratory diet and under hygienic conditions.

Determination of LD50

The LD_{50} of the ethanolic extract of the flowers was calculated according to Karber[14].

In vitro screening for cytotoxic activity

The ethanolic extract of the flowers of *Delonix regia* (Hook.) Raf., its different fractions and the isolated compounds were tested for their cytotoxicity (at the National Cancer Institute, Cairo, Egypt) at different concentrations in DMSO (0-10 μg/ml), against human liver cancer cell line (HEPG2), according to the method of Skehan *et al*[15]. The IC_{50} values were calculated and the results are shown in Table 1.

Assessment of hepatoprotective activity

The ethanolic extract of the flowers as well as the non-polar fraction **A** and the flavonoid rich fraction **B** were tested for

Table 1. *In-vitro* Cytotoxicity of the flowers *Delonix regia (Hook.) Raf.* On human liver cancer cell line (HEPG2).

Compounds	IC_{50} (µg/well)
	HEPG2
The ethanolic extract	9.06
Fraction A	2.67
Fraction B	5.34
Compound 1	3.23
Compound 2	8.09
Compound 3	0.55
Compound 4	5.44
Compound 5	3.96
Compound 6	9.32
Compound 7	-
Compound 8	-
Compound 9	2.51
Doxorubicin®	0.67

Fraction **A,** the non polar fraction of the ethanolic extract. Fraction **B,** the flavonoid rich fraction. **Doxorubicin,** an anticancer reference drug.

their hepatoprotective activity. The tested extracts were administered at two dose levels 50 and 100 mg/kg body weight for one month before induction of liver damage by intraperitoneal injection of 5ml/kg of 25% carbon tetrachloride (CCl_4) in liquid paraffin according to the method of Klassan and Plaa[16], silymarin 25 mg / kg body weight was used as a reference drug. The extracts as well as the reference drug were continued to be administered to the rats for another month after liver damage. The levels of aspartate aminotransferase (AST)[17], alanine aminotransferase (ALT)[17] and alkaline phosphatase (ALP)[18] enzymes were measured in the blood of each group at zero time, after one month of receiving the tested drug, 72 hours after induction of liver damage and after one month of treatment with the tested samples. Results are shown in (Table 2).

RESULTS

Spectral data of the isolated compounds

Nine compounds were isolated from the ethanolic extract of the flowers of *D. regia*. The structures of the isolated compounds were established on the basis of physicochemical properties and spectral analysis (IR, UV, EI/MS, ^{1}H-NMR and ^{13}C-NMR).

Compound 1 (β-sitosterol)

White needle crystals (*n*-hexane)

m.p. 140-141°C

Rf: 0.41 in S_2

MS (EI, 70 eV): *m/z* (%) = 414 $[M]^+$ (100 %), 396 (51 %), 329 (42 %), 303 (44 %), 273 (60 %) and 255 (80 %).

^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.70 (3H, d, *J*=5.4,Me-21), 0.84 (3H, t, *J*=6.3,Me-29), 0.91 (3H, d, *J*=6.3, Me-26), 0.95 (3H,d, *J*=6.3, Me-27), 1.04 (3H, s, Me-18), 1.27 (3H, s, Me-19), 3.51 (1H, m, H-3), 5.38 (1H, br.s., H-6) ppm.

Compound 2 (Stigmasterol)

White needle crystals (*n*-hexane)

m.p. 169-170°C.

Rf 0.41 in S_2.

EIMS (70 eV rel. int.), m/z at 412 $[M]^+$ (100 %), 399 (43 %), 396 (65 %), 369 (73 %), 329 (17 %), 271 (26 %) and 255 (52 %).

^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.69 (3H, d, *J*=5.4 Hz, Me-21), 0.80 (3H, t, *J*=6.3, Me-29), 0.89 (3H, d, *J*=6.4 Hz, Me-26), 0.92 (3H, d, *J*=6.3 Hz, Me-27), 1.04 (3H, s, Me-18), 1.27 (3H, s, Me-19), 3.49 (1H, m, H-3), 5.15 (dd, 1H, J=8.3, 15.4, H-22), 5.21 (1H, dd, *J*=8, 15.2, H-23) and 5.4 (1H, br.s., H-6) ppm.

Compound 3 (Ursolic acid)

White microcrystalline powder.

m.p. 288-290°C.

Rf 0.39 in S_2.

EIMS: (70 eV rel. int.), m/z at 456 $[M]^+$ (12 %), 438 (33 %), 411 (62 %), 248 (100 %), 208 (42 %), 203 (31 %) and 190 (37 %).

^{1}H-NMR: δ (300 MHz, DMSO) 0.68 (3H, s,Me-25), 0.75(3H, s,Me-24), 0.80 (3H, d, *J*=6.6,Me-29), 0.86 (3H, s,Me-26), 0.91 (3H, s,Me-27), 0.91 (3H, d, *J*=6.6,Me-30), 1.04 (3H, s,Me-23), 3 (1H, m, H-18), 4.25 (1H, d, *J*=5.4,H-3) and 5.13 (1H, br. s, H-12) ppm.

^{13} C-NMR: δ (75 MHz, DMSO) 15.76(C-24), 16.68(C-25), 17.32(C-26), 18.09(C-29), 21.24(C-30), 23.06(C-11), 23.43(C-27), 24.26(C-16), 28.22(C-2), 28.30(C-15), 28.75(C-23), 30.45(C-21), 33.39(C-7), 36.67(C-22), 38.13(C-10), 38.44 (C-1), 38.77 (C-4), 38.95(C-20), 39.05 (C-19), 40.88(C-8), 41.39(C-14),47.02(C-17), 51.23(C-18), 55.87(C-5), 77.44(C-3), 125(C-12), 138.92 (C-13) and 179.16(C-28).

Table 2. Effect of *Delonix regia (Hook.) Raf.*on the serum AST, ALT and ALP level on adult main albino rats.

		AST (U/L)				ALT (U/L)				ALP (KAU)			
Group	**Dose**	**Zero**	**30 days**	**72h**	**30 days**	**Zero**	**30 days**	**72h**	**30 days**	**Zero**	**30 days**	**72h**	**30 days**
Control		29.6 ± 0.9	29.2 ± 0.8	139.8 ± 5.3*	151.4 ± 6.9*	33.6 ± 0.7	31.9 ± 0.8	163.7 ± 6.2*□	179.5 ± 8.1*	6.2 ± 0.1	6.3 ± 0.1	35.1 ± 1.1*□	39.8 ± 1.4*•
Silymarin	25 mg/kg	31.8 ± 0.9	30.4 ±0.4	42.6±1.3*□	27.3 ± 0.7*	29.1 ± 0.9	26.3 ± 0.6	37.5 ± 1.4*□	24.8 ± 0.5*•	7.3 ± 0.1	6.9 ± 0.1	10.5 ± 0.8*□	7.0 ± 0.8*•
Ethanolic extract	50 mg/kg	34.1 ± 1.3	33.5 ± 1.2	98.2 ± 3.1	71.4 ± 2.7	31.1 ± 1.2	29.8 ± 0.9	79.2 ± 3.1	52.7 ± 2.9	7.2 ± 0.1	6.8 ± 0.1	29.4 ± 0.9	22.3 ± 0.4
A		33.5 ± 1.2	32.9 ± 1.1	88.9 ± 3.2	76.8 ± 2.6	29.7 ± 0.6	29.4 ± 0.5	81.3 ± 2.6	59.9 ± 1.7	7.1 ± 0.1	6.9 ± 0.1	36.8 ± 1.5	29.7 ± 0.8
B		33.8 ± 1.2	33.1 ± 0.8	73.4 ±	64.3 ± 2.4	30.5 ± 0.9	30.1 ± 1.1	68.9 ± 2.4	58.2 ± 1.4	6.8 ± 0.1	7.1 ± 0.1	31.6 ± 1.1	26.6 ± 0.5
Ethanolic extract	100 mg/kg	31.4 ± 1.1	31.1 ± 1.3	73.6 ± 2.9*□	41.8 ± 1.7*	29.5 ± 0.9	29.1 ± 0.7	58.3 ± 2.1*□	39.8 ± 1.4*•	7.2 ± 0.1	7.3 ± 0.1	19.5 ± 0.9*□	11.7 ± 1.1*•
A		27.3 ± 0.6	27.1 ± 0.9	61.2 ± 2.4*□	41.8 ± 2.3*•	29.8 ± 0.7	28.2 ± 0.4	71.4 ± 2.3*□	43.6 ± 1.3 *•	7.1 ± 0.1	6.8 ± 0.1	25.2 ± 0.6*□	20.4 ± 0.9*•
B		31.6 ± 1.2	30.4 ± 1.1	56.8 ± 1.6*□	39.4 ± 1.2*•	28.9 ± 0.8	29.1 ± 0.7	51.5 ± 1.9*□	49.3 ± 1.2*•	7.3 ± 0.1	7.1 ± 0.1	19.2 ± 0.3*□	14.6 ± 0.5*•

Fraction **A,** is the non polar fraction of the ethanolic extract, Fraction **B,** is the flavonoid rich fraction.
*Statistically significant from zero time $p < 0.01$. • Statistically significant from 72 hours after ccl_4 at $p < 0.01$.
□ Statistically significant from the control group at72 hours after ccl_4 (>20% difference).

Compound 4 (β-sitosterol-3-O-β-D-glucopyranoside)

White microcrystalline powder.

m.p. 290°C.

Rf 0.37 in S_3.

MS (EI, 70 eV): m/z (%) = 414 $[M]^+$ (100 %), 396 (46 %), 329 (36 %), 303 (39 %), 273 (66 %) and 255 (65 %).

^{1}H-NMR: δ (300 MHz, DMSO) 0.66 (3H, d, *J*=5.5 Hz, Me-21), 0.78 (3H, t, *J*=6.3,Me-29), 0.83 (3H, d, *J*=6.2 Hz, Me-26), 0.90 (3H, d, *J*=6.3 Hz, Me-27), 0.92 (3H, s, Me-18), 0.96 (3H, s, Me-19), 3.03 (1H, m, H-3), 4.21 (1H, d, *J*=7.5, H-1`), 5.33 (H, br.s, H-6) ppm.

Compound 5 (Quercetin)

Yellow microcrystalline powder.

m.p. 314-316 °C.

Rf 0.45 in S_4.

UV λ_{max} nm (MeOH): 256, 272 (sh.), 302 (sh.), 372; NaOMe: 248 (sh.), 332; $AlCl_3$: 272, 308 (sh.), 335, 450; $AlCl_3$/HCl: 268, 304 (sh.), 360, 428; NaOAC: 272, 320, 406; NaOAC/H_3BO_3: 260, 306 (sh.), 386.

^{1}H-NMR δ ppm (300 MHz, DMSO) 7.76(1H, d, *J*=1.2 Hz, H-2'), 7.53(1H, d, *J*= 8.1 Hz, H-6') 6.88(1H, d, *J*=8.4Hz, H-5'), 6.41(1H, d, *J*= 1.8Hz, H-8), 6.19 (1H, d, *J*=1.8 Hz, H-6).

^{13}C-NMR δ ppm (125 MHz, DMSO) 175.8 (C-4), 164.0 (C-7), 160.6(C-5), 156.1(C-9), 156.0(C-2), 147.7 (C-4`), 145.0(C-3'), 135.7(C-3), 121.9 (C-1'), 119.9(C-6'), 115.6(C-5'), 115.0 (C-2'), 102.9 (C-10), 98.2 (C-6), 93.3 (C-8).

Compound 6 (Quercetin-3-O-α-L-rhamnoside)

Yellow microcrystalline powder.

m.p. 182-184□C.

Rf 0.7 in S_6.

UV λ_{max} nm (MeOH): 257, 362 ; NaOMe: 272, 327sh, 409; $AlCl_3$: 275, 305sh,438; $AlCl_3$/HCl: 270, 350sh, 395; NaOAC: 273, 324sh,380; NaOAC/H_3BO_3: 262, 298sh,377.

^{1}H-NMR δ ppm (300 MHz, DMSO) 7.60 (1H, d, J=2.1Hz, H-2'), 7.54 (2H,dd, J=2.1& 8Hz, H-2',6`), 6.71(1H, d, J=8.4Hz, H-5'), 6.30(1H, broad singlet, H-8), 6.1 (1H, broad singlet,H-6), 5.41(1H, d, J =1.2 Hz, H-1"), 1.1(3H, d, J =6.3Hz, Me).

^{13} C-NMR δ ppm (125 MHz, DMSO) 177.4 (C-4), 164.0 (C-7), 161.2(C-5), 157.0(C-9), 156.4(C-2), 148.6 (C-4`), 144.8(C-3'), 134.4(C-3), 121.5 (C-1'), 121.0(C-6'), 115.8(C-5'), 115.2 (C-2'), 103.7 (C-10), 101.9 (C-1``), 98.9 (C-6), 93.6 (C-8), 71.5 (C-4``), 70.1 (C-5``),70.5(C-3``),70.4(C-2``), 17.3(C-6``).

Compound 7 (Quercetin-3-O-β-D-glucopyranoside)

Yellow microcrystalline powder.

m.p. 242-243°C.

Rf 0.55 in S_6.

UV λ_{max} nm (MeOH): 257, 362 ; NaOMe: 272, 327sh, 409; $AlCl_3$: 275, 305sh,438; $AlCl_3$/HCl: 270, 350sh, 395; NaOAC: 273, 324sh,380; NaOAC/H_3BO_3: 262, 298sh,377.

^{1}H-NMR δ ppm (300 MHz, DMSO) 7.61 (1H, d, *J*=2.1 Hz, H-2'),7.55(2H, dd, *J*=2.1&8.4Hz, H-2',6`), 6.83(1H, d, *J*=8.4Hz, H-5'), 6.39(1H, d, *J*=1.2Hz, H-8), 6.18 (1H, d, *J*=1.2Hz,H-6), 5.41(1H, d, *J* =6.9Hz, H-1").

^{13} C-NMR δ ppm (125 MHz, DMSO) 177.5 (C-4), 164.0 (C-7),161.2(C-5), 156.5(C-9), 156.4(C-2), 148.4 (C-4`), 144.8(C-3'), 133.9(C-3), 121.4 (C-1'), 121.6(C-6'), 116.5(C-5'), 115.2 (C-2'), 104.2 (C-10), 101.4 (C-1``), 98.8 (C-6), 93.6 (C-8), 77.5 (C-5``),76.8(C-3``),74.3(C-2``), 68.0 (C-4``), 61.3(C-6``).

Compound 8 (Quercetin-3-O-rhamnoglucoside)

Yellow microcrystalline powder.

m.p. 190-192°C.

Rf 0.42 in S_6.

UV λ_{max} nm (MeOH): 258, 300sh, 358 ; NaOMe: 268, 328sh, 410; $AlCl_3$: 270, 306sh, 426; $AlCl_3$/HCl: 268,298sh, 366, 400; NaOAC: 264, 300sh, 382; NaOAC/H_3BO_3: 262, 308sh, 378. ^{1}H-NMR δ ppm (300 MHz, DMSO) 7.70(1H, d, *J*=2.1 Hz, H-2'), 7.55 (2H, dd, *J*=2.1&8.4Hz, H-2',6`), 6.82(1H, d, *J*=9Hz, H-5'), 6.34 (1H, d, *J*= 2.1Hz, H-8), 6.07(1H, d, *J*=2.1Hz, H-6), 5.33(1H, d, *J*=7.5Hz, H-1"), 4.38(1H, d, *J*=1.2 Hz, H-1""), 1.0(3H, d, *J* =6.3Hz, Me).

^{13} C-NMR δ ppm (125 MHz, DMSO) 177.4 (C-4), 164.0 (C-7), 161.1(C-5), 156.5(C-9), 156.4(C-2), 148.4 (C-4`), 144.7(C-3'), 133.6(C-3), 121.5 (C-1'), 121.1(C-6'), 116.2(C-5'), 115.2 (C-2'), 103.8 (C-10), 101.2 (C-1``), 100.7 (C-1```),

98.7 (C-6), 93.6 (C-8), 76.1 (C-5``),76.4(C-3``),72.1(C-4```),74.0(C-2``), 70.2 (C-4``), 70.8 (C-3```), 70.4(C-2```), 68.2(C-5``), 67.0(C-6``), 17.6(C-6```).

Compound 9(L-azeditine-2-carboxylic acid)

Oily yellow liquid.

Rf 0.38 in S_6.

IR(KBr)cm^{-1}: 3450 (N-H) 2600-3000 broad band(OH), 1710 (C=O), 2950, 2860, (C-H).

^{1}H -NMR δ ppm (300 MHz, DMSO) 1.39 (1H, m, H-3), 1.65 (1H, m, H-3), 2.91-3.1(2H, m,H-4), 4.49 (1H,t, H-2), 3.51 (1H,t,N-H), 5.3 (s, 1H,O-H).

^{13}C NMR δ ppm (75 MHz, DMSO) 177.6 (C=O), 60 (C-2), 43.1(C-4), 25.5(C-3).

HPLC analysis

The percentage of the isolated compounds were determined byHPLC analysis and calculated with respect to the dry weight of the plant. β-sitosterol 6.93 %**,** stigmasterol 1.43%, ursolic acid 3.61 %, β-sitosterol-3-O-β-D-glucopyranoside, quercetin 2.92 %, quercetin-3-O-α-L-rhamnoside (quercitrin) 0.59 %, quercetin-3-O-β-D-glucopyranoside (Isoquercitrin) 3.87 % and quercetin-3-O-rhamnoglucoside (rutin) 5.12 %.

THE BIOLOGICAL STUDY

Determination of LD50

The acute toxicity study of the ethanolic extract of the flowers of D. regia revealed no mortality up to a dose level of 7.5 g/kg b.w. thus the anticancer and hepatoprotective activities were assessed.

DISCUSSION

Delonix regia (Hook.) Raf. Flowers contain variety of sterols, triterpenes and flavonoids to be first reported. The isolated compounds were β-sitosterol (**1**)**,** stigmasterol (**2**), ursolic acid (**3**), β-sitosterol-3-O-β-D-glucopyranoside (**4**), quercetin (**5**), quercetin-3-O-α-L-rhamnoside (quercitrin) (**6**), quercetin-3-O-β-D-glucopyranoside (Isoquercitrin) (**7**) and quercetin-3-O-rhamnoglucoside (rutin) (**8**). Their structures were confirmed by comparing their chromatographic, chemical and spectroscopic data (EI/MS, UV, ^{1}H-NMR and ^{13}C-NMR) with the published data[19-29]. The above compounds were not traced in the literature for the genus *Delonix*. Compound **9** was identified as L-azeditine-2-carboxylic acid, its structure was confirmed by comparing its chromatographic data (IR, ^{1}H-NMR and ^{13}C-NMR) with that reported in the literature[30]. Compound **9** was isolated before from the legumes of the same plant[31]. The concentrations of the isolated compounds were determined using HPLC technique it was noticed that β-sitosterol and

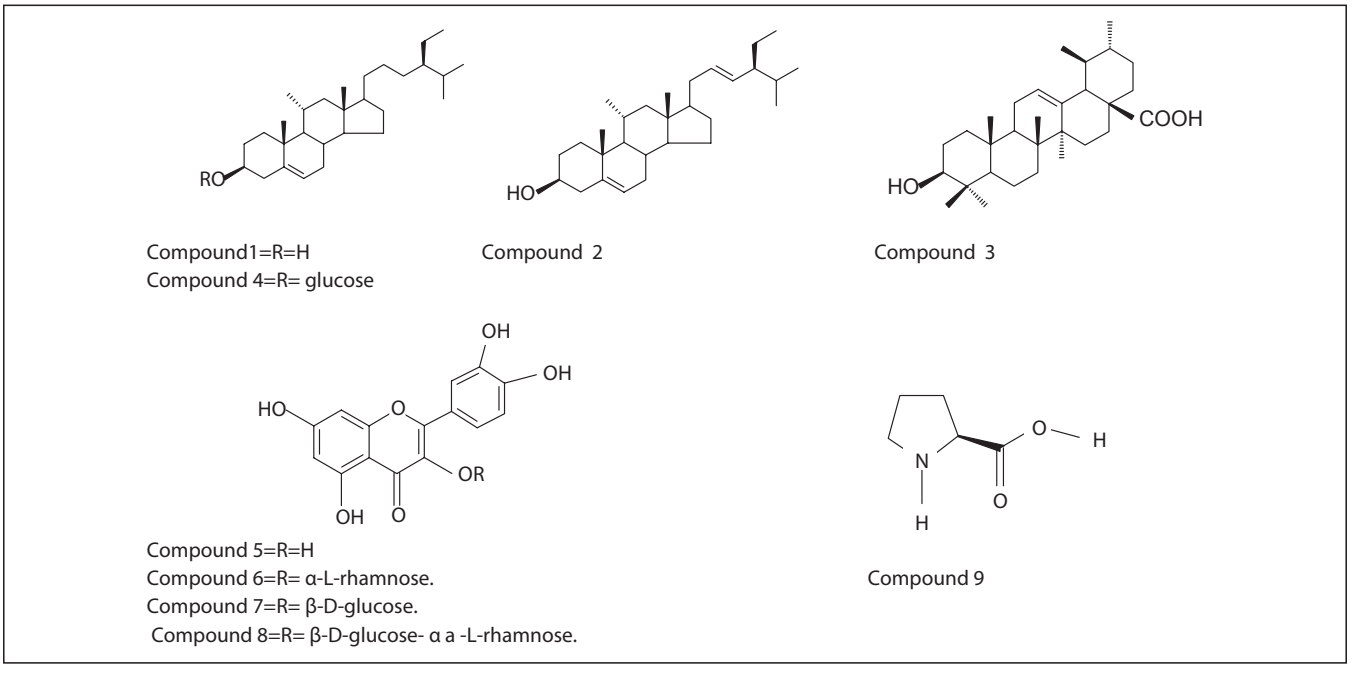


Figure 1. Structures of the isolated compounds

quercetin-3-O-rhamnoglucoside (rutin) were the major compounds in the flowers (6.93 % and 5.12 % , respectively).

