Pharmacognostical Studies on the Leaves of *Eupatorium adenophorum* Spreng

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ABSTRACT

Eupatorium adenophorum Spreng belonging to the family Astercaceae is an annual herb. It grows up to one meter high, bears small white-colored flowers. Even though this plant has gained scientific importance recently, there is a need for the pharmacognostic standardization. Hence, in the present work the leaf and petiol part of the plant were subjected to various microscopical and physical evaluations. In the microscopical studies, the different cell structures and arrangements were studied and in physical evaluation the ash values and extractive values were studied. The various pharmacognostical constants were obtained which could help in the development of a suitable monograph for the plant.

Key words: *Eupatorium adenophorum* Spreng, leaf constants, fluorescencs analysis.

INTRODUCTION

Diseases have been known for thousands of years and many ingenious methods have been followed for the relief of mankind. Almost every source of life and matter surrounding human being has been used in some form or other to treat diseases. Since the beginning of the history of mankind plants have been used for the treatment of various diseases.

E. adenophorum Spreng (Asteraceae), a pantropic weed native to Mexico found in Sikkim, Meghalaya, Tripura, Uttar Pradesh and Tamil Nadu. Stems subterete; leaves elliptic or rhomboid- elliptic, acuminate; flowers in white terminal heads in small corymbs.[1] The plants leaves are used in cuts and wounds, also as Veterinary medicines in cuts and wounds,[2] analgesic[3] and antibacterial.[4] Chemically the plant has been found to be rich in cadenine derivatives and flavnoides; viz., α-cadinnine, naphthalene, 2,3,4,4a,5,6-hexahydro-7-methyl–1-(1-methylethyl)-6- ol, salvigenin, eupifriedelinol, β-amirin, lupeol, stigmasterol, taraxasterol, isohexacosane, n-hexacosanoic acid and stigmastadienone.[1-2] Genus *Eupatorium* has been used traditionally in the treatment of emetic, diaphoretic, emmenagogue, cathartic, stimulant, tonic.[5]

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In spite of the numerous medicinal uses attributed to this plant, however, there is no pharmacognostical report on the leaf or petiol of the plant to determine the anatomical and other physicochemical standards required for quality control of the crude drug. Hence, the present investigation includes morphological and anatomical evaluation, determination of physicochemical constants and the preliminary phytochemical screening of the different extracts of *E. adenophorum* Spreng.

MATERIALS AND METHODS

Plant material

The plant was collected in the month of November and December 2008 from the Forest of Nagdhar, Chamoli Dist., Uttrakhand India. Plant was identified and authenticated from Botanical Survey of India, Dehradun (Acc. no. 1127802).

Chemicals and reagents

All the chemicals and reagents used were of laboratory grade.

Macroscopic and microscopic analysis

The macroscopy and microscopy of the plant were studied according to the method of Brain and Turner.[6] For the microscopical study, cross sections were prepared and stained

as per the procedure of Johansen.[7] The micro powder analysis and leaf constant was done according to the official method.[8-11]

Physicochemical analysis

Physicochemical values such as percentage of ash values and extractive values and loss on drying were performed according to the official methods.[12-13] Fluorescence characteristics of the powder of drug was examined under ultra-violet light according to the methods suggested by Kokoski et al.[14] Behavior of the powder of drug with different reagents observed under ordinary light and UV-radiation according to the methods suggested by Chase, C.R. et al.[15]

Preliminary Phytochemical Screening

Preliminary phytochemical screening was carried out by using standard procedures described by Kokate[16] and Harborne.[17]

RESULTS

Leaf Macroscopic characteristics

The macroscopy of leaf revealed that leaf is paripinnate compound 5.0-14.0 cm long, 2.5 - 5.6cm broad; colour-blakish, lanceolat to ovate lanceolate; margin serrate, apex acute, symmetrical base; both surface glabrous with reticulate (divergent) venation, pinnate veinlets, petiole 1.5- 4-5.0 cm long, characteristic odour and bitter taste. Stem is dark purple with woody base with two opposite leaves; the bark of stem is thin, dark purple. Stem slightly rough to touch; fracture irregular and fibrous, astringent taste with no characteristic odour (Figure 1).