As previous studies had shown that chlorinated hydrocarbons had been demonstrated to induce oxidative stress (imbalance in the pro-oxidant and oxidative status) this oxidative stress and oxidative damage in turn may be responsible for hepatic tumor promoting activity of these compounds[32,33]. Thus, compounds or treatments that lower the oxidative stress can also inhibit tumour promotion[34]. Recent studies also showed that the antioxidant dietary supplementation can result in ablation of the hepatic lesion growth through an increase in apoptosis in hepatic lesion[35]. On the other hand, the presence of significant proportions of quercetin, isoquercitrin and rutin with their reported free radical scavenging, anti-lipid peroxidation[36,37] and hepatoprotective activity[27,29,38], in addition to, the reported antioxidant activity of the ethanolic extract of the flowers under investigation[4] added a support for the authors to evaluate the cytotoxic activity against liver cancer cell line and the hepatoprotective activity of the ethanolic extract and its fractions against CCl_4 induced liver damage.

The ethanolic extract revealed a cytotoxic activity against the human liver cancer cell line (HEPG2) (Table 1), as it had an IC_{50}= 9.06 μg/ml. The two main fractions **A** and **B** showed higher potency displayed by lower IC_{50} values (2.67 and 5.34, respectively). The isolated compounds were also tested for their cytotoxic activity where ursolic acid was the most potent as it revealed the lowest IC_{50} (0.55 μg/ml). β-sitosterol and its glucoside as well as quercetin and its 3-O-rhamnoside showed moderate cytotoxic activity when compared to the reference drug Doxorubicin®. Also, the amino acid L-azeditine-2-carboxylic acid showed a remarkable cytotoxic activity (IC_{50}= 2.51 μg/ml).

Since the ethanolic extract of the flowers as well as its non-polar fraction (**A**) and the flavonoid rich fraction (**B**) showed neither significant change in AST, ALT and ALP levels after one month of administration nor/or weak hepatoprotection against CCl_4 induced liver damage at 50 mg/kg b. wt. (Table 2). Therefore, the extract and its fractions were tested at a higher dose level (100 mg/kg). A daily dose of the ethanolic extract and the two fractions (**A**) and (**B**) (100 mg/kg b. wt.) showed no significant change in AST, ALT and ALP levels after one month of administration (Table 2). On comparing the increase in liver enzymes in the control group at 72 hours after induction of liver damage (by 25% CCl4) with that of the treated groups it was observed that the ethanolic extract, its non-polar fraction **A** as well as its flavonoid rich fraction **B** prevented the increase in the level of AST enzyme by 47.30, 56.20 and 59.80%, respectively and 64.38, 56.38 and 68.54% in ALT enzymatic level and 44.4, 28.20 and 45.29% in that of ALP level, respectively. This protective effect (especially the effect of the ethanol extract and its flavonoid rich fraction **B**) was comparable to that of silymarin which prevented the rise in the levels of AST, ALT and ALP 69.5, 77.09 and 70.1% of, respectively. Furthermore, Administration of the extracts for another one month after induction of liver damage (by 25% CCl_4) led to a significant decrease in the enzyme levels regarding their respective normal values which indicates stabilization of the hepatocyte cell membrane as well as repairing of hepatic tissue damage caused by CCl_4[27]. Since, the preventive action of the liver damage induced by CCL_4 has widely been used as a marker of hepatoprotective activity of drugs in general[39] and as the liver cancer is one of the death leading diseases in worldwide and particularly in Egypt, therefore, the present study has established that the flowers of *D. regia* are holding a great expectation for food and pharmaceutical applications.

CONCLUSION

Our results could justify the use of the ethanolic extract of the flowers of *D. regia* as a chemopreventive agent against the two causes of liver damage which are liver toxicity by chlorinated agents and liver cancer.

REFERENCES

1. Baily L H. Manual Of Cultivated Plants, The Macmillan company. 1958. New York, 222.
2. Allen ON and Allen EK. The Leguminoseae. 1981. Macmillan Publishers Ltd. Londoon and Basingstoke, UK.p.588.
3. Aqil F, Khan MS, Owais M and Ahmad I. Effect of certain bioactive plant extracts on clinical isolates of beta-lactamase producing methicillin resistant *Staphylococcus aureus*. J. Basic. Microbiol. 2005. 45(2):106-14
4. Aqil F, Ahmad I and Mehmood Z. Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. Turk. J. Biol. 2006. 177-83.
5. Mendes NM, de Souza CP, Araujo N, Pereira JP and Katz N. Molluscicide activity of some natural products on *Biomphalaria glabrata*. Mem Inst. Oswaldo Cruz. 1986. 81(1):87-91.
6. Gupta RK and Chandra S.Chemical investigation of *Delonix regia* flowers. Indian J. Pharm. 1971. 33:75.
7. Saleh NAM, Ishak MS. Anthocyanins of some leguminosea flowers and their effect on colour variation. Phytochem. 1976. 15:835-836.
8. Banerjee A and De B. Anthocyanins in some flowers of West Bengal. J. Med. Aromat. Plant. Sci. 2001. 23:600-604.
9. Soltan ME and Sirry SM. Usefulness of some plant flowers as natural acid-base indicators. J. Chin. Chem. Soc. 2002. 49:63-68.
10. Adje F, Lozano YF, Meudec E, Lozano P, Adima A, N'zi GA and Gaydou EM. Anthocyanin characterization of pilot plant water extracts of *Delonix regia* flowers, Molecul. 2008. 13:1238-45.
11. Jungalwala FB and Cama HR. Carotenoids in *Delonix regia* (Gul Mohr) flower. Biochem. J. 1962. 85:1.
12. Mukherjee D. 1975. Keto and imino acids in Delonix regia. Phytochemistry, vol. 14(9):1915-18.
13. Wagner H, Baldt S, Zgainski EM. Plant Drug Analysis. 1984. Translation of German Ed. Springer - Verlag, Berlin.
14. Karber G. Determination of LD50 Arch. Exp. Pathol. Pharma. 1931. 162:480.

15. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. 1990. 82:1107-12.
16. Klassan CD and Plaa GL. Comparison of biochemical alterations in liver of rats treated with carbon tetrachloride. Biochem. Pharmacol. 1969. 1B:2019.
17. Thewfweld W. The enzymatic methods for determination of serum AST and ALT. 1974. Dtsch. Med. 99:343.
18. Kind PR and King EG. Enzymatic determination of of serum alkaline phosphatase (ALP). J. Clin. Path. 1954. 7:322.
19. Markham KR. Techniques of Flavonoid Identification. 1982. Academic Press, London.
20. Nawwar MAM, Ishak MS, Michael HN, Buddrust J.Leaf flavonoid of *Ziziphus spina-christi*. Phytochem. 1984. 23:2110-11.
21. Harborne JB. The Flavonoids: Advances in research since 1986 . 1983. Chapter 10; Chapman & Hall.Ltd., London.
22. Hori K, Satake T, Saiki Y, Kobayashi K, Uzawa J, Fujimoto Y and Murakami T. An external flavonoid and its dimer from the fern od *Oreopteris quelpaertensis.* Phytochem. 1990. 29:1679.
23. Rocha L, Marston A, Potterat OM, Kaplan MC, Evans HS, Hostettmann K. Antibacterial phloroglucinol and flavonoids from *Hypericum brasiliense*. Phytochem. 1995. 40:1447-52.
24. Mabry TJ, Markham KR, Thomas MB. The Systematic Identification of Flavonoids.2nd Ed. 1996. Springer Verlag; New York.
25. Good JL, Akisha T. Analysis of Sterols. 1st ed. Blackie Academic and Proffessional Press; Champan and Hall; 1997.
26. Grace, M. H.; Mohamed, T. K.; Khattab, A. M.Flavonoids of *Carduncellus eriocephalus*. Egypt. J. Pharm. Sci. 1998. 39:409-16.
27. Singab AB, Youssef DTA, Noeman E and Kotb S. Hepatoprotective effect of flavanol rich fraction from Egyptian *Vicia calcarata* Desf. Against CCl4 – induced liver damage in rats" Arch. Pharm. Res. 2005. 28(7):791-798.
28. Agrawal PK. Carbon-13 NMR of Flavonoids. Amsterdam, Oxford, New York and Tokyo: Elsevier; 1989.
29. Awad AS, Maitland DJ and Soliman G A. Hepatoprotective activity of *Schouwia thebica* Webb. Bioorg. and Med. Chem. lett. 2006. 16:4624-28.
30. Futamura Y, Kurokawa M, Obata R, Nishiyama S and Sugai T. Efficient Route to *S* Azeditine-2-carboxylic Acid , Biosci. Biotechnol. Biochem. 2005. 69(10):1892-97.
31. Sung ML and Fowden,L. Azeditine-2- carboxylic acid from the legume *Delonix regia*. Phytochem. 1969.8 (10):2095-96.
32. Bachowski S, Xu Y, Baker TK, Stevenson DE, Walborg EF and Klunig JE. The potential role of oxidative stress in non-genotoxic carcinogensis in the mouse liver. Prog. Clin. Biol. Res. 1995. 391:385-396.
33. Klunig JE, Xu Y, Isenburg JS, Bochowski S, Kolaja KL, Jiazhong J, Stevenson DE and Walborg EJ. The role of oxidative stress in chemical carcinogensis. Environ. Health Perspect. 1998. 106:289-295.
34. Kolaja KL, Bunting KA and Klaunig JE. Inhibition of tumour promotion and hepatocellular growth by dietary restriction in mice. Carcinogensis. 1996. 17:1657-64.
35. Klunig JE and Kamendulis LM. Mechanisms of cancer chemoprevention in hepatic carcinogensis: Modulation of focal lesion growth in mice. Toxicol. Sci. 1999. 52:101-06.
36. Nakagawa M, Kawagoe M, Yoshimura M, Arata H, Minamikawa T, Nakamura M. and Matsumoto A. 2000. Differential effects of flavonoid quercetin on oxidative damages induced by hydrophilic and lipophilic radical generators in hepatic lysosomal fractions of mice. J. Healt. Sci. 2000. 46 (6):509-12.
37. Shibano M, Kakutani K, Taniguchi M, Yasuda M. and Baba K.2008. Antioxidant constituents in the dayflower (*Commelina communis* L.) and their α-glucosidase-inhibitory activity. J Nat. Medic. 2008. 62(3):349-53.
38. Kumar SVS and Mishra SH. Hepatoprotective effect of Pergularia daemia(Forsk.) ethanol extract and its fraction. Ind. J Experim. Biol. 2008. 46:447-52.
39. Wu J and Norton PA. Animals models of liver fibrosis. *Scand. J. Gastroenterol.* 1996. 31:1137-43.

Cyclooxygenase Inhibitory, Cytotoxicity and Free Radical Scavenging Activities of Selected Medicinal Plants Used in Indian Traditional Medicine

Rajesh Gacche*, Rafik Shaikh, Mahesh Pund, Rupesh Deshmukh

School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded - 431 606 (MS), India.

ABSTRACT

Introduction: The enzyme cyclooxygenase (COX) has been implicated to be a key enzyme involved in recruiting inflammations. Developing COX inhibitors has remained one of the important aspects of developing novel and safe anti-inflammatory agents. An attempt has made to find the medicinal plants as alternatives to presently available NSAIDs (Non steroidal anti-inflammatory drugs). **Methods:** In the present study the samples of *Enicostema axillare* (Lam.) Raynal. [Gentianaceae], *Argemone mexicana* L. [Papaveraceae], *Clerodendrum multiflorum* (Burm.f.) O. Ktze. [Verbenaceae], *Withania somnifera* (L.) Dunal. [Solanaceae], *Polyalthia longifolia* (Sonner.) Thw. [Annonaceae] and *Vitex nigundo* L. [Verbenaceae] were sequentially extracted in water, ethanol and hexane and were evaluated i*n-vitro* for COX-1 and 2 inhibitory activities. The free radical scavenging activities were carried out along with cytotoxicity evaluation. **Results:** Among the tested plants, *E. axillare* showed promising COX-2 inhibiting activity in ethanol (48.71 ± 0.035 %), water (42.13 ± 0.030 %) and hexane (12.31 ± 0.040 %) as compared to COX-1 inhibition in ethanol (14.73 ± 0.030 %), water (27.64 ± 0.030 %) and hexane (6.92 ± 0.031 %). The contents of the water extracts of majority of the plant samples were found to interact with DPPH, superoxide and OH radicals. The selected plants did not showed cytoxicity except a poor toxicity demonstrated by ethanol extract of *P. longifolia* (0.15 ± 0.040 %). HPTLC analysis was carried out to study the flavonoids diversity of the selected plant samples. **Conclusion:** The results obtained shows that majority of the plants under study were found to inhibit COX-2 activity significantly as compared to COX-1 activity. However, more detailed studies are required to assess the safety and efficacy of these plants.

Key words: Anti-inflammatory, Cyclooxygenase (COX), Medicinal plants, Antioxidants, Cytotoxicity.

INTRODUCTION

Inflammation is a general name for reactions occurring several types of tissue injuries, infections, or immunologic stimulation as a defence against foreign or altered endogenous substances. The process of inflammation comprises of a series of changes of the terminal tissues, which tend to eliminate the injurious agents and to repair the damage tissue. The enzyme Cyclooxygenase (COX) has been implicated as a key enzyme for recruiting the inflammations. It is a membrane bound glycoprotein found in large amount in the endoplasmic reticulum of prostanoid forming cells.[1] The COX enzyme also known as prostaglandin H synthase (PGHS) or prostaglandin endoperoxide synthase (E.C. 1.14.99.1).[2] It occurs catalytically in two active forms i.e. COX-1 and COX-2. These two isoforms of COX are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations.[3,4] The COX-1 is constitutively expressed and involved in the production of prostaglandins that mediate basic housekeeping functions in the body. However COX-2 is an inducible and expressed in some tissue (e.g. brain and kidney), the expression of which is activated in response to cytokines, mitogenes and endotoxins in a variety of cell types.[5] These enzymes carry out two sequential reactions in spatially distinct but mechanistically coupled active sites: the double dioxygenation of arachidonic acid to prostaglandin G_2 (PGG_2) and the reduction of PGG_2 to PGH_2.

Address for correspondence:
R.N.Gacche, Head Department of Botany,
School of Life Sciences,
Swami Ramanand Teerth Marathwada University,
Nanded - 431 606 (MS), India.
Tel.:+91-02462-229242/43/50. Ext.195, Fax: +91-02462-229325.
E-mail: rngacche@rediffmail.com

DOI: 10.5530/pj.2011.19.11

The arachidonic acid oxygenation occur in the COX active site, and PGG_2 reduction occurs in the peroxidase active site. PGG_2 diffuses from the COX proteins and is transformed by different tissue specific isomerases to prostaglandins which are responsible for inflammation.[6] The reaction catalyzed by COX-1 and COX-2 is shown in (Figure 1). Moreover the process of inflammation is also mediated by number of free radicals. Importantly, studies conducted over the past 15 years indicate that free radical species (superoxide, hydroxyl radicals, nitric oxide, peroxynitrite, and the free radical-derived product hydrogen peroxide) play an important role in inducing and modulating the inflammations.[7]

Currently the non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, naproxen, and rofecoxib are widely used for the treatment of inflammation and inflammatory disorders.[8] Unfortunately, besides the excellent anti-inflammatory potential of the NSAIDs, the severe side effects such as gastrointestinal (GI) ulceration, perforation, obstruction, and bleeding has limited the therapeutic usage of NSAIDs.[9,10] The mucosal irritation occurs due to the acidic nature of most of NSAIDs and inhibition of production of mucosal protective PGE which leads to gastric erosion.[11] It is estimated that in U.S. the annual death rate from NSAID-induced gastrointestinal bleeds is around 0.08 %.[12] This is consistent with the idea that inhibition of COX-1 underlies the gastrointestinal side effects of NSAIDs and that NSAIDs selectivity toward inhibition of COX-1 over COX-2 correlates with their ability to cause gastrointestinal side effects.[13-15] The clinical and epidemiological data indicates that NSAIDs are also attributed with cardiovascular dysfunctions especially in patients with colorectal polyps these results were confirmed by several large pharmacoepidemiological studies.[16-18] Searching selective COX-2 inhibitors without influencing the normal physiological functions COX-1, has remained a major thrust area of anti-inflammatory pharmaceutical research. Nevertheless the anti-inflammatory agents having selective COX-2 inhibition but less reactive towards COX-1 are appreciated as novel anti-inflammatory agents in the mainstream of anti-inflammatory research.[19]

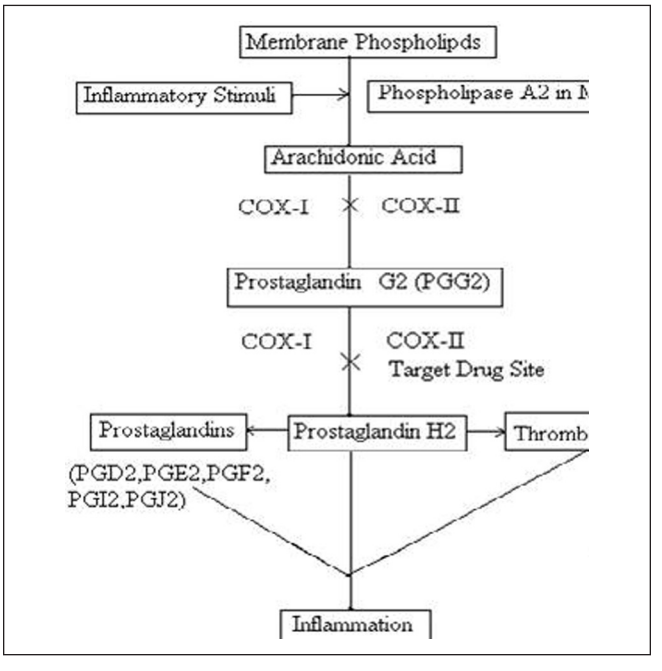


Figure 1: Reaction of COX-1 and COX-2 mediated production of Prostaglandin leading to inflammations.

Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and continue to provide mankind a resource for new remedies for variety of human ailments. There are several records in traditional medicine describing the importance of medicinal plants for relief from pain and inflammation.[20-24] In "Ayurveda": a well known Indian traditional system, various medicinal plants are reported for their use as an anti-inflammatory agent. Since ancient times many people suffering from inflammation were treated with phytochemicals, which is evident from the discovery of first anti-inflammatory, analgesic drug aspirin obtained from the bark of willow-tree. In the last twenty-year period 50 new chemical entities with anti-inflammatory activity have entered the market, of which only 13 are semi synthetically derived from natural products and one, a protein, is of biological origin. This low number of new plant-derived drugs is not reflected in the phytomedical literature.[25] In the recent years, the use of traditional medicine information on plant research has again received considerable interest. The renewed interest in medicinal plants has focused on herbal cures among indigenous populations around the world. Ethanopharmacology and drug discovery using natural products has remained an important issue in the current target-rich, lead-poor scenario.[26]

Considering the above facts and circumstantial clinical and epidemiological evidences, there is an urgent need of exploring plant wealth for the identification of novel, safe and effective anti-inflammatory agents. Nevertheless the standardization of herbal drugs is an essential issue for converting botanicals into therapeutic modalities. The present investigation is an attempt to explore the antioxidant and COX (a key enzyme implicated in inflammation) inhibitory potential of the selected medicinal plants.

MATERIALS AND METHODS

Materials

The COX-1 & 2 (human ovine) inhibitor Screening assay kit [Catalog No. 760111] was obtained from Cayman, U.S.A., DPPH (1,1-diphenyl-2-picryl hydrazyl), MTT (3-(4,5-

dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) were procured from Sigma-Aldrich Co. (St. Louis MO, USA). 1-10 phenanthroline, Phenazine methosulphate (PMS), Nitroblue tetrazolium (NBT) were obtained from s.d. Fine chem. Mumbai. Nicotinamide Adenine Dinucleotide (NADH) was purchased from Spectrochem, Pvt. Lit. Mumbai. Chang Liver cell line was requested from National Centre for Cell Science (NCCS: a National Cell Line Facility) Pune (MS), India. Medicinal plants were collected from the nearby areas of Nanded district (MS), India. All other chemicals and reagents used were of AR grade and were obtained from commercial sources.

Collection, identification and authentication of the selected medicinal plants

The selected plants *Enicostema axillare*, *Argemone mexicana*, *Clerodendrum multiflorum*, *Withania somnifera*, *Polyalthia longifolia*, and *Vitex nigundo* were collected from the nearby regions of Nanded district (MS), in the month of Sept 2009. The plants were identified and authenticated by RNG, Head Department of Botany, School of Life Sciences, Swami Ramanand Teerth Marathwada University Nanded - 431 606 (MS), India, with the help of Flora [27]. Voucher specimens (B13-B18) of the collected plants were deposited in the herbarium centre of the host Institute. The shade dried and powdered plant samples were preserved for further investigations.

Sequential Extraction of the plant samples

The shed dried powdered plant samples (~10gm) were sequentially extracted in water, ethanol and hexane up to 8 hours using Soxhlet's apparatus. The extracted samples were evaporated under reduced pressure at room temperature. The dried extracts were preserved for further analysis.

HPTLC Analysis

HPTLC analysis was performed using CAMAG (Germany) make instrumental thin layer chromatography. TLC plates (Merck silica gel 60 F254, 20 cm × 10 cm) were prewashed with methanol. The plate was activated in an oven at 100 ^{0}C for 10 minutes. Individual plant extracts of 10µl (1mg/ml) were spotted onto the precoated plates using a Linomat 5 application system. Rutin hydrate (5, 10 and 20µg/ml) was used as a marker flavonoid. The flavonoids were separated using ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27) as a mobile phase. Natural product (NP) reagent was used as a flavonoid derivatizing agent and the spots developed were visualized under CAMAG UV cabinet (254 and 366 nm) and were digitized using CAMAG photo documentation system. The HPTLC finger print image of derivatized flavonoids is shown in (figure 2).

COX inhibition assay

The assay was performed by using Colorimetric COX (human ovine) inhibitor Screening assay kit [28]. Briefly, the reaction cocktail contained, 150 µl of assay buffer, 10 µl of heme, 10 µl of enzyme (either COX-1 or COX-2), and 10 µl of plant sample (1mg/ml, in 0.5 % DMSO). The assay utilizes the peroxidase component of the COX catalytic domain. The peroxidase activity can be assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramthyl-*p*-phenylenediamine (TMPD) at 590nm. Aspirin (acetylsalicylic acid, 1 mM) was used as a standard drug. The percent COX inhibition was calculated using following equation:

$$\text{COX inhibition activity}(\%) = 1 - \frac{T}{C} \times 100$$

Where T = Absorbance of the inhibitor well at 590 nm
C = Absorbance of the 100 % initial activity without inhibitor well at 590 nm

DPPH radical scavenging assay

DPPH (1, 1-diphenyl-2- picrylhydrazyl) radical scavenging assay was carried out as per reported method with slight modifications [29,30]. In brief, 1 ml of test solution (individual plant extract) was added to equal quantity of 0.1 mM solution of DPPH in ethanol. After 20 min of incubation at room temperature, the DPPH reduction was measured by reading the absorbance at 517 nm. Ascorbic acid (1mM) was used as reference compound.

OH radical scavenging assay

The OH radicals scavenging activity was demonstrated with Fenton reaction [31]. The reaction mixture contained, 60µl of $FeCl_2$ (1mM), 90µl of 1-10 phenanthroline (1mM),

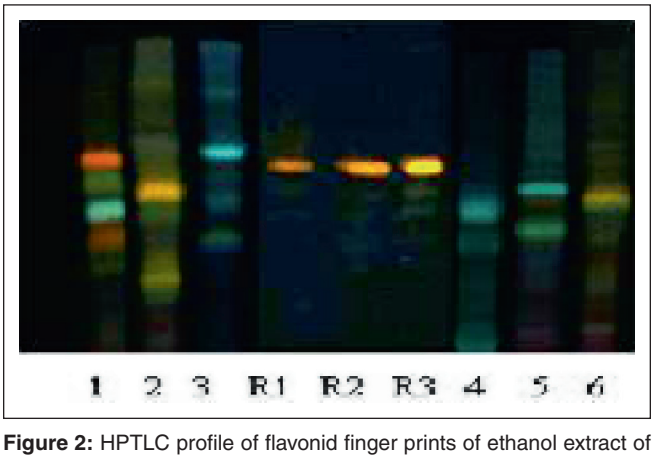


Figure 2: HPTLC profile of flavonid finger prints of ethanol extract of selected medicinal plants using Rutin as a marker compound. Track no. 1- *P. longifolia*, 2- *E. axillare*, 3- *W. somnifera*, 4- *C. multiflorum*, 5- *V. nigundo*, 6- A. mexicana, R1- Rutin (5 µg), R2-10 µg and R3- 20 µg.

2.4 ml of phosphate buffer (0.2M, pH 7.8), 150µl of H_2O_2 (0.17M) and 1.5 ml of individual plant extract (1mg/ml). The reaction was started by adding H_2O_2. After 5 min. incubation at room temperature, the absorbance was recorded at 560nm. Ascorbic acid (1mM) was used as reference compound.

Superoxide radical (SOR) scavenging assay

The superoxide anion scavenging assay was performed by the published method [32]. Superoxide anion radicals were generated in a non-enzymatic Phenazine methosulphate - Nicotinamide Adenine Dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and Oxygen. It was assayed by the reduction of Nitroblue tetrazolium (NBT). In this experiment superoxide anion was generated in 3ml of Tris HCL buffer (100mM, pH 7.4) containing 0.75ml of NBT (300mM), 0.75ml of NADH (936 mM), and 0.3ml of plant sample (1mg/ml). The reaction was initiated by adding 0.75ml of PMS (120 mM) to the mixture. After 5min. of incubation at room temperature the absorbance at 560nm was measured in spectrophotometer. Ascorbic acid (1mM) was used as reference compound.

MTT Cytotoxicity assay

The MTT cytotoxicity assay was performed as reported previously.[33-35] The Chang liver cells were harvested (4.5×10^4 cells/well) and inoculated in 96 well microtiter plates. The cells were washed with phosphate buffered saline (PBS) and the cultured cells were then inoculated with and without the individual ethanolic plant extract (1mg/ml). After 72 hrs incubation, the medium was aspirated followed by addition of 150 µL of MTT solution (5 mg/mL in PBS, pH 7.2) to each well and the plates were reincubated for 4 hrs at 37 °C. After incubation time, 800 µL of DMSO was added to the wells followed by gentle shaking to solubilize the formazan crystal for 15 min. Absorbance was read at 540 nm using Thermo make Automatic Ex-Microplate Reader (M 51118170) and the % cell viability was calculated. The H_2O_2 (1mM) was used as cytotoxic cytotoxic agent. The DPPH, OH, SOR scavenging activity (%) and cell viability inhibition (%) was calculated using following formula.

$$\text{COX inhibition activity (\%)} = 1 - \frac{T}{C} \times 100,$$

Where T = Absorbance of the test sample
C = Absorbance of the control sample

RESULTS

HPTLC Profiling

As a part of standardization of selected herbal samples HPTLC analysis was performed for making a finger print of ethanol soluble flavonoids using rutin as a marker flavonoid (Figure 2). The results of the HPTLC analysis shows the more diversity of flavonoid content in *P. longifolia* moreover this is the only sample containing 1.5 % of rutin concentration, while all other sample were devoid of rutin content.

Effect of selected plant extracts on COX-1 & 2 activities

The profile of COX-1 and 2 inhibition studies shows that the majority of plant samples show considerable COX-2 inhibition as compared to COX-1 (Table 1 & 2). Amongst the tested plants the *E. axillare* factions in water, ethanol and hexane extract (1mg/ml) showed more inhibition of COX-2 than COX-1. It was also observed that the ethanolic extract of most of the plant samples, except *P. longifolia* showed more inhibitory effect on COX-2 than the COX-1. The ethanolic extract of *C. multiflorum* showed maximum inhibitory effect on COX-2 (63.24 ± 0.040 %) while *V. nigundo* had a minimum effect on COX-2 activity (07.37 ± 0.020 %). The water extract of all the plant samples

Table 1: Effect of selected medicinal plant fractions on COX-1 Inhibition.

	Name of the plant	COX-1 inhibition (%)		
		Water	Ethanol	Hexane
1	*Enicostema axillare*	27.64 ± 0.030	14.73 ± 0.030	06.92 ± 0.031
2	*Argemone mexicana*.	15.58 ± 0.032	27.43 ± 0.025	11.32 ± 0.037
3	*Clerodendrum multiflorum*	12.83 ± 0.035	24.47 ± 0.030	09.71 ± 0.035
4	*Withania somnifera*	20.27 ± 0.030	23.52 ± 0.020	13.42 ± 0.030
5	*Polyalthia longifolia*	17.33 ± 0.032	37.26 ± 0.030	15.93 ± 0.040
6	*Vitex nigundo*	25.73 ± 0.030	NR	NR
7	Aspirin		08.82 ± 0.028	

Results presented here are the mean values from three independent experiments ± S.D.,
NR = No reaction under experimental condition

inhibited COX-2 & 1 activity in a range of 08.42 ± 0.020 - 42.13 ± 0.030 % and 12.83 ± 0.035 - 27.64 ± 0.030 % respectively. Aspirin, a reference compound was found to inhibit COX-2 activity (11.15 ± 0.021 %) more as compared to COX-1 (08.82 ± 0.028 %).

DPPH radical scavenging activity

The results of DPPH reduction are summarized in (Table 3). It is clear from the results that the water extracts of all plant samples (1mg/ml) were found to interact with the stable free radical DPPH, which indicates their radical scavenging ability. The overall range of DPPH radical scavenging activity was found to be 06.24 ± 0.030 - 68.75 ± 0.030 %. Moreover the ethanolic extracts of all the samples except, *C. multiflorum* showed significant DPPH radical scavenging activity in a range of 07.18 ± 0.030 - 64.24 ± 0.040 % as compared to ascorbic acid (82.54 ± 0.021 %). It was observed that, the hexane extracts of only two plant samples *A. mexicana* (36.14 ± 0.035 %) and *C. multiflorum* (33.36 ± 0.020%) were found to interact with DPPH radicals. The maximum activity (68.75 ± 0.030 %) was observed in water extract of *A. mexicana*, while minimum (6.24 ± 0.030) interaction was recorded in water extract of *V. nigundo*.

OH radical scavenging activity

The summary of OH radical scavenging activities has been shown in (Table 4). In general, except the water extract of *V. nigundo*, all other water extracts (1mg/ml) of the selected plant samples showed effective OH radical stabilizing potentials in a range of 03.85 ± 0.040 - 26.92 ± 0.030 % . None of the hexane extract of plant sample showed OH radical scavenging activity. The ethanolic extract of *C. multiflorum* (28.85 ± 0.025 %) was found to be hyper reactive towards OH radicals whereas the water extract of *A. mexicana* (03.85 ± 0.040 %) showed minimum effect on OH radicals.

SOR scavenging activity

The results of SOR scavenging activities are summarized in (Table 5). Again, the water extracts of all plants were observed to be effective towards stabilizing the SOR, which demonstrated the SOR scavenging activity in the range of 48.34 ± 0.036 - 60.94 ± 0.035 %. Surprisingly, none of the ethanolic extract of plant samples showed SOR scavenging activity. The water extract of *A. mexicana* (60.94 ± 0.035 %) showed maximum SOR scavenging effect, while hexane extract of the same plant demonstrated minimum effect (17.26 ± 0.045%).

Table 2: Profile of COX-2 inhibition by selected plant samples.

		COX-2 inhibition (%)		
	Name of the plant	Water	Ethanol	Hexane
1	*Enicostema axillare*	42.13 ± 0.030	48.71 ± 0.035	12.31 ± 0.040
2	*Argemone Mexicana*	18.70 ± 0.032	51.27 ± 0.020	10.90 ± 0.030
3	*Clerodendrum multiflorum*	08.42 ± 0.020	63.24 ± 0.040	18.44 ± 0.036
4	*Withania somnifera*	18.45 ± 0.020	54.71 ± 0.035	21.15 ± 0.035
5	*Polyalthia longifolia*	29.92 ± 0.025	17.94 ± 0.020	18.26 ± 0.040
6	*Vitex nigundo*	32.18 ± 0.030	NR	07.37 ± 0.020
7	Aspirin		11.15 ± 0.021	

Results summarized here are the mean values from three independent experiments ± S.D.,
NR = No reaction under experimental condition

Table 3: DPPH radical scavenging activity of selected plant samples in different solvents.

		DPPH radical scavenging activity (%)		
	Name of the plant	Water	Ethanol	Hexane
1	*Enicostema axillare*	12.49 ± 0.036	07.18 ± 0.030	NR
2	*Argemone mexicana*	68.75 ± 0.030	42.85 ± 0.034	36.14 ± 0.035
3	*Clerodendrum multiflorum*	25.02 ± 0.030	NR	33.36 ± 0.020
4	*Withania somnifera*	06.28 ± 0.026	64.24 ± 0.040	NR
5	*Polyalthia longifolia*	25.05 ± 0.051	42.83 ± 0.025	NR
6	*Vitex nigundo*	06.24 ± 0.030	21.46 ± 0.031	NR
7	Ascorbic acid		82.54 ± 0.021	

Results presented herein are the mean values of n = 3, ± S.D., NR = No reaction under experimental condition

Cytotoxic effect of plant extracts

Ethanolic extracts (1mg/ml) of the selected plant samples were evaluated for their cytotoxic effects on Chang Liver cells using MTT assay. The results of the cytotoxic activity of crude extracts of these plants are summarized in (Table 6). At 1mg/ml concentration, none of the plant extract showed cytotoxic effects except the negligible cytotoxicity demonstrated by the extract of *P. longifolia* (0.15 ± 0.040 %) as compared H_2O_2 (4.92 ± 0.021 %).

DISCUSSION

The present work was designed for evaluating the potential of selected medicinal plants as anti-inflammatory agents in the midst of searching novel alternatives to NSAIDs.The medicinal plants are proved to be important elements of indigenous medicinal system in India and all over the world. The medicinal plants have provided a rich resource for natural drug research and development of novel therapeutic agents. It is reported that the phytochemicals present in

Table 4: OH radical scavenging potential of the selected plant extract in different solvents.

		OH radical scavenging activity (%)		
	Name of the plant	Water	Ethanol	Hexane
1	*Enicostema axillare*	23.12 ± 0.036	15.34 ± 0.030	NR
2	*Argemone mexicana*	03.85 ± 0.040	11.54 ± 0.042	NR
3	*Clerodendrum multiflorum*	21.14 ± 0.045	28.85 ± 0.025	NR
4	*Withania somnifera*	26.92 ± 0.030	NR	NR
5	*Polyalthia longifolia*	09.65 ± 0.035	NR	NR
6	*Vitex nigundo*	NR	NR	NR
7	Ascorbic acid		02.82 ± 0.021	

Results shown here are the mean values from three independent experiments ± S.D.,
NR = No reaction under experimental condition

Table 5: Superoxide radical scavenging activity of various fractions of the selected medicinal plants.

		SOR scavenging activity (%)		
	Name of the Plant	Water	Ethanol	Hexane
1	*Enicostema axillare*	57.08 ± 0.040	NR	NR
2	*Argemone mexicana*	60.94 ± 0.035	NR	17.26 ± 0.045
3	*Clerodendrum multiflorum*	54.65 ± 0.045	NR	NR
4	*Withania somnifera*	48.34 ± 0.036	NR	NR
5	*Polyalthia longifolia*	60.45 ± 0.035	NR	23.25 ± 0.035
6	*Vitex nigundo*	60.43 ± 0.375	NR	NR
7	Ascorbic acid		51.24 ± 0.028	

Results presented here are the mean values from three independent experiments ± S.D.,
NR = No reaction under experimental condition.

Table 6: Effect of selected plant samples on the viability of Chang Liver cells (human).

	Name of the plant	Inhibition of Cell Viability (%) at 1mg/ml
1	*Enicostema axillare*	-1.56 ± 0.305
2	*Argemone mexicana*	-2.35 ± 0.035
3	*Clerodendrum multiflorum*	-2.64 ± 0.036
4	*Withania somnifera*	-4.84 ± 0.030
5	*Polyalthia longifolia*	0.15 ± 0.040
6	*Vitex nigundo*	-6.53 ± 0.030
7	H_2O_2	4.92 ± 0.021

The cytotoxicity was determined by performing MTT assay. The results presented are the percent inhibition values obtained from n = 3 ± S.D.

plants like terpenoids and flavonoids has significant anti-inflammatory activity by inhibiting COX.[36-38] However, several plant iridoids, iridoid glucosides (aucubin, catalpol, gentiopicroside, swertiamarin, geniposide, geniposidic acid and loganin) and an iridoid aglycone (genipin) were investigated as anti-inflammatory agents. [39] The Indian spice turmeric, *Curcuma longa* L. possessing curcumin (and synthetic analogs) have established reputation as an anti-inflammatory agent by inhibiting COX-1 and COX-2 [40]. However several records have found that the plants as, *Siphonochilus aethiopicus* [41], *Erythrina sp.*[42], *Eucomis autumnalis*[43] posses a significant COX inhibitory activity. The COX-1 and 2 inhibition activities demonstrated by various fractions of the selected plant samples might be due to presence of aforesaid phytochemicals, moreover the HPTLC flavonoids finger prints (Figure 2) indicates the presence of diverse mixture of flavonoids present in the selected medicinal plants.

Free radicals especially the reactive oxygen species (ROS) are proposed to be the key players in the pathophysiological mechanisms associated with various inflammatory disorders. [44] There are several compounds (hydrogen donor) that interact with DPPH radicals and reduce them to corresponding hydrazines.[45] It has been reported that the compounds possessing significant reducing potential can be considered as potential candidate for designing a novel anti-inflammatory agent that may adversely interact with enzyme COX.[46] Equally, the OH and SOR radicals have been implicated to play a critical role in the physiological control of cell function.[47] These radicals react indiscriminately with high rate constant with almost every type of biomolecules found in living cell such as, sugars, amino acids, phospholipids, DNA bases, organic acids and may deviate the cells from its normal physiological functions. [48] The active antioxidants like ascorbate, glutathione, urate, flavonoids, tocopherols, carotenoids and hydrooycinnamic acids present in plants may be the contributing factors for scavenging of DPPH, OH and SOR radicals.[49] The herbal toxicity is a key issue to be addressed to the consumers; nevertheless it is a basic prerequisite for converting botanicals into therapeutic modalities. The plants described in the present investigations can be considered safe for making other herbal formulations for health amelioration.

CONCLUSION

In conclusion, the result of the present studies indicates the potential of selected botanicals as a possible resource for the design or development of novel safe and effective anti-inflammatory agents. As some of the above examples illustrate often activity cannot be linked to a single compound, the activity results from several active compounds. There might be additive or synergistic effects of the compounds, something we have not tested, and which generally is an under investigated field in phytomedicine. Overall, many plants are more suited as phytomedicines, where patients benefit from the complex mixture of compounds, rather than as sources of purified drugs. The present findings may also supplement and strengthen the process of standardization and validation of herbal drugs containing active ingredients derived from the selected medicinal plants.

ACKNOWLEDGMENT

Authors are thankful to University Grant Commission (UGC), New Delhi (India), for financial assistance (F. No. 33-157/2007 (SR). RUS thanks UGC for JRF.

REFERENCES

1. Smith WL. Prostaglandin biosynthesis and its compartmentation in vascular smooth muscle and endothelial cells. Annual Rev of Physiol. 1986; 48: 251-62.
2. Falkay GJ, Herczeg, Sas M. Prostaglandin synthesis and metabolism in the human uterus and midtrimester fetal tissue. J of Repr & Fert. 1980; 59: 525-529.
3. Jouzeau JY, Terlain B, Abid A, Nedelec E, Netter P. Cyclooxygenase isoenzymes. Drugs. 1997; 53(4): 563-82.
4. Vane JR, Botting R.M. Anti-inflammatory drugs and their mechanism of action. Inflamm Res. 1998; 47(2): S78-S87.
5. Smith WL, De Witt DL, Garavito RM. Cyclooxygenase: structural, cellular and molecular biology. Annual Rev of Biochem. 2000; 69:145-182.
6. Anna LB, Lawrence JM. Structural and functional basis of cyclooxygenase inhibition. J of Med Chem. 2007; 50(7): 1427-1441.
7. Gerald SS, Leigh A.C. Free radical mediated skeletal muscle dysfunction in inflammatory conditions. J of Appl Physiol. 2007; 102: 2056-2063.
8. Mitchell JA, Lucas R, Vojnovic I, Hasan K, Pepper JR. Stronger inhibition by non-steroidal anti-inflammatory drugs of cyclooxygenase-1 in endothelial cells than platelets offers an explanation for increased risk of thrombotic events. The FASEB Journal. 2006; 20: 2468-2475.
9. Gierse JK, Hauser SD, Creely DP. Expression and selective inhibition of the constitutive and inducible forms of human cyclooxygenase. Biochem J. 1995; 305: 479-84.
10. Silverstein FE, Faich G, Goldstein JL. Gastrointestinal toxicity with celecoxib vs non-steroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. J of Ame Med Asso. 2000; 284: 1247-55.
11. Dogne JM, Supuran CT, Pratico D. Adverse cardiovascular effect of the Coxibs. J of Med Chem. 2005; 48(7): 2251-2257.
12. Lazzaroni M, Porro BG. Gastrointestinal side-effects of traditional non-steroidal anti-inflammatory drugs and new formulations. Alimen & Pharmacol Ther. 2004; 20(2)**:** 48–58.
13. Seibert K, Zhang Y, Leahy K. Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. Proce of Nat Acad of Sci. 1994; 91: 12013-17.
14. Lee JL, Mukhtar H, Bickers DR, Kopelovich L, Athar M. Cyclooxygenases in the skin: pharmacological and toxicological implications. Toxicol & Appl Pharmacol. 2003; 192: 294-306.
15. Warner TD, Mitchell JA. Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. FASEB Journal. 2004; 18: 790–804.
16. Solomon DH, Schneeweiss S, Glynn RJ. Relationship between selective cyclooxygenase-2 inhibitors and acute myocardial infarction in older adults. Circulation. 2004; 109: 2068-2073.
17. Graham DJ, Campen D, Hui R. Risk of acute myocardial infarction and sudden cardiac death in patients treated with cyclooxygenase-2 selective

and nonselective non-steroidal anti-inflammatory drugs: nested case-control study. Lancet. 2005; 365: 475-481.