Leaf Microscopic characters

Midrib: The T.S. of midrib (figure2A) shows dorsiventral structure and a distinct biconvex out line in the basal and middle regions where as in the apical region becomes Plano convex. The T.S. show single layered epidermis covered with distinct characteristic striated cuticle. Epidermal cells of the ventral side and dorsal side are more or less round to oval in shape and distinct thinking on radial walls. Some of the epidermal cells on the ventral sides elongate to form covering trichomes which are 3-4 cells long and show mostly pointed end whereas the epidermal cells on dorsal side show more number of trichome as compared to ventral side which are similar to the trichome of ventral side. Beneath the epidermal cells on both the sides the layers of collenchymatous cells is wider towards the ventral side and

Figure 1: A young plant of *Eupatorium adenophorum* Spreng

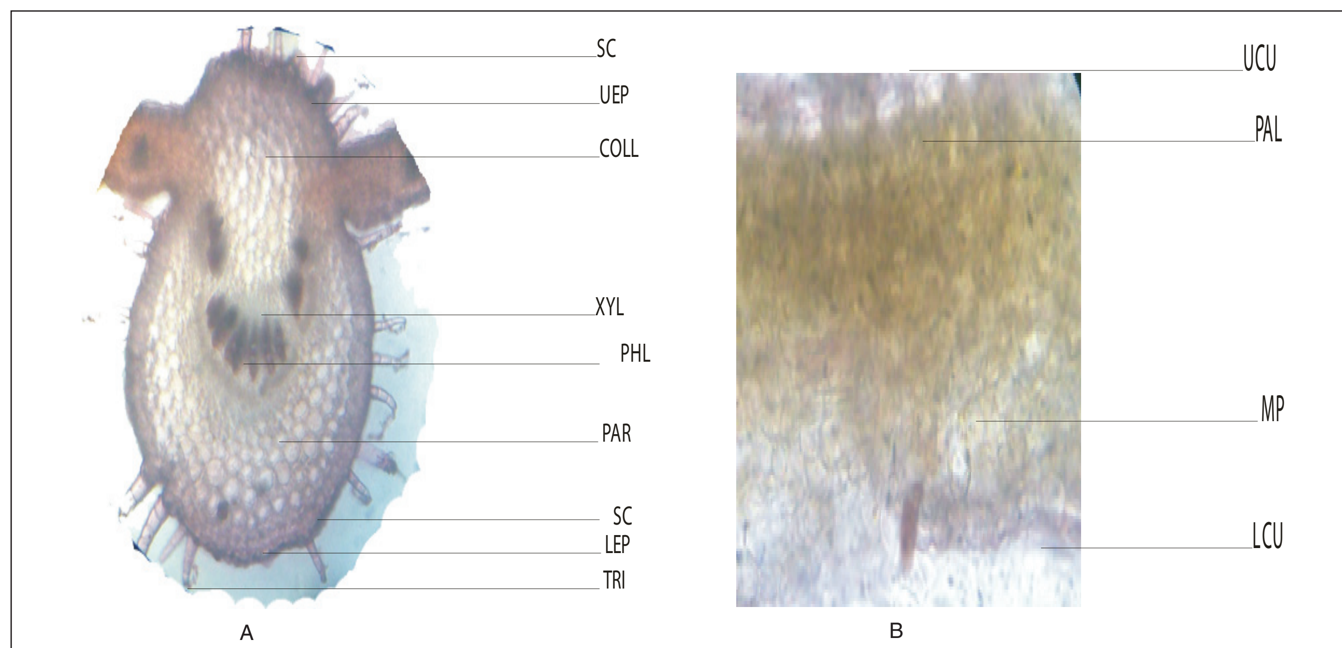

Figure 2: (A) T.S.of leaf through mid –rib; (B) T.S.of leaf through lamina at 100X; SC- striated cuticle, UEP- upper epidermis, COLL- collenchyma, XYL-xylem, PHL-phloem, PAR-parenchyma, LEP-lower epidermis, TRI- trichome, UCU-upper cuticle, PAL-palisade, MP-mesophyll, LCU- lower cuticle.

more or less double in width that of dorsal side. All the collenchyamtous cells show distinct angular thinking on their walls which becomes lesser towards the central region. The parenchymatous cells are mostly round or oval in shape but the parenchymatous cells on dorsal side have distinctly bigger space as compared to ventral side. The central region of the mid-rib is occupied by vascular tissues which are arch shape showing, phloem on the dorsal side capping the xylem. The phloem consist sieve-tubes, compaining cell and phloem parenchyma. The xylem consists of vessels, tracheids and xylem parenchyma which show distinctly thick wall lignification and wide lumen. Below the arch of the xylem is a wide region of parenchymatous cell which also appear similar to parenchymatous cells described earlier.

Lamina: The T.S. of lamina (figure2B) shows a single layered epidermis composed of either tangentially or radial elongated cells. The epidermal cells of the ventral sides are distinctly bigger than the epidermal cells of dorsal side. Large numbers of epidermal cells get modified into stomata on both dorsal and ventral side which is usually paracytic. However, they are more in number towards the dorsal side. Epidermis on dorsal and ventral side is covered with distinct striated cuticle. The palisade cells are followed by 3-5 layers of spongy parenchyma which are usually round to oval in shape showing large spaces. Some of the spongy parenchymatous cells show prismatic crystal of calcium-oxalate which varies in shape and size.

Petiole: T.S.of Petiole show (figure 3A, 3B) Epidermis is single layered with large to small epidermal cells towards the plane region where as towards the convex region the epidermal cells are oval and same in size. However, epidermal cells on both sides a strong thickening on their radial wall. The numbers of epidermal cells elongated to

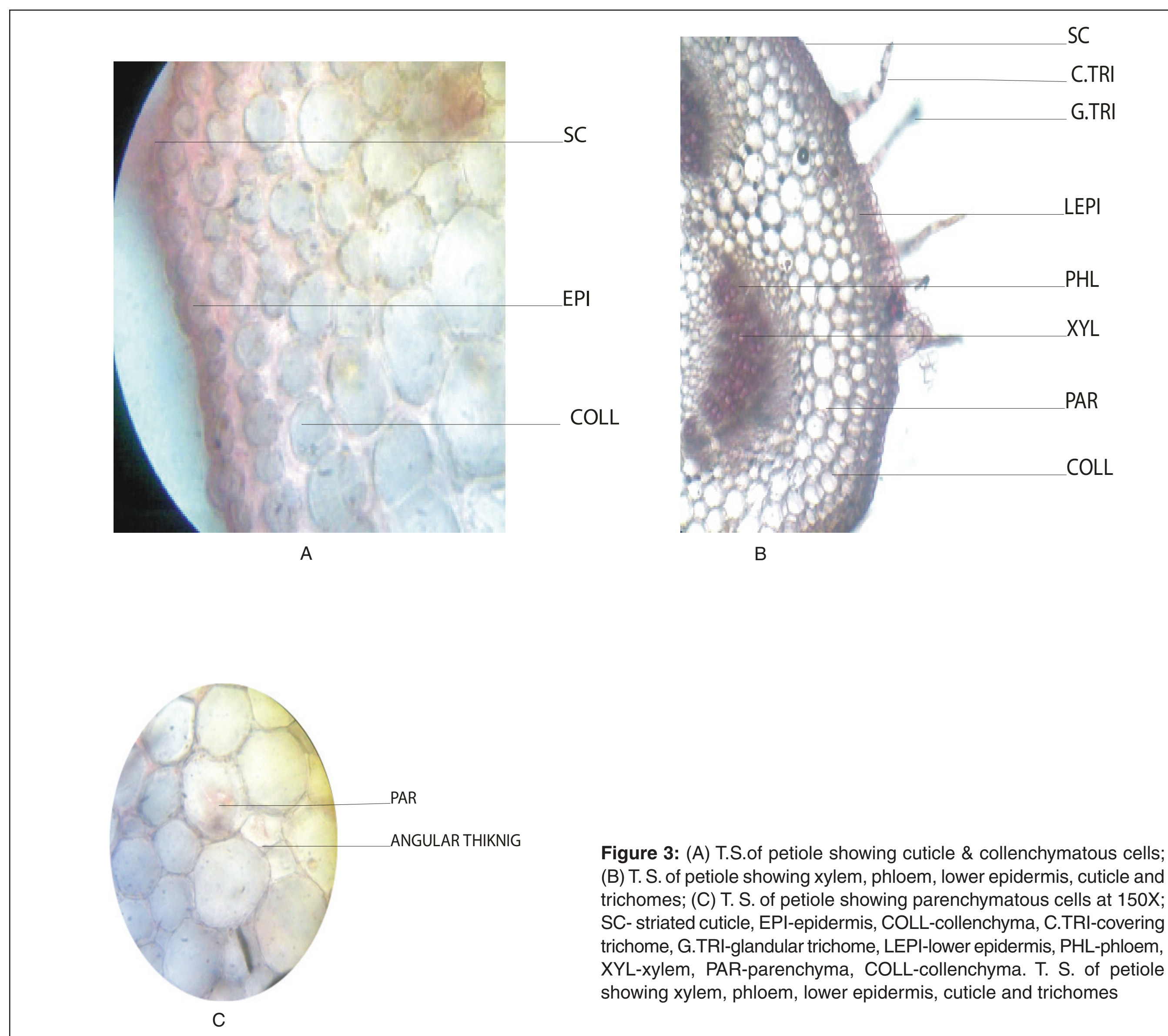

Figure 3: (A) T.S.of petiole showing cuticle & collenchymatous cells; (B) T. S. of petiole showing xylem, phloem, lower epidermis, cuticle and trichomes; (C) T. S. of petiole showing parenchymatous cells at 150X; SC- striated cuticle, EPI-epidermis, COLL-collenchyma, C.TRI-covering trichome, G.TRI-glandular trichome, LEPI-lower epidermis, PHL-phloem, XYL-xylem, PAR-parenchyma, COLL-collenchyma. T. S. of petiole showing xylem, phloem, lower epidermis, cuticle and trichomes