18. Hippisley CJ, Coupland C. Risk of myocardial infarction in patients taking cyclooxygenase inhibitors or conventional non-steroidal anti-inflammatory drugs: population based nested case-control analysis. Brit Med J. 2005; 330: 1366-1372.
19. Bandgar BP, Patil SA, Gacche RN, Korbad BL, Hote BS. Synthesis and biological evaluation of nitrogen-containing chalcones as possible anti-inflammatory and antioxidant agents. Bioor & Med Chem Lett. 2010; 20: 730–733.
20. Lee D. A novel cycloxyegenase inhibitory stilbenolignan from the seeds of *Aiphanes aculeatal*. Org Lett. 2001; 3(4): 2169.
21. Su B. Constituent of the bark and twigs of *Artocarpus dadah* with cyclooxygenase inhibitory activity. J of Nat Prod. 2002; 65(2): 163.
22. Garrido G. In- vivo and In-vitro anti-inflammatory activity of *Mangifera indica* L. extract. Pharmacol Res. 2004; 50(2): 143.
23. Rollinger J. Discovering COX inhibiting constituent from Morus bark: activity guided versus computer aided methods. Planta Med. 2005; 71(5): 399-405.
24. Zou J. Selective cyclooxygenase-2 inhibitors from *Calophyllum membranaceum*. J of Nat Prod. 2005; 68(10): 1514.
25. Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981–2002. J of Nat Prod. 2003; 66: 1022–1037.
26. Selvum C, Jachak S. A cclooxygenase inhibitory biflavonoid from the seeds of *Semecarpus anacardium*. J of Ethanopharmacol. 2004; 95: 209-212.
27. Naik VN. Flora of Marathwada (vol-I, II). Aurangabad (MS). Amrut Prakashan; 1998.
28. Maurias M. Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure activity relationship. Bioor & Med Chem. 2004; 12: 5571-5578.
29. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958; 181: 1199-1150.
30. Roberta R. Luciana GM, Luciana CC, Glaucia P. Evaluation of the antioxidant Properties of the Brazilian Cerrado fruit *Annona crassiflora* (Araticum). J of Food Sci. 2006; 71**:** 102-107.
31. Rollet-Labelle E, Gragne MS, Elbim C, Marquetty C, Gougerot-Pocidalo MA. Hydroxyl radicals as a potantial intracellular mediator of polymorpho nuclear neutrophil apoptosis. Free Rad Biol & Med. 1998; 24: 563-572.
32. Liu F, Ooi VEC, Chang ST. Free radical scavenging activity of mushroom polysaccharide extracts. Life Sci. 1997; 60: 763-771.
33. Mosmann T. Rapid colorimetric assay for cellular grow and survival: application to proliferation and cytotoxicity assays. J of Immuno & Method. 1983; 65: 55-63.
34. Mantani N, Imanishii N, Kawamata H, Terasawa K, Ochiai H. Inhibitory effect of (+)-catechin on the growth of influenza A/PR/8 virus in MDCK cells. Planta Med. 2001; 67: 240-243.
35. Kawase M, Motohashi N, Satoh K, Sakagami H, Nakashima H. Biological activity of Persimmon (*Diospyros kaki*) peel extracts. Phytother Res. 2003; 17: 495- 500.
36. Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. J of Pharmacol Sci. 2004; 96: 229-245.
37. Gacche RN, Dhole NA. Evaluation of an antioxidant and possible anti-inflammatory potential of selected medicinal plants prescribed in Indian traditional system of medicine. J of Pharm Biol. 2006; 44(5): 1-7.
38. Alberto MR, Zampini IC, Isla MI. Inhibition of cyclooxygenase activity by standardized hydroalcoholic extracts of four Asteraceae species from the Argentine Puna. Braz J of Med and Biol Res. 2009; 42: 787-790.
39. Recio MC, Giner RM, Manez S, Rios JL. Structural considerations on the iridoids as anti-inflammatory agents. Planta Med. 1994; 60: 232–234.
40. Feldman MD, Mc Mohan AT. Do cyclooxygenase-2 inhibitors provide benefits similar to those of traditional non steroidal anti-inflammatory drugs, with less gastrointestinal toxicity. Annal of Inter Med. 2000; 132: 134-143.
41. Lindsey KL, Jagar AK, Viljoen A. Cyclooxygenase inhibitory activity of Aloes. S Afr J of Bot. 2002; 68: 47-50.
42. Pillay CCN, Jagar AK, Mulholland DA, van Standen J. Anti-inflammatory and anti-bacterial activities of South African *Erythrina* species. J of Ethanopharmacol. 2001; 74: 231-237.
43. Taylor JLS, van Staden J. COX-1 and COX-2 inhibitory activity in extracts prepared from *Eucomis* species, with further reference to extracts from *E. autumnalis* . S Afr J of Bot. 2002; 68: 80-85.
44. Trouba KJ, Hamadeh HK, Amin RP, Germolec DR. Oxidative stress and its role in skin disease. Antiox & Red Signal. 2002; 4: 665–673.
45. Contreas-Guzman ES, Strong FC. Determination of tocopherol (vitamin E) by reduction of cupric ion. J of Asso of Anal Chem. 1982; 65: 1215-1222.
46. Demetrios NN, Konstantinos EL, Dimitra HL. Synthesis and biological evaluation of several coumarin-4-carboxamidoxime and 3-(coumarin-4yl)-1,2,4-oxadiazole derivatives. Euro J of Med Chem. 1998; 33: 715-724.
47. Drouge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002; 82: 47-95.
48. Barry H, John MCG. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J. 1984; 219: 1-14.
49. Eastwood MA. Interaction of dietary antioxidants in vivo: How fruit and vegetables prevent disease? Quart. J of Med. 1999; 92: 527-530.

Anti-obesity (Pancreatic lipase inhibitory) activity of *Everniastrum cirrhatum* (Fr.) Hale (Parmeliaceae)

Anil Kumar HS[1*], Prashith Kekuda TR[2], Vinayaka KS[3], Swathi D[2], Venugopal TM[2]

[1] *Department of Biotechnology Engineering, NMAM Institute of Technology, Nitte-574110, Udupi, Karnataka, India*

[2] *Dept. of PG Studies and Research in Microbiology, Sahyadri Science College (Autonomous), Shivamogga-577203, Karnataka, India*

[3] *Dept. of PG Studies and Research in Botany, Kuvempu University, Jnanasahyadri, Shankaraghatta-577451, Karnataka, India*

ABSTRACT

Introduction: Obesity has increased at an alarming rate and is now a worldwide health problem. *Everniastrum cirrhatum* (Fr.) Hale (Parmeliaceae) is a foliose lichen and grow luxuriantly in tropical Himalayas, central India and higher altitudes of southern India. In this study, we report Anti-obesity activity, in terms of pancreatic lipase inhibitory activity, of methanol extract of *E. cirrhatum* for the first time. **Methods:** The powdered lichen material was extracted with methanol in soxhlet apparatus. The extract was tested for secondary metabolites by standard phytochemical tests and thin layer chromatography (TLC). Lipase enzyme was obtained from the chicken pancreas. Lipase inhibitory activity of different concentrations of methanol extract was determined in terms of inhibition of lipase activity using olive oil as substrate. **Results:** The extract was found to inhibit activity of chicken pancreatic lipase and the effect was found to be concentration dependent. Phytochemical analysis revealed the presence of alkaloids, saponins, tannins and terpenoids. TLC revealed Atranorin, Salazinic acid and Protolichesterinic acid. **Conclusion:** The result of lipase inhibitory activity of the lichen in this study is promising. The inhibitory activity may be attributed to the presence of secondary metabolites. In suitable form, the lichen could find its application as anti-obesity agent. Further studies on isolation of active principles from the extract and their enzyme inhibitory activity are under investigation.

Keywords: *Everniastrum cirrhatum* (Fr.) Hale, Anti-obesity activity, Pancreatic lipase, Lipase inhibitory activity, *Gallus domesticus*, Olive oil

INTRODUCTION

Lichens are symbiotic organisms composed of a fungus (mycobiont) and an alga (photobiont). They produce characteristic secondary metabolites, lichen substances, which seldom occur in other organisms.[1] *Everniastrum cirrhatum* (Fr.) Hale (Parmeliaceae) is a foliose lichen in which phycobiont is a chlorolichen alga. It usually grows on the barks of trees in temperate regions.[2,3] It grows luxuriantly in tropical Himalayas, central India and higher altitudes of southern India. It is characterized by linear, laciniate lobes which are grey in color. Thallus is tapering apically, 2-6mm wide and 10cm long. Thallus is loosely attached to the substratum and pendulous in nature. Apothecia are laminal, margins are inflexed, cilia are black in color, and spores are large.[4] In Ayurveda, it is mentioned as astringent, resolvent, laxative, carminative and aphrodisiac. It is also useful in bleeding piles, leprosy and excessive salivation. It is used as spices in Madhya Pradesh. Whole boiled material used as vegetable in Nepal and north Sikkim.[5,6] *E. cirrhatum* is traditionally used as antiseptic, used to heal wound and bronchitis in Sikkim.[7] *E. cirrhatum* has been used as material for sacrificial fire by Gaddi tribe of Kangra valley. It is as a spice and flavoring agent for meat and vegetables by Bhaiga, Bhil, Bhilala, Gond, Korka, Muria of Madhya Pradesh. It is used as vegetables in Lepchas and Nepalese of Sakyong valley, North Sikkim. In Uttaranchal, Uttar Pradesh and Sikkim, it is commercially sold as spice.[8] The mycobiont (lichen forming fungus) of *E. cirrhatum* was shown to cause mycelial growth inhibition of hot pepper

Address for correspondence:
Anil Kumar HS, Department of Biotechnology Engineering, NMAM Institute of Technology, Nitte-574110, Udupi, Karnataka, India.
Ph: +919845372379
Email: anilkumarhs1979@rediffmail.com

DOI: 10.5530/pj.2011.19.12

anthracnose pathogen, *Colletortichum acutatum*.[1] Ethanol extract of *E. cirrhatum* has shown antimycobacteral properties against *Mycobacterium tuberculosis* H37Rv and H37Ra strains with minimum inhibitory concentration of 500µg/ml.[3] In a study, it was reported that the whole thallus of *E. cirrhatum* yielded a red brown dye.[9] Although it is a used as traditional medicine in Indian subcontinent, literature surveys reveals that many of the bioactivities including Anti-obesity activity of this lichen has not yet been documented. In this study, we report for the first time the anti-obesity activity (in terms of lipase inhibitory activity) of methanol extract of *E. cirrhatum* from Bhadra wildlife sanctuary, Karnataka, India.

MATERIALS AND METHODS

Collection and identification of lichen

The lichen *E. cirrhatum*, growing on barks of trees, was collected from the Bhadra wildlife sanctuary, Karnataka, India, during August 2010. The lichen specimen was identified by morphological, anatomical, chemical tests.[10] The voucher specimen of the lichen (Voucher no. KU00703) was deposited in the University herbaria, Department of PG Studies and Research in Botany, Shankaraghatta-577451, Karnataka, India for future reference.

Detection of secondary metabolites by thin layer chromatography (TLC)

The shade dried powdered lichen material was extracted with methanol, spotted on the silica plate and developed with solvent A (180 ml toluene: 60 ml 1-4, dioxine: 8 ml acetic acid) to detect secondary metabolites using standard protocols.[11,12]

Preparation of extract using methanol

The lichen was dried at room temperature under shade. After drying, the lichen material was ground to fine powder and extracted by soxhlet apparatus using methanol as solvent. The extract was filtered using Whatman filter paper no. 1 and concentrated at 40°C under reduced pressure. The condensed methanol extract was stored at 4°C until use.[13]

Phytochemical analysis of methanol extract

The extract obtained after solvent evaporation was subjected to standard tests for detection of alkaloids (Dragendorff's reagent and Mayer's reagent), tannins (ferric chloride test), saponins (frothing test and hemolysis test), glycosides (Salkawski test and Keller-Kiliani test), sterols (Burchard test), flavonoids (Shinoda test) and terpenoids (Salkowski test).[14,15]

Anti-lipase activity of methanol extract of *E. cirrhatum*

Extraction of lipase from Chicken (Gallus domesticus) pancreas:

Pancreas of freshly slaughtered chicken were collected, washed and placed in ice cold sucrose solution (0.01M). The pancreas was homogenized in 0.01M sucrose, centrifuged, supernatant was separated and subjected to ammonium sulphate precipitation (50% saturation). The pellet obtained after centrifugation was dissolved in sucrose solution and again saturated to 50% ammonium sulphate saturation and centrifuged. The pellet obtained was dissolved in Phosphate buffer and used as enzyme source.[16]

Determination of Chicken Pancreatic Lipase activity:

The activity of lipase was determined by incubating an emulsion containing 8ml of olive oil, 0.4ml of phosphate buffer and 1ml of chicken pancreatic lipase for an hour in rotary shaker, followed by stopping the reaction by addition of 1.5ml of a mixture solution containing acetone and 95% ethanol (1:1). The liberated fatty acids were determined by titrating the solution against 0.02M NaOH (standardized by 0.01M oxalic acid) using phenolphthalein as an indicator. [17,18]

Lipase Inhibitory activity of methanol extract of E. cirrhatum:

Lipase inhibitory activity of different concentrations of methanol extract was tested by mixing 100µl of each concentration of methanol extract, 8ml of oil emulsion and 1ml of chicken pancreatic lipase followed by incubation of 60 minutes. The reaction was stopped by adding 1.5 ml of a mixture solution containing acetone and 95% ethanol (1:1). The liberated fatty acids were determined by titrating the solution against 0.02M NaOH (standardized by 0.01M oxalic acid) using phenolphthalein as an indicator. [17-19] Percentage inhibition of lipase activity was calculated using the formula:

Lipase inhibition = A − B/A × 100, where A is lipase activity, B is activity of lipase when incubated with the extract.

Statistical Analysis

All data were expressed as mean ± SD of the number of experiments (n =6). Past software version 1.92 was used.

RESULTS AND DISCUSSION

Secondary metabolites detected in *E. cirrhatum*

Preliminary phytochemical analysis of methanol extract of *E. cirrhatum* was determined by chemical tests. Phytoconstituents

namely alkaloids, saponins, tannins and terpenoids were detected in the extract (Table-1). TLC in solvent A showed the presence of Atranorin, Salazinic acid and Protolichesterinic acid in the lichen material.

Lipase inhibitory activity of methanol extract of *E. cirrhatum*

Inhibitory activity on chicken pancreatic lipase of different concentrations of methanol extract of *E. cirrhatum* was determined using olive oil as the substrate. The activity of pancreatic lipase was checked. It was found that the activity of lipase was affected when incubated with the methanol extract. The inhibitory activity was found to be dose dependent i.e., higher inhibition of enzyme was observed on increasing the concentration of extract. A marked inhibition of enzyme activity was observed with extract concentration 5mg/ml and higher (Figure 1).

Table 1: Phytoconstituents detected in methanol extract of *E. cirrhatum*

Phytoconstituent	Test	Result
Alkaloids	Dragendorff's test	+
	Mayer's test	+
Saponins	Frothing test	+
	Hemolysis tst	+
Flavonoids	Shinoda test	-
Glycosides	Salkowski test	-
	Keller-Kiliani test	-
Tannins	Ferric chloride test	+
Sterols	Burchard test	-
Terpenoids	Salkowski test	+

'+' Present; '-' Absent

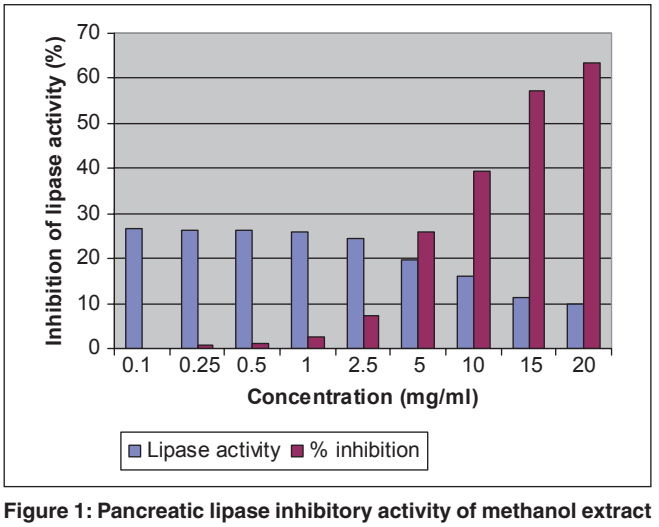


Figure 1: Pancreatic lipase inhibitory activity of methanol extract of *E. cirrhatum*

Obesity has increased at an alarming rate and is now a worldwide health problem. It is widely accepted that obesity results from disequilibrium between energy intake and expenditure. Obesity is found to be strong risk factor for type 2 diabetes. Dietary lipids represent the major source of unwanted calories; therefore, lipid metabolism is a vital and subtle balance that maintains energy homeostasis. Once this balance is lost, obesity or hyperlipidemia develops, followed by a series of severe diseases, including atherosclerosis, hypertension, diabetes, and dysfunction of certain organs. Obviously, drug control of lipid metabolism offers a possible way to prevent or treat these diseases. The identification and characterization of several enzymes involved in lipid metabolism have yielded a rich pool of potential targets for drugs to treat obesity and other metabolic disorders. [20-24] Pancreatic lipase, the main lipid-digesting enzyme, removes fatty acids from the α and α' position of dietary triglycerides, which yields the lipolytic product β-monoglyceride and long-chain saturated and polyunsaturated fatty acids. Inhibition of pancreatic lipase is an attractive targeted approach for the treatment of obesity. [22] Naturally occurring compounds present an exciting opportunity for the discovery of newer anti-obesity agents. A number of studies have been carried on Anti-lipase activity of natural products. In a study, 95% ethanol extract of *Taraxacum officinale* inhibited porcine pancreatic lipase activity by 86.3% at a concentration of 250µg/ml. [25] The seed extract of *Vitis vinifera* was found to cause 80% inhibition of porcine pancreatic lipase at concentration of 1 mg/ml. [26] An extract of *Nomame herba* was found to possess inhibitory activity against porcine pancreatic lipase in a dose-dependent manner. [27] In our study also, a marked inhibition of chicken pancreatic lipase by the extract was observed which is consistent with earlier reports on lipase inhibition by natural products.

CONCLUSION

Obesity is one of the main public health problems in developed countries. It is considered to be a risk factor associated with the genesis or development of major chronic diseases, including cardiovascular disease, diabetes, and cancer. A marked inhibition of activity of pancreatic lipase by methanol extract of *E. cirrhatum* was observed in this study. The inhibitory role could be attributed to the presence of various secondary metabolites in the extract. The extract may be used as anti-obesity agent in suitable form. Further studies on isolation of active principles from the extract and their inhibitory efficacy against lipases are under investigation.

REFERENCES

1. Wei X, Jeon H, Han KS, Koh YJ, H J. Antifungal activity of Lichen-forming fungi against *Colletotrichum acutatum* on Hot pepper. Plant Pathology Journal 2008; 24(2):202-206

2. Nayaka S, Ranjan S, Saxena P, Pathre UV, Upreti DK and Singh R. Assessing the vitality of Himalayan lichens by measuring their photosynthetic performances using chlorophyll fluorescence technique. Current Science 2009; 97(4):538-545
3. Gupta VK, Darokar MP, Saikia D, Pal A, Fatima A and Khanuja SPS. Antimycobacterial Activity of Lichens. Pharmaceutical Biology 2007; 45(3):200-204
4. Divakar PK and Upreti DK. Parmelioid lichens in India. Bishen Singh Mahendra Pal Singh publisher. Dehra Dun, India, 2005, Pp-97-98.
5. Lal B, Upreti DK and Kalakoti BS. Ethnobotanical utilization of lichens by the tribals of Madhya Pradesh. J Eco Tax Bot 1985; 7: 203-204
6. Chandra S and Singh A. A lichen drug from India. J Res Ind Med 1971; 6:209-225
7. Saklani A, Upreti DK. Folk uses of some lichens in Sikkim. J Ethnopharmacol 1992; 37:229-233.
8. Upreti DK, Divakar PK, Nayaka S. Commercial and Ethnic use of lichens in India. Economic Botany 2005; 59(3):269-273
9. Gaur RD. Traditional dye yielding plants of Uttarakhand, India. Natural Product Radiance 2008; 7(2):154-165
10. Awasthi DD. A Compendium of the Macrolichens from India, Nepal and Sri Lanka. Dehra Dun: Bishen Singh Mahendra Pal Singh Publishers and Distributors of Scientific books, 2000, 1-580.
11. Culberson CF. Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. J Chromatogr 1972; 72:113-125.
12. Walker FJ and James PW. A revised guide to microchemical technique for the identification of lichen products. *Bull Brit Lich Soc 1980;* 46: 13-29 (Supplement).
13. Manjunatha BK, Patil HSR, Vidya SM, Kekuda TRP, Mukunda S and Divakar R. Studies on the antibacterial activity of *Mucuna monosperma* DC. Indian Drugs. 2006; 43:150-152
14. Mallikarjuna PB, Rajanna LN, Seetharam YN and Sharanabasappa GK. Phytochemical studies of *Strychnos potatorum* L.f.- A medicinal plant. E-Journal of Chemistry 2007; 4(4):510-518
15. George NJ, Obot JB, Ikot AN, Akpan AE and Obi-Egbedi NO. Phytochemical and Antimicrobial properties of leaves of Alchonea cordifolia. E-Journal of Chemistry 2010; 7(3):1071-1079
16. Sahani KM, Khan IM, Chandan RC. Bovine Pancreatic Lipase: Isolation, Homogeneity, and Characterization. J Dairy Sci 1976; 59(3):369-375
17. Hasan F, Shah AA, Hameed A. Methods for detection and characterization of lipases A comprehensive review. Biotechnology Advances 2009; 27(6): 782-798
18. Werasit K, Boonpan A. Screening of Halophilic Lipase-Producing Bacteria and Characterization of Enzyme for Fish Sauce Quality Improvement. Nat Sci 2007; 41:576-585.
19. Zheng C, Duan Y, Gao J, Ruan Z. Screening for Anti-lipase Properties of 37 Traditional Chinese Medicinal Herbs. J Chin Med Assoc 2010; 73(6):319-324
20. Shi Y, Burn P. Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. Nat Rev Drug Discov 2004; 3: 695–710.
21. Mukherjee M. Human digestive and metabolic lipases: a brief review. J Mol Catal B Enzym 2003; 22:369-76.
22. Thomson AB, De Pover A, Keelan M, Jarocka-Cyrta E, Clandinin MT. Inhibition of lipid absorption as an approach to the treatment of obesity. Meth Enzymol 1997; 286:3-41.
23. Larsson B, Bjorntorp P, Tibblin G. The health consequences of moderate obesity. Intl J Obesity 1981; 5:97-116.
24. Hartz AJ, Rupley Jr DC, Kalkhoff RD, Rimm AA. Relationship of obesity to diabetes: influence of obesity level and body fat distribution. Preventive Medicine 1983; 12:351-357.
25. Zhang J, Kang M, Kim M, Kim M, Song J, Lee Y, Kim J. Pancreatic lipase inhibitory activity of *Taraxacum officinale in vitro* and *in vivo.* Nutr Res Pract 2008; 2(4):200-203
26. Moreno DA, Ilic N, Poulev A, Brasaemle DL, Fried SK, Raskin I. Inhibitory Effects of Grape Seed Extract on Lipases. Nutrition 2003; 19:876-879
27. Yamamoto M, Shimura S, Itoh Y, Ohsaka T, Egawa M, Inoue S. Anti-obesity effects of lipase inhibitor CT-II, an extract from edible herbs, Nomame herba, on rats fed a high-fat diet. Int J Obes Relat Metab Disord 2000; 24(6):758-764

In vitro Effectiveness of *Acacia concinna* Extract against Dermatomycotic Pathogens

Mansuang Wuthi-udomlert[1*] and Omboon Vallisuta[2]

[1]Department of Microbiology, [2]Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand.

ABSTRACT

Acacia concinna (Wild.) D.C. is an important medicinal plant in Thailand and throughout Asian countries. Its dried pods are traditionally utilized as herbal medicine to treat many health symptoms e.g. laxative, cough, antidandruff and skin diseases. This investigation was performed in order to demonstrate the antimicrobial potential of different *A. concinna* extracts against the fungal causative agents of ringworm and opportunistic infections of immunocompromised populations. Phytochemical study showed that the crude extract of *A. concinna* pod consisted of alkaloids, flavonoids, saponin and tannin but none of antraquinone and cyanotic glycosides. The extracts e.g. ethanolic Soxhlet extract and chloroform extract from Soxhlet extractor, and lyophilized extract and macerated ethanolic extract were tested using diffusion method against 35 isolates of dermatophytes and each 20 isolates of *Candida albicans, Cryptococcus neoformans* and *Penicillium marneffei.* The inhibitory effects were observed by considering their average inhibitory zone diameter (IZD) compared to that of ketoconazole's. From the averaged IZDs of all fungal isolates, the antifungal effectiveness of lyophilized, chloroform, ethanolic (Soxhlet) and ethanolic (macerated) extract was at 18.38, 18.34, 16.67 and 14.06 mm, respectively.

Key words: *Candida*, *Cryptococcus*, dermatophytes, traditional medicine, lyophilized extract, soap pod

INTRODUCTION

Dermatophytes are a group of three keratinophilic fungal genera, *Trichophyton, Epidermophyton* and *Microsporum*, that cause daily found dermatomycotic symptoms called tinea or ringworm on different parts of human body, as well as animals'. This contagious mycopathogens may spread among human or from animal or soil to human.[1] Although dermatophytoses do not express any life threatening but their clinical appearances are one of cosmetic problems,[2-3] mental annoyance and can be regarded as everyday troublesomeness. Most of modern topical drugs available are not always affordable by people who required a long term treatment to clear those disturbing features, these cost a nation high budget for the effective treatment of the daily found mycoses.

Address for correspondence:
Mansuang Wuthi-udomlert, Department of Microbiology,
Faculty of Pharmacy, Mahidol University,
447 Sri Ayuthaya Rd; Rajthevi, Bangkok 10400, Thailand.
Email: pymwt@mahidol.ac.th

DOI: 10.5530/pj.2011.19.13

Apart from these troubles, *Candida albicans* and *Cryptococcus neoformans* are involved as frequently found mycoses in tropical regions, alongside with acquisition of penicillosis from *Penicillium marneffei* which disseminated in patients infected by human immunodeficient virus.[4-7] The interesting essentials from plant extracts that possess activities against dermatophytes might express similar effects upon these yeast-like fungi and dimorphic fungi.

Fortunately, many of those who faced these difficulties reside in regions where herbs are utilized as traditional medicines to which formularies and recipes have been accustomed. For this reason, using of traditional herbal medicines is an alternative mode in biodiversity rich countries, with the support of Government and by regulations, scientific researches in related area are increasing accordingly. Therefore, the effectiveness of native medicinal plants and their extracts by various methods of extraction can be used as alternative manipulation to treat many common infections.

Acacia concinna (Wild.) D.C. or soap pod, is naturally found and being cultivated throughout South Asian countries. The grind legume fruit powder is good to clean hair for its superior cleansing quality to give lustrous hair, promoting

hair growth and also reducing dandruff. Besides, other medicinal activities of this pod fruit are for phlegm expellant, laxative, cough syrup, appetizer, skin disease and fever treatment. *A. concinna* is recorded on the use as traditional medicines i.e. the root is to treat fever and intestinal disorder, stem and leaves for laxative, stem bark to expel phlegm and spoilage, flowers to treat ligamentous dysfunction. Therefore, this plant is now being cultivated in large quantities for commercial purpose in India and the Far East because of its versatile properties.[8-10]

However, various approaches on medicinal plant usages render several varieties of essential ingredients from plant. Because medicinal plants are used on the basis of traditional knowledge in which conventional household consumption is made from fresh plant naturally growth or kitchen garden cultivation. Most of the utilizations are squeezing to obtain juice, boiling with water, maceration in local made alcohol or modifying into preserved part of plant, honey pill, tablet, powder, sachet etc. To fulfill the immediate requirement of traditional medicines and to preserve the over-supply of fresh cultivation of seasonal products, simple expediency to keep raw products for long term storage gives rise to the community the benefit in managerial skill of local raw products. Therefore, instead of freshly used, the specific part of plant can be macerated in a short time and aqueous supernatant is lyophilized, the dried material obtained can be conveniently reconstituted to readily deliver the exact quantitative amount. The activities of this lyophilized extract are compared with those from the use of conventional Soxhlet apparatus.

Biological activity of medicinal plant extracts can be assessed by various techniques, from qualitative diffusion in agar plates, quantitative assay of dilution in broth or in agar, and the commercial E Test which combines those two methods' principles. Research studies reported the congenial results between qualitative diffusion test and quantitative dilution test. Not only for unicellular fungi e.g. *Candida,*[11-16] or *Geotrichum,*[17] but also for filamentous fungi e.g. dermatophytes,[18] *Aspergillus,*[19-20] opportunistic hyphomycetes i.e. species of *Fusarium, Cladosporium,* and ascomycetes i.e. *Chaetomium* spp. as well.[21] However, these were evaluated for modern drugs activities, different protocols and several conditions were employed and presented in various interesting aspects. These scientific evidences revealed the relationship of disc diffusion test and those of quantitative tests; therefore, the likewise protocol should be effective in the evaluation for bioactivity of medicinal plant extracts to manifest the required substantial properties which lead to further modification of extracts into novelty herbal drugs.

This research is aimed to find out whether the medicinal plants appeared in the records of Thai traditional medicines demonstrate scientific proof for antifungal activities. The pods of *A. concinna* were macerated in ethanol, maceration in water and further dried out by the use of lyophilizer and the extraction by using Soxhlet apparatus. Therefore, ethanolic extract, aqueous lyophilized extracts and the Soxhlet ethanolic and chloroform extracts, respectively were obtained. All were tested against 35 isolates of dermatophytes and each of 20 isolates of *Candida albicans, Cryptococcus neoformans* and *Penicillium marneffei* by the use of diffusion method.

MATERIALS AND METHODS

Plant materials and plant extracts

Matured and dried fruits of *Acacia concinna* (Wild.) DC. was purchased from local suppliers and identified according to the voucher specimens deposited in Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, and the forest herbarium of the Royal Forest Department, Bangkok, Thailand.

Ethanolic extract and chloroform extract: using Soxhlet extractor

Dried ground powder of *A. concinna* was submitted to sequential extraction with chloroform and methanol using Soxhlet apparatus, the extract obtained was vacuum evaporated.

Ethanolic extract: maceration

A. concinna powder was overnight macerated under aseptic condition in 95% ethanol (1:4, w/v); after filtered through sterile gauze, the filtrate was vacuum evaporated.

Aqueous lyophilized extract: using lyophilizer

All steps in the following procedures were aseptically done. Clean, dried ground fruit of *A. concinna* was macerated in sterile distilled water at 1:4 w/v for 24 h. The supernatant after filtering was quantitatively collected and further processed with lyophilizer (Leybold-Haerareus, Lyovac GT-2, Germany). Aqueous lyophilized extract was kept in bottle with tight closed lid at -20° C.

Antifungal activity test

The antimicrobial activity was determined by agar diffusion assay described as the followings.

Microbial cultures

Clinical strains of 35 isolates of dermatophytes and each of 20 isolates of *Penicillium marneffei, Candida albicans* and

Cryptococcus neoformans were obtained from Department of Microbiology, Faculty of Pharmacy, Mahidol University. All pure isolates were kept as stock strain at -20° C on Sabouraud dextrose agar (SDA) (Pronadisa, S.A. Spain).

Inoculum preparation

Active growth of dermatophytes isolates and *P. marneffei* with the appropriate selected characteristics was suspended in Sabouraud dextrose broth (SDB) (Pronadisa, S.A. Spain), to give the fungal density equivalent to turbidimetric No. 1 McFarland. Culture of *Candida albicans* and *Cryptococcus neoformans* were done likewise but to the density of No. 0.5 McFarland.

Extracts and reference drug preparation

A. concinna extracts were prepared as followings: ethanolic extract and chloroform extract from Soxhlet extractor were diluted 1:1 with their corresponding solvent; aqueous lyophilized extract was treated in similar manner with sterile distilled water. All test extracts were incorporated onto 6 mm in diameter sterile blank disc (Schleicher & Schuell, Germany) at 20 µl/disc. Ketoconazole (Siemsgluss & Sohn, Germany) was dissolved in methanol to give the net amount of drug at 20 µg ketoconazole/disc. Test control discs were ethanol and chloroform at 20 µl/disc each. Test discs were prepared and used immediately after optimally dried out in sterile condition.

Test methods

Suspension of all isolates of dermatophytes and *P. marneffei,* 20 µl each, was top layer cultivated onto total amount of 20 ml SDA plates whereas suspension of *C. albicans* and *C. neoformans* was aseptically swabbed onto 20 ml SDA plates. After the surfaces of all test plates were properly dried, at least duplicates of prepared discs of each extract and ketoconazole were laid onto the surface of inoculated plates. Inoculated plate of dermatophytes and *P. marneffei* were incubated at room temperature while *C. albicans* and *C. neoformans* plates at 37° C. Diameter of each inhibitory zone was 3-time measured at different radial positions and arithmetically averaged. The figures obtained were considered as the activity of test materials. Culture control of each microorganism was included in similar manner to deliver the perfect timing in measuring the inhibitory zones.

RESULTS

Two samples of ethanolic extract were accessed from evaporating macerated sample and from Soxhlet extractor yielded different percentages of extracts. The first mentioned extract gave nearly twice amount compared to the Soxhlet extract whereas aqueous extract gave the highest yield among all (Table 1).

Phytochemical Analysis

Phytochemical properties of *A. concinna* fruit were alkaloids, saponin, tannin, flavonoids and cardiac glycosides but no antraquinone and cyanotic glycosides (Table2).

However, using equal amount of suspension of each extract incorporated onto each disc, the different amount of ground material of *A. concinna* employed for one single disc could be calculated (Table 3).

Table 1: Percentage yield of *A. concinna* extract from different extraction methods.

Extract	% yield
Ethanolic	14.18
Lyophilized	26.22
Chloroform	10.67
Ethanolic (Soxhlet)	8.73

Table 2: Phytochemical properties of *A. concinna* fruit

Plant	Alkaloids	Saponin	Tannin	Flavonoids
A.. concinna fruit	+	+	+	+

Table 3: Antifungal activity of *A. concinna* extracts as inhibitory zone diameter (IZD)

	Inhibitory Zone Diameter (mean ± SD, mm)				
Organisms (n)	Ethanolic Extract (0.14)*	Lyophilized Extract (0.08)*	Ethanolic (Soxhlet) extract (0.23)*	Chloroform Extract (0.19)*	Ketoconazole 20 µg/disc**
Dermatophytes (35)	14.88 ± 0.48	21.66 ± 0.49	18.50 ± 0.45	27.69 ± 0. 52	32.22 ± 0.92
P. marneffei (20)	18.71 ± 0.55	19.80 ± 0.66	23.31 ± 0.66	17.90 ± 0.85	35.25 ± 0.59
C. albicans(20)	10.56 ± 0.71	10.67 ± 0.83	10.75 ± 1.05	12.33 ± 0.67	26.60 ± 0.48
C. neoformans(20)	12.09 ± 1.07	21.39 ± 0.55	14.13 ± 0.43	15.42 ± 1.07	31.89 ± 0.41
Arithemetic mean	14.06	18.38	16.67	18.34	31.49

*mg/disc = calculated mg of raw grinding *A. concinna* **approx. eqv. to 1 mg of 2% topical drug

DISCUSSION

Phytochemical ingredients are continuously employed as natural essentials which are being promoted in many regions to preserve and to remunerate local resources. The versatile utilities of *A. concinna* has appeared in diverse traditional records with different stresses in certain property e.g. the use as laxative is accepted and being included as the traditional use in the National List of Essential Drugs and herbal crude drugs in Thai traditional household remedies since 2004.[22]

This study employed various extraction methods of *A. concinna* fruit and demonstrated the activity of those extracts by a selected test on the basis of return value to support further traditional plant use in public health care. Hence, agar-based method or diffusion test was the method of choice for the fact that this test is simple, less time consuming in performing the test, less labor intensive, low cost, readable result at different times without subculture confirmation, and being reproducible method.

Considering the IZDs of overall isolates, dermatophytes and *P. marneffei* exhibited the respective highest susceptibility to chloroform extract and ethanolic extract from Soxhlet extractor, respectively. The result from lyophilized extract gave the best result on *C. neoformans*. All extracts exerted less than one half efficacy on *C. albicans* in comparison with those of reference drugs, ketoconazole. Nevertheless, these results exhibited the effectiveness of all *A. concinna* extracts to be selected for further formulation.

On calculation of the raw material used for each disc i.e. calculation of the incorporated extract on disc, extract yield and the first weight of raw material used for extraction, the positive inhibitory reaction revealed that aqueous lyophilized extract was the most economically advantageous (0.08 mg raw powder per disc) compared with other extracts. The second was macerated ethanolic extract (0.14 mg raw powder per disc). This implied the fact that the use of *A. concinna* was so simple and able to verify the mode of utilization of ancient medicine e.g. using herbs with water and/or alcohol.

From various extraction methods, the use of Soxhlet apparatus which is generally employed and basically familiar in pharmacognosy area requires a lot of resources: chemicals, instrument, time consumed and personal expertise. However, while the antifungal activity of *A. concinna* extracts are apparently demonstrated, it is of interest to investigate whether those activities are attributed to any of phytochemical components e.g. alkaloids, saponin, tannin or flavonoids.

ACKNOWLEDGMENTS

The content of this manuscript is a part of project granted by The National Research Council of Thailand (NRCT). We would like to thank the NRCT for their permission to publish this article.

REFERENCES

1. Rippon JW. Medical mycology. The pathogenic fungi and the pathogenic actinomycetes, 3rd ed. WB Saunders, Philadelphia, 1988; Pp 134.
2. Gupta AK, Albreski D, Del Rosso JQ, Konnikov N. The use of the new oral antifungal agents, Itraconazole, Terbinafine, and Fluconazole, to treat onychomycosis and other dermatomycoses. *Current Problems in Dermatology* 2001; 13(4):213-246.
3. Scher RK. Onychomycosis is more than a cosmetic problem. *Br. J. Dermatol*. 1994; 130(Suppl. 43):15.
4. Vithayasai P, Vithayasai V. Atlas of HIV infection, 2nd ed. Support The Children Foundation. O.S. Printing.1994; Pp 1,8,41.
5. Andrianopoulos A. Control of morphogenesis in the human fungal pathogen *Penicillium marneffei*. *Int. J Med Microbiol*. 2002; 292; 331-347.
6. Chayakulkeeree M, Perfect JR. Cryptococcosis. *Infect Dis Clin North Am*. 2006;20(3):507-44.
7. Supparatpinyo K, Khamwan C, Baosoung V, Sirisanthana T, Nelson KE. Disseminated *Penicillium marneffei* infection in Southeast Asia. *The Lancet* 1994; 344(8915):110-113.
8. Poonam K, Singh GS. Ethanobotanical study of medicinal plants used by the Taungya community in Terai Arc Landscape, India. *J Ethnopharmacol*. 2009; 123:167-176.
9. Natarajan V, Natarajan S. Antidermatophytic activity of *Acacia concinna*. *Global J of Pharmacol.* 2009; 3 (1):06-07.
10. Singh S, Pandy P, Kumar S. Traditional knowledge on the medicinal plants of Ayurveda. Central Institute of Medicinal and Aromatic Plants(CIMAP), India, Lucknow, 2000; Pp 281.
11. Barry AL, Pfaller MA, Rennie RP, Fuchs PC, Brown SD. Precision and accuracy of fluconazole susceptibility testing by broth microdilution, Etest, and disk diffusion methods. *Antimicrob. Agents Chemother*. 2002; 46(6):1781-1784.
12. Matar MJ, Ostrosky-Zeichner L, Paetznick VL, Rodriguez JR, Chen E, Rex JH. Correlation between E-Test, disk diffusion, and microdilution methods for antifungal susceptibility testing of fluconazole and voriconazole. *Antimicrob. Agents Chemother*. 2003; 47(5):1647-1651.
13. Odabasi Z, Paetznick V, Goldstein BP, Rex JH, Ostrosky-Zeichner L. Disk Diffusion-based methods for determining *Candida parapsilosis* susceptibility to anidulafungin. *Antimicrob. Agents Chemother*. 2003; 47(9):3018-3020.
14. Sims CR, Paetznick VL, Rodriguez JR, Chen E, Ostrosky-Zeichner L. Correlation between microdilution, E-test, and disk diffusion methods for antifungal susceptibility testing of posaconazole against *Candida* spp. *J Clin Microbiol*. 2006; 44(6):2105-2108.
15. Kiraz N, Dag˘ I, Yamac M, Kiremitci A, Kas¸ifog˘lu N, Akgun Y. Antifungal activity of caspofungin in combination with amphotericin B against *Candida glabrata*: comparison of disk diffusion, Etest, and time-kill methods. *Antimicrob. Agents Chemother*. 2009:53(2): 788-790.
16. Mandras N, Tullio V, Allizond V, Scalas D, Banche G, Roana J, et al. *In Vitro* Activities of fluconazole and voriconazole against clinical isolates of *Candida* spp. Determined by disk diffusion testing in Turin, Italy. *Atimicrob. Agents Chemother*. 2009; 53(4):1657-1659.
17. Girmenia C, Pizzarelli G, D'Antonio D, Cristini F, Martino P. *In Vitro* Susceptibility testing of *Geotrichum capitatum*: comparison of the E-Test, disk diffusion, and sensititre colorimetric methods with the NCCLS M27-A2 broth microdilution reference method. *Antimicrob. Agents Chemother*. 2003; 47(12):3985-3988.

18. Ferna´ndez-Torres B, Carrillo-Mun˜oz A, Inza I, Guarro J. Effect of culture medium on the disk diffusion method for determining antifungal Susceptibilities of dermatophytes. *Antimicrob. Agents Chemother.* 2006; 50(6); 2222-2224.
19. Arikan S, Yurdakul P, Hascelik G. Comparison of two methods and three end points in determination of *in vitro* activity of micafungin against *Aspergillus* spp. Antimicrob. Agents Chemother. 2003; 47(8):2640-2643.
20. Lo´pez-Oviedo E, Aller AI, Martı´n C, Castro C, Ramirez M, Pema´n JM, et al. Evaluation of disk diffusion method for determining posaconazole susceptibility of filamentous fungi: comparison with CLSI broth microdilution method. *Antimicrob. Agents Chemother.* 2006; 50(3):1108-1111.
21. Pujol I, Guarro J, Llop C, Soler L, Ferna´ndez-Ballart J. Comparison study of broth macrodilution and microdilution antifungal susceptibility tests for the filamentous fungi. *Antimicrob. Agents Chemother*. 1996; 40:2106-2110.
22. Chokevivat V, Chuthaputti A. The role of Thai traditional medicine in health promotion. 6GCHP Bangkok, Thailand; 2005.

Antibacterial and Larvicidal Activities of Sri Lankan Endemic Plant, *Vateria copallifera*

S. P. D. Senadeera[1*], C. D. Wijayarathna[2], I. S. Weerasinghe[3], K. A. K. C. Kulatunga[4]

[1]*Department of Natural Products, Medical Research Institute, No. 527, Dr. Danister De Silva Mawatha, Colombo 08, Sri Lanka.*

[2]*Department of Chemistry, Faculty of Science, University of Colombo, Sri Lanka.*

[3]*Department of Entomology, Medical Research Institute, No. 527, Dr. Danister De Silva Mawatha, Colombo 08, Sri Lanka.*

[4]*Department of Bacteriology, Medical Research Institute, No. 527, Dr. Danister De Silva Mawatha, Colombo 08, Sri Lanka.*

ABSTRACT

Crude acetone extracts of leaf, bark, pericarp and seeds of Sri Lankan endemic plant, *Vateria copallifera* were evaluated for antibacterial and larvicidal activities against ten clinically important pathogenic microorganisms and 3rd instar mosquito larvae of *Culex quinquefasciatus* and *Aedes aegypti*. The highest antibacterial activity with zone of inhibition 19.66 ± 0.65 mm was reported from the seeds against *Methicilin resistant staphylococcus aureus* (MRSA) as evident from minimum inhibitory concentration (MIC) > 0.01526 mg/ml and minimum bactericidal concentration (MBC) > 0.125 mg/ml. The lowest antibacterial activity was reported from the pericarp against *Methicilin resistant staphylococcus aureus* with MIC > 0.125 mg/ml and MBC > 4 mg/ml. Seed extract which reported the highest antibacterial activity was subjected to bioassay guided fractionation and obtained five fractions. (F1, F2, F3, F4, F5). From five fractions of seed extract, F2 reported the highest antibacterial activity. Commercially available antibiotic discs sulphamethoxazole trimethoprim (25 µg), ampicilin (10 µg), erythromycin (15 µg) were used as positive controls. Absolute ethanol was the negative control with zone of inhibition 0 ± 0. Larvicidal activity was reported only from seed extract with LC_{50} and LC_{90} values 465.3mg/ml, 915.8mg.ml for *Culex quinquefasciatus* and 661.3mg/ml and 1105.9mg/ml for *Aedes aegypti* respectively.

Key words: antibacterial activity, minimum inhibitory concentration, minimum bactericidal concentration, larvicidal activity, *Vateria copallifera*

INTRODUCTION

Dipterocarpaceae is an important Asiatic tree family with three sub families, Dipterocarpoideae, Monotoideae and Pakaraimoideae, containing seventeen genera and approximately five hundred species. A number of stilbenoid (C_6 – C_2- C_6) oligomers also called resveratrol oligimers have been isolated from this family.[1] The oligomers in this family have a variety of molecular frameworks resulting from different oxidative condensation of the nucleus in the dihydrofuran ring, benzocyclopentane ring, dibenzol, heptadiene system and dibenzobicyclo octadiene system. They include di-, tri-, tetra-, hexa-, hepta-, and octa stilbenoids. Stilbenoid derivatives have biological activities such as anticancer, antibacterial, antiviral and anti-inflammatory.[2, 3] Plants of the sub family Dipterocarpoideae are the main sources of stilbenoid oligomers. In addition to stilbenoid oligomers, triterpenes and sesquiterpenes are found in plants of this sub family.[4] Thus, Dipterocarpacea plants are very promising for chemical research in natural products and the pharmaceutical industry.

Vateria copallifera is a plant endemic to Sri Lanka that belongs to the family Dipterocarpacae.[5] Decoction of the bark is used in the treatment of rheumatic pains, ulcers, diarrhea, and diabetes mellitus in traditional medicine.[6] Flowers and fruits are used to treat various ailments such as nervous system diseases, vision problems, ear diseases, gastrointestinal tract infections, cardiovascular diseases and skin diseases. The oil extracted from seeds is used in the treatment of

Address for correspondence:
S. P. D. Senadeera, Department of Natural Products,
Medical Research Institute,
No. 527, Dr. Danister De Silva Mawatha,
Colombo 08, Sri Lanka.
Tel: +942693532-4 ext 451, +66832352127,
Fax: +942691495,
Email: spdsenadeera@gmail.com

DOI: 10.5530/pj.2011.19.14

diseases caused by phlegm, bile and in rheumatism. Smoke of the resin exuded by the plant is used in the treatment of hemorrhoids and hemorrhages.[7] The bark is also used to arrest fermentation and the flour of seeds is used to prepare food.[8] Previously isolated constituents of the plant are sitosterol, β-amyrin acetate, β-amyrin, dipterocarpol, copalliferol A and copalliferol B.[4,9]

Infectious diseases account for a high proportion of health problems, especially in developing countries.[10] Microorganisms have developed resistance to many antibiotics and this has created immense clinical problems in the treatment of infectious diseases.[11] The increasing resistance and side effects of popularly used chemotherapeutics has led to the search for new compounds with antimicrobial activity.[12] Secondary metabolites produced by plants constitute bioactive substances and scientific interest has increased in the search for new drugs from medicinal plants.

Dengue virus is a mosquito-borne flavivirus and the most prevalent arbovirus in tropical and subtropical regions of the world.[13] This infectious disease is now widely distributed in many countries in South-East and South Asia, Central and South America and the Western Pacific. As estimated 50 million dengue infections occur every year, including 50,000 cases of Dengue haemorrhagic fever (DHF) that require hospitalization – equivalent to one young life lost with DHF every 25 minutes. The dengue virus is transmitted by the mosquitoes, i.e. *Aedes aegypti* and *Aedes albopictus*. Today in Sri Lanka Dengue fever with its severe manifestation such as dengue haemorrhagic fever and dengue shock syndrome has emerged as a major mosquito-borne viral disease. Dengue is endemic in Sri Lanka and has become a major public health problem with frequent epidemics, which is getting worse each year.

Filariasis is one of the major vector borne diseases transmitted by *Culex quinquefasciatus* mosquito. Based on WHO report 2006a about 120 million people in 83 countries had been physically disabled. It has a cosmopolitan distribution and predominantly found in tropics and warm temperate regions.[14] *Culex quinquefasciatus* is a local vector of filariasis in Sri Lanka and they breed especially in water polluted with organic materials such as refuse and excreta or rotting plants, i.e. soak away pits, septic tanks, pit latrines, blocked drains, canals and abandoned wells. The only efficacious approach to minimize the incidence of filariasis is to eradicate and control *Culex quinquefasciatus* mosquito vector.

Vector control has been mainly affected by use of conventional insecticides but these have caused their own problems, such as adverse effects on the environment and the development of pesticide resistance in some mosquitoes. These problems have stimulated a search for safer alternative mosquito control methods. It has been found that herbal extracts are one such safer alternative for mosquito control, especially the extracts of certain medicinal plants. Not only medicinal plant extracts can be effective but also they greatly reduce the risk of adverse ecological effects and they do not induce pesticide resistance in mosquitoes. Since these chemicals are taken from medicinal plants, they are expected to have low human toxicity and a high degree of biodegradation.[15] Recently many studies have been conducted in Sri Lanka and around the world have shown that medicinal plants have larvicidal, pupaecidal and adulticidal effects on mosquitoes.[16]

The purpose of the present study was to investigate the antibacterial and larvaecidal potential of *Vateria copallifera* at the level of doses. Acetone extracts of various parts of the plant were evaluated against ten clinically important pathogenic bacterial strains. Commercially available antibiotics were used for comparison. The extract with the highest antibacterial activity was determined using zones of inhibition, MIC and MBC values and fractionated using column chromatography. Fractions were evaluated for antibacterial activity. Larvicidal activity of the plant was tested against third instar mosquito larvae of *Culex quinquefasciatus* and *Aedes aegypti* using various concentrations of acetone extracts of seed, bark, pericarp and leaves.

MATERIALS AND METHODS

Collection of plant material

Plants were collected from the Gampaha District, Sri Lanka and the plant was identified using morphological characters and the identity was confirmed as *Vateria copallifera* by comparing with herbarium specimens at Royal Botanical Garden, Peradeniya, Sri Lanka. Voucher specimens were deposited at the Botany Department, Bandaranayke Memorial Ayurvedic Research Institute, Nawinna, Maharagama, Sri Lanka.

Preparation of plant extracts

The collected plant materials, leaf, bark, seeds and pericarp (500 g from each) were washed and cleaned. The cleaned plant materials were chopped into small pieces; air dried and finely ground using a grinder. Acetone 99% (Fluka) was added to the ground plant materials and kept overnight at room temperature. The supernatant was decanted and filtered through Whatman # one filter paper. The filtrates were rotary evaporated to dryness under reduced pressure below 45 ºC. The resulting crude extracts were stored at -40 ºC until used.

Test organisms

Seven standard cultures *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *and Streptococcus faecalis* ATCC 29212, *Escherichia Coli* 0-157, *Staphylococcus aureus* NCTC 4163 and *Pseudomonas aeruginosa var erythrogen* NCTC 6749 and three hospital isolates *Salmonella typhi, Methicillin resistant staphylococcus aureus* (MRSA), *Methicilin sensitive staphylococcus aureus* (MSSA) were obtained from the Bacteriology Department of Medical Research Institute, Sri Lanka. The bacterial strains were maintained on nutrient agar (Oxoid) at 4ºC. Two vector mosquito larvaes, *Culex quinquefasciatus* and *Aedes aegypti* were used to test larvicidal activity. Separate mosquito colonies were maintained in the insectary at the Department of Entomology, Medical Research Institute, Sri Lanka to obtain 3rd instar larvae for bio assays.

Determination of antibacterial activity by disc diffusion method

The disc diffusion assay was carried out to determine the growth of inhibition of bacteria by plant extracts. Each extract (100 mg) was dissolved in 1 ml of absolute ethanol (Fisher scientific) and 20 µl (equivalent to 2 mg of the dried extract) were impregnated on sterile 6 mm discs. Once ethanol had evaporated, the discs were kept in a refrigerator at 4º C for the duration of the assay. Muller Hinton agar media (Himedia) and petri dishes (9 cm) were sterilized at 120 ºC for 15 minutes. Plates were prepared by pouring 25 ml Muller Hinton agar medium into petri dishes and allowing it to set. Fresh bacterial strains cultured on Muller Hinton agar at 37 ºC were used for plate inoculation. Density of inoculums used was 10^6 cells/ml as compared to the Mc Farland standard solution of $BaSO_4$. Previously prepared four discs from each seed, bark, leaf and pericarp were placed on the agar plates inoculated with bacteria. Air dried ethanol saturated discs were used as negative controls and sulphamethoxazole trimethoprim (25 µg) (Oxoid), ampicilin (10 µg) (Oxoid), erythromycin (15 µg) (Oxoid) were used as positive controls. The plates were incubated at 37 ºC overnight. Zone of inhibition caused by plant extracts, negative controls and positive controls were recorded.

Determination of MIC and MBC

Bacterial strains which showed a growth of inhibition for plant extracts were used to determine the MIC. The MIC of extracts was determined according to the macro dilution method as described by the National Committee for Clinical Laboratory Standards (1993). Nutrient broth (0.5ml) (Himedia) was added to test tubes one to eleven. An equal volume (0.5 ml) of extract (4 mg/ml) and was mixed well in the first test tube and transferred (0.5 ml) to the second test tube through tenth tube to obtain 4 mg/ml to 0.0078125 mg/ml concentrations of plant extracts. The eleventh test tube served as the control with no extract. Fresh cultures were diluted in sterile distilled water at a density adjusted to turbidity of 0.5 Mc Farland standards and added (50 µl) to each test tube. The test was carried out in duplicate and the tubes were incubated at 37 ºC overnight. The concentrations of absolute ethanol in tubes were kept below 10%. Pre experimental procedure showed that 10% absolute ethanol concentration did not inhibit growth of microorganisms. The lowest concentration of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tube was regarded as the MIC. The tubes where the growth of microorganism was not visible were used to sub culture on Muller Hinton agar medium and the MBC was determined.

Bio assay guided fractionation of seed extract

The extract with highest antibacterial activity was subjected to thin layer chromatography (TLC) to determine the solvent system which gave the best separation of the extract. The extract was subjected to column chromatography with MeOH: $CHCl_3$: EtOAC (1: 4: 3) solvent system. Portions of the eluent (25 ml) were collected into clean separate test tubes. The same procedure was repeated until 1.2 liters were collected. The most polar fraction was eluted using 100% MeOH. Portions with the same TLC patterns were pooled together. Five different fractions, F1, F2, F3, F4, F5, were obtained and they were rotary evaporated to dryness. Antibacterial activities of five fractions were determined against the microorganisms that showed inhibition for the crude extract. The fraction that showed the highest antibacterial activity was chosen to study the effect of concentration on antibacterial activity.

Mosquito larvicidal bioassay

Mosquito larvicidal bioassays were carried out according to the WHO (2005) guidelines for laboratory and field testing of mosquito larvicides against *Culex quinquefasciatus* and *Aedes aegypti*. A known amount of each extract (leaf, bark, pericarp and seed) was dissolved in absolute ethanol to provide stock solutions. To determine the larvicidal activity of plant extracts, initially the mosquito larvae were exposed to 1000ppm concentration of plant extracts. Twenty five late 3rd instar larvae were introduced in to clean disposable cups each containing 198 ml of distilled water and 2 ml of test solution. The final concentration of the test solution in the cups was 1000 ppm. Each experiment set contained three replicate controls which consisted of 2 ml ethanol and 198ml of distilled water. All the cups

were kept at room temperature and mortality was recorded after 48 hours. Plant extracts which showed mosquito larvicidal activity to 1000 ppm concentration were selected for further testing with lower concentrations (750 ppm, 500 ppm, 250 ppm, 125 ppm). The concentrations lethal to 50% (LC_{50}) and 90% (LC_{90}) of test organisms, 95% confidence interval and the slopes of probit regression line were determined by probit analysis to compare the effectiveness of extracts.

Statistical analysis

All the experiments were carried out in replicates of three. Results were expressed as Mean ± Standard Error of the mean or graphically, unless specified.

RESULTS

Zones of inhibition of specific concentrations (20 µl from 1000 µg/ml) of acetone extracts of various parts of *Vateria copallifera*, positive controls and negative control against ten pathogenic microorganisms are shown in Tables 1 and 2 respectively. Tables 3 and 4 present MIC and MBC of active plant extracts respectively. Tables 5 and 6 show the antibacterial activity of fractions (F1, F2, F3, F4 and F5) of seed extract and zones of growth inhibition of bacteria at different levels of fraction F2**.** Figure 1 represents the variation of zones of inhibition of microorganisms at different levels of fraction F2. Tables 7 and 8 present the larvicidal activity of each extract as LC_{50} (mg/l) and LC_{90} (mg/l).

Table 1: Zone diameter of inhibition by various plant parts of *Vateria copallifera* (mm)

Organism	Plant Part			
	Seed	Bark	Pericarp	Leaf
Escherichia coli ATCC 25922	n. a.	n. a.	n. a.	n. a.
Pseudomonas aeruginosa ATCC 27853	n. a.	7 ± 0	n. a.	n. a.
Staphylococcus aureus ATCC 25923	20 ± 0	19.66 ± 0.51	12 ± 0	19.8 ± 0.42
Streptococcus faecalis ATCC 29212	18 ± 0	15.44 ± 0.72	11.75 ± 0.62	21.66 ± 0.65
E. Coli 0-157	n. a.	n. a.	n. a.	n. a.
Salmonella typhi	n. a.	n. a.	n. a.	n. a.
Pseudomonas aeruginosa NCTC 6749	n. a.	n. a.	n. a.	7 ± 0
Staphylococcus aureus NCTC 4163	18.33 ± 0.65	17.08 ± 0.51	11.25 ± 0.62	20.00 ± 00
MRSA	19.66 ± 0.65	15.0 ± 0.65	9.58 ± 0.51	17.66 ± 0.49
MSSA	15.00 ± 0	17.66 ± 0.49	15.00 ± 0	7.75 ± 0.45

n. a.: no activity

Table 2: Zone diameter of inhibition by positive and negative controls (mm)

Organism	Antibiotic			
	Ampicillin	Erythromycin	ST	Ethanol
Escherichia coli ATCC 25922	7 ± 0	n. a.	30 ± 0	0 ± 0
*Pseudomonas aeruginosa*ATCC27853	n. a.	n. a.	n. a.	0 ± 0
Staphylococcus aureus ATCC25923	30 ± 0	30 ± 0	35 ± 0	0 ± 0
Streptococcus faecalis ATCC29212	25 ± 0	27 ± 0	35 ± 0	0 ± 0
E. Coli 0-157	12 ± 0	n. a.	30 ± 0	0 ± 0
Salmonella typhi	25 ± 0	n. a.	30 ± 0	0 ± 0
Pseudomonas aeruginosa NCTC 6749	n. a.	n. a.	n. a.	0 ± 0
Staphylococcus aureus NCTC 4163	35 ± 0	30 ± 0	30 ± 0	0 ± 0
MRSA	n. a.	n. a.	n. a.	0 ± 0
MSSA	5 ± 0	26 ± 0	30 ± 0	0 ± 0

ST- Sulphamethoxazole Trimethoprim, n. a.-no activity

DISCUSSION

In the present study, the antibacterial activity of leaf, bark, seeds and pericarp of *Vateria copallifera* was carried out against ten clinically important pathogenic bacterial strains. Most studies on the family of Dipterocarpaceae have been limited only to the bark. Five of the bacterial strains used in this study were Gram-negative and the other five were Gram-positive. All the extracts showed activity mainly against Gram-positive strains. Only Gram-negative bacteria *Pseudomonas aeruginosa* NCTC 6749 and *Pseudomonas aeruginosa* ATCC 27853 showed inhibition with 7 mm ± 0 zones against leaf and bark respectively. Geevananda *et al*.1986 report that copalliferol B isolated from the bark exhibits pronounced antibacterial activity against *Oxford staphylococcus* and *E. coli*. However, the results obtained in the present work do not show such activity against *Escherichia coli* ATCC 25922 and *E. coli* O-157. This inconsistency may be due to the low concentration of the compound responsible for the inhibition of Gram-negative bacteria or the geographical source of the material. In general, Gram-negative bacteria are more resistant than Gram-positive bacteria. There is a morphological basis for the difference in susceptibilities. Gram-negative bacteria have an outer membrane composed mainly of lipopolysaccharide, which is rather impermeable to lipophilic molecules. Gram-positive bacteria lack this outer membrane but have a very much thicker peptidoglycan layer which is not an effective permeability barrier to hydrophilic solutes. [17]

Tables 1 and 2 present the zones of inhibition of extracts, positive controls and negative control against tested bacterial strains. In general, all the extracts showed high antibacterial activity against Gram- positive bacteria. Zones of inhibition of extracts ranged from 7 ± 0 mm – 22 ± 0.65 mm for all the inhibited bacteria. The highest zone of inhibition, 21.66 ± 0 mm, was reported from leaf against *Streptococcus faecalis* ATCC 29212. According to the zones of inhibition *Staphylococcus aureus* NCTC 4163 was the most susceptible bacteria to all the plant extracts. On the contrary the lowest zone of inhibition for Gram-positive bacteria, 9.58 ± 0.51 mm, was reported against MRSA. The activity of pericarp was not that significant. It was difficult to determine which plant part had the highest antibacterial activity by considering only the zones of inhibition since the zones of inhibition are more or less the same for three of the extracts (except for pericarp). The negative control, ethanol, did not show inhibitory zones for any of the tested bacterial strains. *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa var erythrogen* NCTC 6749 and MRSA were resistant to all the positive controls, sulphamethoxazole trimethoprim (25 µg), ampicilin (10 µg) and erythromycin (15 µg). From these three organisms, growth of MRSA was highly inhibited by all the plant extracts while growth of *Pseudomonas aeruginosa* ATCC 2785 and, *Pseudomonas aeruginosa var erythrogen* NCTC 6749 was slightly inhibited by bark and leaf respectively. Since MRSA is methicilin resistant bacteria, these active plant parts may provide interesting alternatives to the antibiotics now used clinically. Erythromycin showed activity only on *Staphylococcus aureus* ATCC 25923, *Streptococcus faecalis* ATCC 29212, *Staph aureus* NCTC 4163 and *Methicilin sensitive staphylococcus aureus* (MSSA).

The results obtained for *Staphylococcus aureus* were interesting. According to the guidelines on standard operation procedures for microbiology, ampicilin, erythromycin and sulphamethoxazole trimethoprim are used to test the susceptibility or resistance of *Staphylococcus aureus*. The standard zone diameter of growth inhibition for *Staphylococcus aureus*

Table 3: MIC of various parts of *Vateria copallifera* (mg/ml)

Organism	Seed	Bark	Pericarp	Leaf
Staphylococcus aureus ATCC 25923	> 0.0625	> 0.03125	> 0.0625	> 0.0625
Streptococcus faecalis ATCC 29212	> 0.03125	> 0.03125	> 0.0625	> 0.0.0625
Staphylococcus aures NCTC 4163	> 0.03125	> 0.03125	> 0.0625	> 0.03125
MSSA	> 0.0625	> 0.125	> 0.0625	> 0.0625
MRSA	> 0.015625	> 0.0156255	> 0.25	> 0.015625

Table 4: MBC of various parts of *Vateria copallifera* (mg/ml)

Organism	seed	Bark	Pericarp	Leaf
*Staphylococcus aureus*ATCC25923	> 0.5	> 1	> 1	> 0.5
Streptococcus faecalis ATCC29212	> 0.25	> 2	> 1	> 0.5
Staphylococcus aureus NCTC 4163	> 0.125	> 2	> 2	> 0.5
MSSA	> 1	> 2	> 2	> 2
MRSA	> 0.125	> 0.5	> 4	> 0.5

of ampicilin (10 µg), Erythromycin (15 µg) Sulphamethoxazole trimethoprim (25 µg) are 27-35 mm, 22-30 mm, 24-32 mm respectively. [18] By comparison, the standard zone diameter of inhibition obtained with the crude extracts of leaf, bark, seeds and pericarp suggest that they are promising antimicrobial agents against *Staphylococcus aureus*.

The MIC was determined only on microorganisms that were found to be sensitive in the disc diffusion method. The lowest MIC value corresponding to the seeds was > 0.015625 mg/ml against MRSA. The highest MIC value of 0.25 mg/ml was reported against MRSA from pericarp. The lowest MBC values were reported from seeds against MRSA and *Staphylococcus aureus* NCTC 4163. This may indicate that the compound responsible for the antimicrobial activity is present in each extract as a mixture of compounds. On considering the zones of inhibition, MIC values and MBC values, it can be concluded that the highest antibacterial activity is shown by the seeds *of Vateria copallifera*. Hence, the acetone extract of seed was selected for further studies. For the separation of major and minor compounds in the seed extract thin layer chromatography was carried out and methanol: chloroform: ethyl acetate (1:4:3) solvent system was found to be the most effective. Five major components were observed on the basis of spots and retention factor (R_f) values and the extract was fractionated eluting the silica column with the same solvent system. The most polar fraction was eluted with 100% methanol. The fractions with similar R_f values were pooled together and labeled accordingly. A total of five fractions were obtained and labeled as F1, F2, F3, F4 and F5. Varying degrees of growth inhibitions were shown by the five fractions against the same five pathogenic organisms which were inhibited by the crude (Table 5). Based on the zone diameter of growth inhibition, fraction F2 was found to have the maximum growth inhibition activity against the tested microorganisms. Moderate growth inhibition was reported from fractions F3, F4 and F5. Fraction F1 slightly inhibited the growth of only two microorganisms, namely, *Staphylococcus aureus* NCTC 4163 and MRSA. Since F2 reported the highest growth of inhibition against five tested pathogenic microorganisms, the variation of growth inhibition was studied as a function of amount F2. The results are shown in Table 6 and Figure 1. The results indicate that the growth inhibition increases with the increasing amount of F2.

Table 5: Zone diameter of inhibition of fractions F1, F2, F3, F4 and F5 of seed extract (mm)

Organism	F1	F2	F3	F4	F5
Staphylococcus aureus ATCC25923	n. a.	20 ± 0	16.5 ± 0.58	12.75 ± 0.5	15.625 ± 0.48
Streptococcus faecalis ATCC29212	n. a.	19.5 ± 1	17 ± 0	12 ± 0	19.75 ± 0.86
Staphylococcus aureus NCTC 4163	9.5 ± 0.58	20 ± 0	16.25 ± 0.5	13 ± 0	16.5 ± 0.58
MSSA	n. a.	20 ± 0	16.5 ± 0.58	12 ± 0	13 ± 0
MRSA	6.25 ± 0.5	20 ± 1.41	17.25 ± 0.5	11 ± 1.15	16.125 ± 0.25

n. a. - no activity

Table 6: Zone diameter of inhibition at different levels of fraction F2 of seed extract (mm)

Organism	0.00mg	0.01mg	0.1mg	1mg	2mg	3mg
*Staphylococcus aureus*ATCC25923	0.00 ± 0	8 ± 0	17 ± 0	18 ± 0	19.5 ± 0	21 ± 0
*Streptococcus faecalis*ATCC29212	0.00 ± 0	12.75 ± 0.5	17 ± 0	21 ± 0	24 ± 0	25 ± 0
Staphylococcus aureus NCTC 4163	0.00 ± 0	10 ± 0	17.25 ± 0.5	20 ± 0	22 ± 0	25 ± 0
MSSA	0.00 ± 0	8 ± 0	15 ± 0	16 ± 0	18 ± 0	19 ± 0
MRSA	0.00 ± 0	8 ± 0	13.5 ± 0.58	17 ± 0	18 ± 0	20 ± 0

Table 7: Mosquito larvicidal activity of various parts of *Vateria copallifera* - LC_{50} (mg/l)

Plant part	Activity LC_{50} (mg/l)	
	Cx. quinquefasciatus	*Aedes aegypti*
Seed	465.3	661.3
Bark	n. a.	n. a.
Leaf	n. a.	n. a.
Pericarp	n. a.	n. a.

Cx.-Culex, n. a. - no activity

Table 8: Mosquito larvicidal activity of various parts of *Vateria copallifera* - LC_{90} (mg/l)

Plant part	Activity LC_{90} (mg/l)	
	Cx. quinquefasciatus	*Aedes aegypti*
Seed	915.8	1105.9
Bark	n. a.	n. a.
Leaf	n. a.	n. a.
Pericarp	n. a.	n. a.

Cx.-Culex, n. a. - no activity

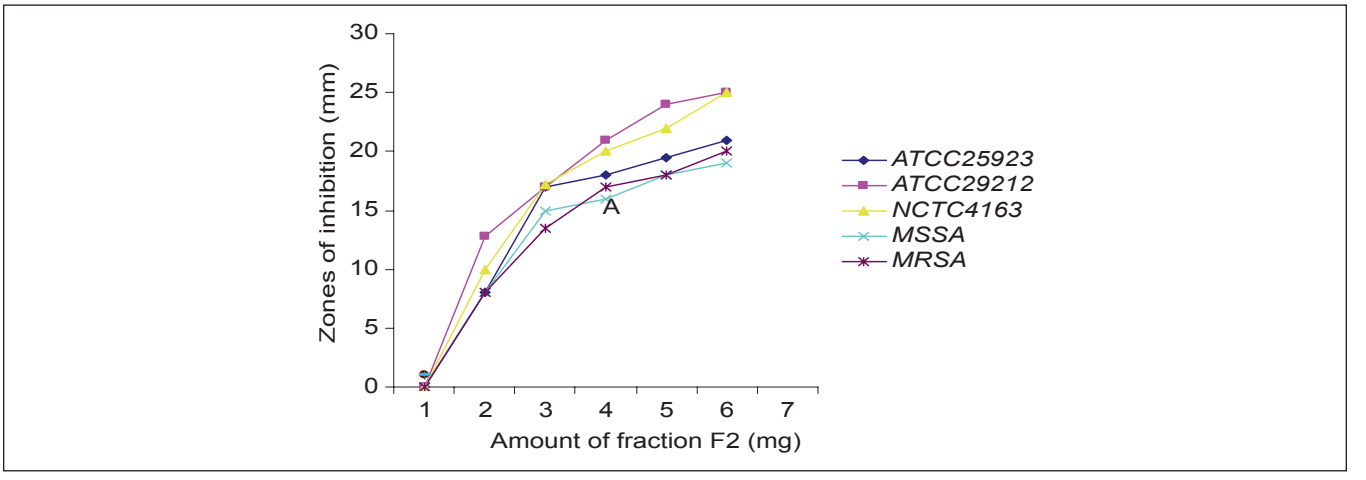


Figure 1: Variation of zones of inhibition of microorganisms at different levels of fraction F2 of seed extract

The larvicidal study of *Vateria copallifera* was initiated to find better plant based mosquito larvicidal agents. In this study various parts of *Vateria copallifera* were tested against *Culex quinquefasciatus* and *Aedes aegypti* larvae. Extracts which possessed larvicidal activity for 1000 ppm concentrations were selected for further analysis. Out of seed, bark, pericarp and leaves tested, only seed showed larvicidal activity against *Culex quinquefasciatus* and *Aedes aegypti*. LC_{50} and LC_{90} values of seeds against *Culex quinquefasciatus* are 465.3 mg/l and 915.8 mg/l respectively and that against *Aedes aegypti* are 661.3 mg/l and 1015.9 mg/l. Therefore the seed extract of *Vateria copallifera* will be a promising approach for the control of *Culex quinquefasciatus and Aedes aegypti* larvae.

CONCLUSION

Based on the current results it can be concluded that *Vateria copallifera* shows antibacterial and larvicidal activities. Antibacterial activity is as potent as standard antibacterial drugs against some clinically important microorganisms, especially against *Staphylococcus aureus* strains.

ACKNOLEDGEMENTS

We would like to thank staff of Medical Research Institute of Sri Lanka and the Department of Chemistry, University of Colombo, Sri Lanka for the technical support given for the study.

REFERENCES

1. Atun S, Aznam N, Arianingrum R, Takaya Y, Masatake N. Resveratrol Derivatives from Stem Bark of Hopea and Their Biological Activity Test. J. Phy. Sci. 2008;19 (2):7-21.
2. Mishima S, Matsumoto K, Futamura Y, Araki Y, Ito T, Tanaka T, et al. Antitumor effect of stilbenoids from Vateria indica against allografted sarcoma S-180 in animal model. J. Exp. Therapeut. Oncol. 2003; 3(5):283-8.
3. Muhtadi, Hakim EH, Syah YM, Juliawaty LD, Latip J. Resveratrol oligomers from Dipterocarpus Hasseltii: Cytotoxic effect and Chemotaxonomic Significance. Jurnal Ilmiah Farmasi. 2008; 5(1):18-24.
4. Geevananda YA, Gunawardana P, S. Sultanbawa MU, Balasubramaniam S. Distribution of some triterpenes and phenolic compounds in the extractives of endemic dipterocarpaceae species of Sri Lanka. Phytochemistry. 1980; 19(6):1099-102.
5. Vivekananda K. The Sri Lanka Forester (The Ceylon Forester), . Published by the Sri Lanka Forest Department. 1974; 11(3,4):119.
6. Ratnasooriya WD, Lelwala LHDS, Kannangara KNK, Sandanayaka SDD, Ediriweera ERHSS. Sedative activity of stem bark of the Sri Lankan endemic plant, Vateria copallifera. Fitoterapia. 2006; 77(4):331-2.
7. Ayurveda Pharmacopoeia, The Department of Ayurveda, Colombo, Sri Lanka; 1985; 357-358.
8. Revised hand book to the flora of Ceylon, New Delhi: Amerind Co. Pvt. Ltd.; 1985
9. Geewananda YA, Gunawardena P, Sotheeswaran S, Uvais M, Sultanbawa S, Surendrakumar S, et al. Another antibacterial polyphenol, copalliferol B, from Vateria copallifera(dipterocarpaceae) Phytochemistry. 1986; 25(6):1498-500.
10. Paiva SRD, Figueiredo MR, Aragão TV, Kaplan MAC. Antimicrobial Activity in Vitro of Plumbagin Isolated from Plumbago Species. Mem Inst Oswaldo Cruz, Rio de Janeiro. 2003; 1998 (7):959-61.
11. Davies J. Inactivation of antibiotics and the dissemination of resistance genes. Science. 1994; 264:375-82.
12. Ghosh A, Das BK, Chatterjee SK, Chandra G. Antibacterial potentiality and phytochemical analysis of mature leaves of *Polyalthia longifolia* (Magnoliales: Annonaceae). The south Pacific J. Nat. Sci. 2008; 26(1):68-72.
13. Sriprom M, Pongsumpun P, Yoksan S, Barbazan P, Gonzalez JP, Tang IM. Dengue Hemorrhagic fever in Thailand, 1998-2003: Primary or Secondary Infection. Dengue Bulletin. 2003; 27:39-45.
14. Samarasekara R, Weerasinghe IS. Advances in Natural Products. Ahnted, Z. and Dar,A. (ED) Delhi: Daya publishing house; 2008.
15. Choochote W, Chaithong U, Kamsuk K, Rattanachanpichai E, Jitpakdi A,. Adulticidal activity against *Stegomyia aegypti*, (Diptera, Culicidae) of three piper spp, Rev. Inst. Med. Trop. S. Paulo. 2006; 48(1):33-7.
16. Ranaweera SS. Mosquito larvicidal activity of some Sri Lankan medicinal plants,. J. Natn. Coun. Sri Lanka. 1996; 24(2):63-70.
17. Rabe T, Van SJ. Antibacterial activity of South African plants used for medicinal purposes. J. Ethnopharmacol. 1997; 56(1):81-7.
18. Kumari S, Ichhpujani RL. Guidelines on standard operating procedures for microbiology: Published by World Health Organisation; 2000.

A Complete Review on *Oxystelma esculentum* R. Br.

Pandya DJ[1]*, Anand IS[2]

[1]R K College of Pharmacy, Rajkot, Gujarat. [2]Shri Sarvajanik Pharmacy College, Mehasana, Gujarat.

ABSTRACT

Many plants which are found commonly and are mentioned in texts of traditional Indian medicine have not been investigated thoroughly. It is necessary to conduct systematic evaluation, standardization, documentation and patenting of these plants. *Oxystelma esculentum* R. Br. (Family – Asclepiadaceae), commonly known as 'Jaldudhi', is one such plant which has not been studied sufficiently. It has many potential therapeutic uses which are of vital importance in curing the diseases of the modern world like cancer, hepatitis, kidney disorders, stress-related disorders and microbial infections. It contains two very important classes of phytoconstituents: cardenolides and pregnane glycosides, which are easily obtained from this plant and can act as precursors of many therapeutically important compounds. The study of this plant will be important in the future for bioactivity-guided fractionation of medicinal phytoconstituents, for conducting pre-clinical or clinical trials and for preparing formulations or semi-synthetic compounds. The present review, based on an extensive literature search of reputed books, scientific websites and high-impact journals, sheds light on the research done on this plant so far, thereby providing informative guidelines regarding the work that can be done in the future.

Key words: Asclepiadaceae, Cardenolides, Diuretic, Jaldudhi, *Oxystelma esculentum*, Pregnane glycosides

INTRODUCTION

Oxystelma esculentum R. Br. (Family – Asclepiadaceae), known as 'Jaldudhi', is a common Ayurvedic herb which has not been sufficiently explored. It is one of the few plants to contain cardenolides and pregnane glycosides, which are major classes of therapeutically important phytoconstituents. *O. esculentum* has been reported to possess good therapeutic action against many ailments of the current world. The present review, based on an extensive literature search of reputed books, websites and journals, remarks on the study done on this plant so far, thereby providing a direction for future research.

Synonyms

Oxystelma secamone Linn., *Periploca esculenta* Roxb., *Periploca secamone* Linn., *Sarcostemma secamone* Bennet, *Sarcostemma esculentum* Linn., *Asclepias rosea* R. Br.[1]

Address for correspondence:
Devang J. Pandya,
R K College of Pharmacy, Rajkot, Gujarat.
Email: pandyadevang82@yahoo.com

DOI: 10.5530/pj.2011.19.16

Vernacular names[2]

Sanskrit	: Dugdhika, Tiktadugdha
Hindi	: Dudhlata
Gujarati	: Jaldudhi
Bengali	: Khirai, Dudhialata
Marathi	: Dudhani, Dudhika
Telugu	: Dudipala
Tamil	: Usipallai

Distribution[2]

Throughout plains and lower elevation areas of India, usually near water. Also found in Pakistan, Sri Lanka, Burma and extends to China and Indonesia.

Taxonomic classification[3]

KINGDOM	: Plant
DIVISION	: Phanerogams
SUBDIVISION	: Angiosperms

CLASS	: Dicotyledons
SUBCLASS	: Sympetalae/Gamopetalae
ORDER	: Gentianales
FAMILY	: Asclepiadaceae
GENUS	: *Oxystelma*
SPECIES	: *Oxystelma esculentum* R. Br.

Description of the plant

The plant genus derives its name from two words: *Oxys* (sharp) and *Stelma* (crown), which describes the acute lobes of the corolla.[4] It is a twining herb or undershrub whose stem is cylindrical, glabrous, long, slender and much branched. Leaf is simple, opposite, dorsiventral, deciduous, usually 8cm x 0.5cm, linear lanceolate with acute apex and symmetrical base, having long and slender petiole. Inflorescence is racemose, subumbellate cyme or solitary. Flowers are widely open, white with purple veins, 2.5-3cm in diameter, drooping, pedunculate, lateral subumbellate or racemose flowered cymes. Calyx is pentasepalous, connate, glabrous, oblong-lanceolate, acute, glandular inside. Corolla is pentapetalous, connate, 2.5cm wide, glabrous, saucer-shaped, broadly rotate, lobed half-way down, having a densely pubescent corolline corolla, double corona. Corolla lobes are triangular, acute, ciliate, purple veined, valvate at base and shortly overlapping to the right. Androecium consists of five stamens, adnate near base of corolla, having connate filaments, anthers with inflexed membranous deltoid tips and waxy, pendulous, elongate-clavate, solitary pollen in each cell. Gynoecium is bicarpellary, with style apex truncate or convex, stigma depressed or sub-convex. Follicles are 4.5-7.5cm long, often solitary, ovate-lanceolate, glabrous, having acute apex and containing numerous black, broadly ovate seeds [Fig.1]. Other species of *Oxystelma*, which is rarely found in India, is *O. esculentum* var. *wallichii*. Its follicles are shorter, 2.5-4cm long, oblong and rounded at both ends. The only morphological difference between the two varieties lies in its follicles.[5]

Uses

The plant has the property of being *Ushna* (hot), *Guru* (heavy), *Ruksha* (dry) and *Katu* (bitter). It is a diuretic, laxative, spermatogenetic, antitussive, anthelmintic and antileprotic. It increases *Vatta* and stimulates female fertility.[6] Entire plant is used as diuretic, laxative, antiseptic, anthelmintic, antiulcer, aphrodisiac, hepatoprotective and useful in leucoderma and bronchitis. Decoction of plant is used in ulcer, sore-throat and itches. Milky juice is used as galactogogue, antiperiodic, antiulcer and as a vulnerary. Root is used ethnomedicinally in jaundice by the people of Orissa, India. Fruit is bitter tonic, expectorant, anthelmintic. Fruit juice is used in muscle pain, gonorrhoea, cough and leucoderma, and given to children as astringent. [7,8]

Fig.1 *Oxystelma esculentum* R. Br.

PHYTOCHEMICAL REVIEW

General chemical analysis

Researchers first carried out the chemical analysis of *O. esculentum* which revealed the presence of water, fibers, proteins, lipids and carbohydrates. [9]

Isolation of pregnane glycosides

A group of researchers isolated a pregnane ester glycoside Oxystine from the roots of *O. esculentum*. Powdered roots were extracted with solvents of different polarities. Repeated column chromatography of the diethyl ether extract over silica gel using chloroform: methanol (96:4) as eluent afforded oxystine, which was found to be a tetraglycoside of 12-*O*-cinnamoyl desacylmetaplexigenin.[10] Another pregnane glycoside Oxysine was isolated from the roots. Column chromatography of the chloroform extract using chloroform: methanol (24:1) as eluent afforded oxysine, a triglycoside of calogenin.[11] A pregnane glycoside Esculentin was also isolated from the roots. Column chromatography of the methanolic

extract using chloroform: methanol (24:1) as eluent afforded esculentin, a triglycoside of sarcogenin.[12] Researchers isolated polyhydroxypregnane glycosides Alpinosides A, B and C from the leaves of *O. esculentum*. Dried aerial parts were exhaustively extracted with ethanol: water (7:3) in a Soxhlet apparatus. The extract was condensed under reduced pressure to a syrupy consistency. Crude extract was dissolved in methanol: water (2:1) and transferred into a separator funnel. The extract was shaken with hexane, chloroform and n-butanol respectively till exhaustion. The chloroform fraction was loaded on silica gel column. Fractions eluted with chloroform: methanol yielded three compounds of kidjolanin: Alpinoside A (pentaglycoside), Alpinoside B (tetraglycoside) and Apinoside C (pentaglycoside).[13]

Isolation of cardenolides

Three cardenolides, Oxystelmine, Oxyline and Oxystelmoside, have so far been isolated from the roots of *O. esculentum*. Oxyline was found to be a tetraglycoside of 3-epi-uzarigenin, oxystelmoside is a diglycoside of uzarigenin whereas oxystelmine is a diglycoside of periplogenin. [14,15]

PHARMACOLOGICAL REVIEW

Diuretic activity

Considering the claims in the traditional medicinal texts, researchers studied the effects of methanolic extract of leaves of *O. esculentum*. on diuresis in male Wistar albino rats. Urinary output and excretion of electrolytes (Na^+, K^+, Ca^{2+} and Cl^-) were measured. The methanolic extract significantly increased the urine output and had a significant effect on the electrolyte balance in a dose dependent manner, indicating that *O. esculentum* is an effective hypernatremic, hyperkalaemic, hypercalcemic and hyperchloremic diuretic.[16]

Antioxidant activity

A group of researchers performed the evaluation of antioxidant and free radical scavenging activities of methanolic extracts of leaves of *O. esculentum* in various *in vitro* models. It was discovered that the total antioxidant activity increased with increasing concentration. The reducing capability and free radical scavenging activity in DPPH also increased in a dose dependent manner. The methanolic extract was found to scavenge the superoxide generated by PMS/NADH/NBT system. Moreover, the extract was found to inhibit the nitric oxide radical generated from sodium nitroprusside. The extract was also found to inhibit the hydroxyl radical generated by Fe^{3+}/ascorbate/EDTA/water system. The extract scavenged the hydrogen peroxide in a dose dependent manner. These results give a clear indication that *O. esculentum* has a strong antioxidant activity and can be used as a natural antioxidant.[17]

Anticancer activity

Antineoplastic activities of methanolic leaf extracts of *O. esculentum* on Swiss albino mice bearing Ehrlich's ascites carcinoma were studied. Decrease in tumor volume, packed cell volume, and viable cell count were observed in methanolic extract-treated mice. The extract also decreased the body weight of the EAC-bearing mice. Hematological profiles indicated decrease in white blood cells, increase in red blood cells and increase in Hemoglobin content. The methanolic extract restored all the parameters of hematological profiles to normal. Treatment with methanolic extract decreased the levels of LPO and increased the levels of GSH, SOD and CAT. These data indicate that the methanolic extract of leaves of *O. esculentum* exhibits significant antitumor activity.[18]

Antimicrobial activity

Antibacterial activity of leaves of *O. esculentum* against some hospital isolated human pathogenic bacterial strains was studied. From the results it is clear that leaves of *O. esculentum* are effective in controlling both gram positive and gram negative bacterial pathogens. The most effective crude extracts were ethyl acetate and methanolic fractions. Aqueous extract also showed sensitivity against all test organisms. Petrol and benzene extracts of the leaves showed weak antimicrobial action.[19] Antimicrobial activity of methanolic extract of leaves *O. esculentum* was studied further. The antibacterial studies confirmed that the methanolic extract had a zone of inhibition, but the MIC in two fold serial dilution method ensured no prominent action on the tested bacterial strains. The antifungal studies confirmed that methanolic extract had an effective zone of inhibition against *C. albicans* and *C. neoformans*. In MIC studies, the methanolic extract had more effect on *C. albicans*, thus giving a lead for further *in vivo* anticandidal studies. In future, the active constituent can be formulated into a topical dosage form.[20]

DISCUSSION

Oxystelma esculentum is one of the few plants to contain cardenolides and pregnane glycosides, which can be obtained by simple and inexpensive methods from this plant. Also, these phytoconstituents can act as precursors of many other therapeutically important compounds. Due to the changing climate and lifestyle, health disorders like cancer, hepatitis, stress-related disorders, urinary disorders and bacterial infections have emerged as serious global issues. This plant has been reported to possess good therapeutic action against

many of such diseases. The present review can pave a way for a thorough evaluation, standardization, documentation and patenting of this plant. An exhaustive pharmacognostical, phytochemical, pre-clinical, clinical and formulation-based research on this plant can prove to be very fruitful for mankind.

REFERENCES

1. Guha DN, Bakshi BK. Flora of Murshidabad District. Jodhpur: Scientific Publishers; 1984.
2. Nadkarni KM. Indian Materia Medica. Mumbai: Popular Prakashan; 1976.
3. Bendre A, Kumar A. A Text Book of Practical Botany, Vol. II. 6th ed. Meerut: Rastogi Publication; 1996.
4. Drury H. Handbook of Indian Flora, Vol. II. New Delhi: M/S Bishen Singh Mahendrapal Singh; 1982.
5. Cooke T. Flora of Presidency of Bombay, Vol. II, Part I. Mumbai: Taylor & Francis; 1904.
6. Bhavmishra. Bhavmishrakrut Haritkyadi Nighantu. Mumbai: Shrivenkateshwar Steam Press; 1926.
7. Kirtikar KR, Basu BD. Indian Medicinal Plants, Vol. III. New Delhi: Periodical Experts; 1975.
8. Watt G. Dictionary of Economic Products of India, Vol. V. Delhi: Periodical Experts; 1891.
9. Memon AR, Bhatti KM. Chemical Analysis of *Caralluma edulis* Benth. and *Oxystelma esculentum* R. Br. J Chem Soc Pak. 1984; 6(1):71-2.
10. Trivedi R, Khare A, Khare MP. A pregnane ester tetraglycoside from *Oxystelma esculentum*. Phytochemistry. 1988; 27(7):2297-300.
11. Trivedi R, Khare A, Khare MP. A pregnane ester oligoglycoside from *Oxystelma esculentum.* Phytochemistry. 1989; 28(4):1211-3.
12. Trivedi R, Khare A, Khare MP. A pregnane triglycoside from *Oxystelma esculentum.* Phytochemistry. 1990; 29(12):3967-70.
13. Hamed AI, Sheded MG, Shaheen AM, Hamada FA. Polyhydroxypregnane glycosides from *O. esculentum*. Phytochemistry. 2004; 65(7):975-80.
14. Srivastava S, Khare MP, Khare A. A cardenolide tetraglycoside from *Oxystelma esculentum*. Phytochemistry. 1991; 30(1):301-3.
15. Srivastava S, Khare MP, Khare A. Cardenolide diglycosides from *Oxystelma esculentum*. Phytochemistry. 1993; 32(4):1019-21.
16. Durairaj A, Mazumder UK, Gupta M, Ray SK. Effects of methanolic extract of *Oxystelma esculentum* on diuresis and urinary electrolytes excretion in rats. Iranian Journal of Pharmacology and Therapeutics. 2007; 6(2):207-11.
17. Durairaj A, Mazumder UK, Gupta M, Senthilkumar GP, Selvan VT. Evaluation of antioxidant and free radical scavenging activities of *Oxystelma esculentum* in various in vitro models. J Complement Integr Med. 2008; 5:1-6.
18. Durairaj A, Mazumder UK, Gupta M, Selvan VT. Antineoplastic and antioxidant activities of *Oxystelma esculentum* on Swiss albino mice bearing Ehrlich's ascites carcinoma. Pharm Biol. 2009; 47(3):195-202.
19. Khan AV, Ahmad R, Khan AA, Shukla I. Antibacterial activity of *Oxystelma esculentum* leaf extracts against some hospital isolated human pathogenic bacterial strains. Journal of Herbal and Medicinal Toxicology. 2008; 2(2):67-70.
20. Babu AD, Reddy UM. Antimicrobial activity of methanol extract of *Oxystelma esculentum*. J Pharm Sci Tech. 2010; 2(2):119-23.

Indian Medicinal Plants Used in Liver disease: A Short Review

Chaudhary Sachin[1]*, Pal Ajay[2]

[1]Moradabad Educational Trust Group of Institutions, Faculty of Pharmacy, MIT campus, Ram Ganga Vihar Phase-2, Moradabad-244001, Uttar Pradesh, India [2]Faculty of pharmacy, Garyounis University, Benghazi, Libya.

ABSTRACT

Liver disease is one of the serious health problems. Herbal drugs play a major role in the treatment of hepatic disorders. In the absence of reliable liver hepatoprotective drugs in modern medicine, in India a number of medicinal plants and their formulations are used to cure hepatic disorders in traditional system of medicines In Himalayan region there are many plants which are use in liver diseases. In this review, Indian medicinal plants having hepatoprotective property are summarized in terms of their biological source, active constituents and biological activity.

Key words: Hepatoprotective, Herbal drugs.

INTRODUCTION

Medicinal plants have been a major source for the cure of human diseases since time immemorial. Today, one fourth of the world population depends on traditional medicines. Side effects of conventional medicine, efficiency of plant-derived drugs and growing interest in natural products has increased scientific interest in medicinal plants. There is a no rational therapy available for liver disorder, and it is a still challenge to modern medicine. In olden times herbal products were used for medicinal purposes, both internally as well as externally. Herbal drugs were used as juice, latex or in dried powder form[1]. In India, about 40 polyherbal commercial formulations reputed to have hepatoprotective action are being used. It has been reported that 160 phyto-constituents from 101 plants have hepatoprotective activity[2]. Liver protective herbal drugs contain a variety of chemical constituents like phenols, coumarins, lignans, essential oils, monoterpenes, carotinoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthines. Plant extracts of many crue drugs are also used for the treatment of liver disorders. Extracts of different plants have been reported to cure liver disorders[3].

Address for correspondence:
Sachin Chaudhary, Asst. Professor,
Moradabad Educational Trust Group of Institutions,
Faculty of Pharmacy, MIT campus,
Ram Ganga Vihar Phase-2, Moradabad-244001,
Uttar Pradesh, India.

DOI: 10.5530/pj.2011.19.17

DIFFERENT HERBS USED AS HEPATOPROTECTIVES

Punarnava (*Boerhaavia diffussa*)

Punarnava (*Boerhaavia diffussa,* family-Nyctaginaceae) occurs as dried herb of abundantly as a weed throughout India, up to an altitude of 2,000 m in the Himalayas. It is also cultivated to some extent in West Bengal. The roots contain alkaloids ,triacontanol hentriacontane, ß-sitosterol, ursolic acid, flavone, and an unidentified ketone, rotenoid boeravinones A1, B1, C2, D, E and F besides the new dihydroisofurenoxanthin, borhavine and, punarnavoside. Two lignans, liriodendrin and syringaresinol mono-ß-D-glucoside, have also been reported in the roots. The herb is used as a diuretic and an expectorant, stomachic and is prescribed in the treatment of jaundice. It is also given in the loss of digestive power, enlargement of spleen and for abdominal pains. The roots are used by a large number of tribes in India for the treatment of various hepatic disorders[4].

Arjuna (*Terminalia arjuna* Rob)

Arjuna (*Terminalia arjuna* Rob, family Combretaceae) consists of dried stem bark of the plant, it grows in most parts of India and is also planted in many parts for shade

and ornament. Arjuna contains ß-sitosterol, ellagic acid, and arjunic acid.The bark is useful as an anti-ischemic and cardio protective agent in hypertension and ischemic heart diseases, especially in disturbed cardiac rhythm, angina or myocardial infarction. It apparently has a diuretic and a general tonic effect in cases of cirrhosis of the liver[5].

kutki (Picrorrhiza Kurrao)

Kutki (*Picrorrhiza kurrao*, Family- Scrophulariaceae) consists of dried rhizomes of the plant found in Himalayas, Jammu & Kashmir and Sikkim region. Root contains a glucoside called 'Kutkin", a glycosidal bitter principal, which is a mixture of two iridoid glycosides viz. Picroside I and kutkoside. The herb also contains other substances such as glucose, wax, cathartic acid etc.The plant is mainly used as therapy for liver and lung diseases. (Kutki) has focused on its hepatoprotective (liver protection), anticholestatic, antioxidant, and immune-modulating activity, improves the gall bladder secretions and helps in digestion and metabolism of fats. It is very useful in treating fatty liver and also regulates the fat metabolism in liver[6]

Amla (Emblica officinalis)

Amla (Emblica officinalis, family-Euphorbiaceae) is deciduous tree widely found in India at the height of 350 m. It is often cultivated at commercial level in Uttar Pradesh, Gujarat, Maharashtra and Rajasthan. Amla contains 5-6% of tannins such as gallic acid, ellagic acid and phyllembelin. Fresh fruit is refrigerant, diuretic and laxative, carminative and stomachic.The herb is also aphrodisiac, haemostatic, nutritive tonic and rejuvenative. It increases red blood cell count. A very recent study with alloxan-induced diabetic rats given an aqueous amla fruit extract has shown significant decrease of the blood glucose as well as triglyceridemic levels and an improvement of the liver function caused by a normalization of the liver-specific enzyme alanine transaminase (ALT) activity[7].

Shatavari (Asparagus racemosus)

Shatavari (Asparagus racemosus family- Liliaceae.) consists of dried roots and leaves found through out tropical Africa, Java, Australia, India, Sri Lanka and southern parts of China. In India it is found in plains to 4,000 ft high, in tropical, sub-tropical, dry and deciduous forests and in the Himalayas. Shatavari contains saponins, alkaloids, proteins, starch, tannin, mucilage and diosgenin. The plant contains triterpene saponins - Shatavarin I - IV, which are phytoestrogen compounds. The roots have oleaginous, cooling, antispasmodic, indigestible, appetizer, alliterative, stomach, tonic, aphrodisiac, galactagogue, astringent, antidiarrhoeatic, laxative properties and is useful in tumors, inflammations, diseases of blood and eye, throat complaints, tuberculosis, leprosy, epilepsy, night blindness and kidney troubles. A. racemosus has also been used successfully by some Ayurvedic practitioners for nervous disorders, inflammation, liver diseases and certain infectious diseases[8].

Chirata (Swertia chirayita)

Chirata (Swertia chirayita, family- Gentianacea.) is the entire herb found in the temperate Himalayas at altitudes of 1,200-3,000 m. from Kashmir to Bhutan, and in the Khasi hills in Meghalaya at 1,200-1,500 m. Chiratta is reported to contain a yellow bitter acid, ophelic acid, two bitter glycosides, chiratin and amarogentin, gentiopicrin, two yellow crystalline phenols, a neutral, yellow crystalline compound, and a new xanthone, swerchirin. Amarogentin is one of the bitterest substances known According to Ayurveda, this herb is a bitter tonic, stomachic. It is useful in liver disorders, eyes, heart remedy for a weak stomach, especially when this gives rise to nausea, indigestion and bloating and it has also been shown to protect the liver[9].

Milk thistle (Silybum marianum L.)

Silybum marianum (L.) family Asteraceae Gaerth is found in Western Himalayas at 1800 and Kashmir at 2400 m also grow in gardens. The active ingredient that protects the liver in milk thistle is known as silymarin. Silymarin is actually a group of flavonoids (silibinin, silidianin, and silicristin), which help in repairing liver cells damaged by alcohol and other toxic substances. Silymarin also keeps new liver cells from being destroyed by these same toxins. Silymarin have multiple mechanism of action that may be hepatoprotective, including anti-oxidant activity, toxin blockade, enhanced protein synthesis, and anti-fibrotic activity[10].

Dandelion *(Taraxacum officinale)*

Dandelion (Taraxacum officinale family- Asteraceae) is a herbaceous perennial plant found growing in temperate regions of the world, in lawns, on roadsides, on disturbed banks and shores of water ways, and other areas with moist soils.Dandelion root is a registered drug in Canada, sold principally as a diuretic. A leaf decoction can be drunk to "purify the blood", for the treatment of anemia, jaundice and also for nervousness. Drunk before meals, dandelion root coffee is claimed to stimulate digestive functions and function as a liver tonic[11].

Saffron (Crocus sativus)

Saffron (Crocus sativus Linn. Family- Iridaceae) consists of the dried stigmas and upper parts of styles of plant; cultivation in India is mostly confined to the table-land of

Pampur (5,300 ft. above sea level) in Jammu & Kashmir. Saffron contains glycosides crocin and picrocrocin together with lycopene, ß-carotene, g-carotene. The essential oil of saffron deposits, on standing, stearoptene, probably a tertiary alcohol. It is used as a nerve sedative and emmenagogue. It is used in fevers, melancholia and enlargement of the liver. It also has stimulant and stomachic properties and is considered to be a remedy for catarrhal affections of children. The stigmas in overdoses are narcotic.[12]

Nettle (Urtica parviflora)

Nettle (Urtica parviflora, family- Urticaceae) is a perennial shrub used in traditional medicine in Sikkim, Darjeeling and in North Bengal .The roots are employed for the treatment of fractures of bone and dislocations of joints. The leaves are used in dysentery, joint pain and liver disorders[13].

Fire flame bush (Woodfordia Fruticosa)

Fire flame bush (Woodfordia Fruticosa, family Lythraceae) is found throughout India but abundantly found in north India up to 1600 m. The chemical constituents like Woodfordins A, B, C, D, E, F, G, H, I and were identified from the flowers. The flowers are acrid, astringent, styptic, depurative, uterine sedative, constipating and antibacterial. They are useful in the conditions diarrhea, dysentery, fever, headache, hemorrhoids, herpes, internal hemorrhage, leucorrhea, liver disorders. Juice of leaves is used in bilious sickness[14].

Daruharidra (Berberis aristata)

Daruharidra (Berberis aristata, family Berberidaceae) the plant is native of the whole range of Himalaya Mountains at an elevation 2000 to 3500 metres. It also occurs in Nilagiri range in Southern India. The bark contains alkaloids like berberine, berbamine, aromoline, karachine, palmatine, oxyacanthine and oxyberberine. The roots possess antibacterial and anti-inflammatory activities. The drug is regarded as a bitter tonic and is apparently used as a cholagogue, stomachic, laxative, diaphoretic, antipyretic and antiseptic[15].

Himalayan May Apple (Podophyllum hexandrum)

Himalayan May apple (Podophyllum hexandrum, family- Berberideaceae) is a perennial herb, 15-40 cm tall, native to the Himalayas. The active principle of podophyllum is contained in the resinous mixture known as podophyllin. The other constituent of the root is podophyllotoxin. The rhizomes yield podophyllol, a sticky resin, quercetin and podophyllotoxin. The principal use of Podophyllum is in liver infections, primarily, it induces a large flow of bile. It is indicated in torpid or chronically congested liver, when diarrhea is present[16].

Jatamansi (Nardostachys jatamansi)

Jatamansi (Nardostachys jatamansi, family- Valerianceae) consist of dried rhizhomes of plant grown alpine Himalayas from Punjab to Sikkim and Bhutan, at altitudes of between 3,000 and 5,000 m. The Chemical constituents found are Jatamansone, Jatamanshic acid, Virolin and its diastereomers. Traditionally the drug has been used in the treatment of epilepsy, hysteria, and convulsions. The oil possesses anti-arrhythmic activity with possible therapeutic usefulness in cases of auricular flutter[17].

Kapur Kachri (Hedychium spicatum)

Kapur Kachri (Hedychium spicatum, family- Zingiberaceae) grows in sub tropical regions of Himalayas. It contains α-Pinene, β-pinene, limonene, 1, 8-cineole, 2-alkanones, linalool, camphor, linalyl acetate, ethyl-p-methoxy cinnamate, ethyl cinnamate, D-sabinene and sesquiterpene-cadinene etc. It is used in nausea, bronchial asthma, halitosis and vomiting, in diminished appetite, hiccups, local inflammation, in the treatment of liver complaints/ in the treatment of indigestion and poor circulation due to thickening of the blood[18].

Saussurea costus

It consists of dried roots of *Saussurea costus,* family- Compositae. Different pharmacological experiments in a number of *in vitro* and *in vivo* models have convincingly demonstrated the ability of *Saussurea costus* to exhibit anti-inflammatory, anti-ulcer, anticancer and hepatoprotective activities. Roots are tonic, stomachic, stimulant, carminative, diuretic, antiseptic, anthelmintic in nature[19].

Berberry *(Berberis lyceum)*

Berberry *(Berberis lyceum, family*-Berberidaceae) found from Kashmir to Garhwal on the outer Northern Western Himalayas on clearances and along road side between 800 to 2500 m. In Himachal Pradesh it is present in Chamba, Kangra, Kinnaur, Kullu, Shimla, Sirmour. Berberry contains Berberine and Umbellitine as the main alkaloids. The fruit contains tartaric, citric acids and tannins.Barberry's roots are used as remedy for swollen and sore eyes, broken bones, wounds, gonorrhea, curative piles and ulcer. Leaves are given in treatment of jaundice[12].

ACKNOWLEDGEMENT

The author is thankful to Prof.A.K Wahi, for his kind support and guidance.

REFERENCES

1. Clark AM. Natural products as a resource for new drugs. *Pharm. Res*.1996; **13**:1133-1144.
2. Gupta AK, Mishra N. Hepatoprotective activity of aqueous ethanolic extract of *Chamomile capitula* in paracetamol intoxicated albino rats. *American Journal of Pharmacology and Toxicology.* 2006; **1**:17-20
3. Grabley S, Thiericke R. Bioactive agents from natural sources: trends in discovery and application. *Adv. Biochem. Eng. Biotechnol*. 1999; **64**:101-154.
4. Rawat AK, Tripathi SC, Shome U. Some hepatoprotective activity of *Boerhaavia diffusa* L. roots- a popular Indian ethno medicine. *Journal of ethno Pharmacology*. 1997; **56**:61-66.
5. Girish C, Koner BC, Jayanthi S, Rao KR, Rajesh B. Hepatoprotective activity of six polyherbal formulations in paracetamol induced liver toxicity in mice. *Indian J Med Res* .2009; **129**:569-578.
6. Satyapal US, Kadam VJ, Ghosh R. Hepatoprotective activity of livobond a poly herbal formulation against CCl_4 induced Hepatotoxicity in rats. *International Journal of Pharmacology.* 2008; **4(6)**:472-476.
7. Khan KH. Roles of *Emblica officinalis* in medicine-a review. *Botany Research International*.2009; **2(4)**:218-228.
8. Madhavan V, Tijare RD, Mythreyi R, Gurudeva MR, Yoganarasimhan SN. Pharmacognostical studies on the root tubers of *Asparagus gonoclados* Baker- alternate source for the ayurvedic drugs shatavari. Indian Journal of Natural Products and Resources.2010; **1**:57-62.
9. Joshi P, Dhawan V. *Swertia chirayita*- an overview. *Current Science*; 2005; **89(4)**:635-640.
10. Ball. KarenRD, Kowdley. A review of *Ilybum marianum* (Milk Thistle) as treatment for alcoholic liver disease. *Journal of Clinical Gastroenterology*.2005; **39(6)**:520-528.
11. You Y, Yoo S, Yoon HG Park J, Lee YH. In vitro and in vivo hepatoprotective effects of the aqueous extracts from *Taraxacum officinale* (dandelion) root against alcohol-induced oxidative stress. *Food Chem Toxicol*. 2010; **48(6)**:1632-1637.
12. Sharma A, Shanker C, Tyagi L, Singh M, Rao CV. *Herbal medicines for market potential in India: an overview. Academic Journal of Plant Sciences.* 2008; **1(2)**:26-36.
13. Kar PS, Nath L, Dash S, Bhagabat N. Hepatoprotective effect of the ethanolic extract of *Urtica parviflora* Roxb. in CCl_4 treated rats. *International Journal of Pharmacology*. 2007; **3(4)**:362-366.
14. Das PK, Goswami S, Panda N, Banerjee S, Sahu NP. *Woodforia fruticosa:* Traditional uses and recent findings. *Journal of Ethnopharmacology.* 2007; **110(2):**189-199.
15. Sharma YK, Singh H, Mehra BL. Hepatoprotective effect of few avurvedic herbs in patients receiving antituberculus treatment. *Indian Journal of Traditional Knowledge*. 2004; **3(4)**:391-396
16. Rahal A, Mehra D, Rajesh S, Singh V, Ahmad AH. Prophylactic efficacy of *Podophyllum hexandrum* in alleviation of immobilization stress- induced oxidative damage in rat. *Journal of Natural products.* 2009; **2**:110-115.
17. Dandagi PM, Patil MB, Gadad AP, Dhumansure RH. Development and evaluation of hepatoprotective polyherbal formulation containing some indigenous medicinal plants. *Indian Journal of Pharmaceutical Sciences.* 2008; **70(2)**:265-268.
18. Gangwar KK, Deepali, Gangwar RS. Ethnomedicinal plants diversity in kumaun himalaya of uttarakhand , India . *Nature and Science*. 2010; **8(5)**:66-78.
19. Kaul MK. High altitude botanicals in integrative medicine-case studies from northwest himalaya. *Indian Journal of Traditional Knowledge*. 2010; **9(1)**:18-25.