forms trichomes. Epidermis on both sides is followed by the collenchyma composed of 7-10 layers but the collenchymatous cells towards the plane surface distinctly are bigger than the dorsal side. The collenchymatous cells show strong angular thickening on their walls and they vary in shape and size. Beneath the collenchymatous region, there is continuous layer of parenchyma composed of 7-10 layers. The numbers of distinct layers of parenchyma are more below the collenchyma towards the plane region. The parenchymatous cells (figure 3C) are mostly round to circular in shape beneath the collenchyma of plane region. Central region of the petiole is occupied by arc shape vascular tissues showing phloem towards the dorsal side xylem. The phloem appears wider as compare to xylem and is composed of sieve-tubes, phloem parenchyma and companion cells.

Trichomes: The trichomes (figure 4A, 3B, 3C) are two type, covering, and glandular. The covering trichome are 5-7 cells long showing tapering ends or bifurcated ends and they shows thick or thin walls. Covering trichome are more common towards the plane region as compared to dorsal side. The glandular trichomes show the base consisting of 2-4 cells with unicellular head. However the cell of stalk of glandular trichome also show thick wall. The glandular trichome are absent toward the plane region but are few in number on the dorsal side.

Characteristics of Leaf Powder

Powder characteristic of the leaf revealed the presence of vessel with simple pitted and scleriform thickenings, groups of prismatic calcium oxalate crystals and fragments of epidermal cells with paracytic stomata (figure 5A- F).

Phytochemical Screening

Preliminary phytochemical screening mainly revealed the presence of carbohydrates, alkaloids, phytosterols, fixed oils, flavnoides, terpenoides, and phenolic compounds Table 1.

Fluorescence Analysis

The powder was subject to fluorescence analysis as per the standard procedure and shown in Table 2.

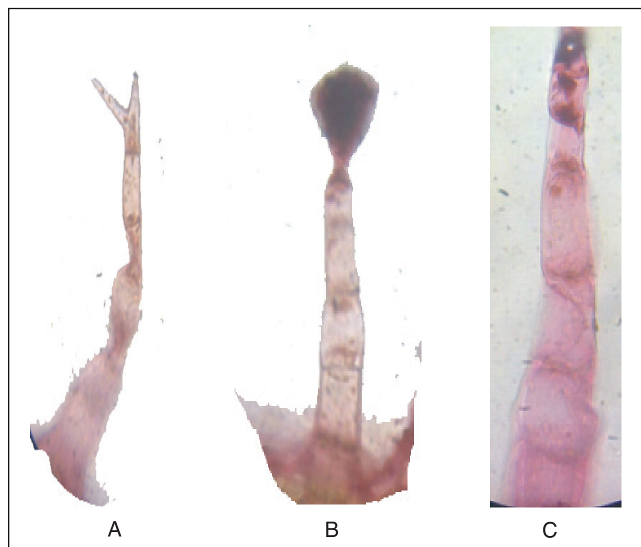

Figure 4: (A) Covering trichome with bifurcated end; (B) Glandular trichome; (C) Covering trichome with tapering end at 150X

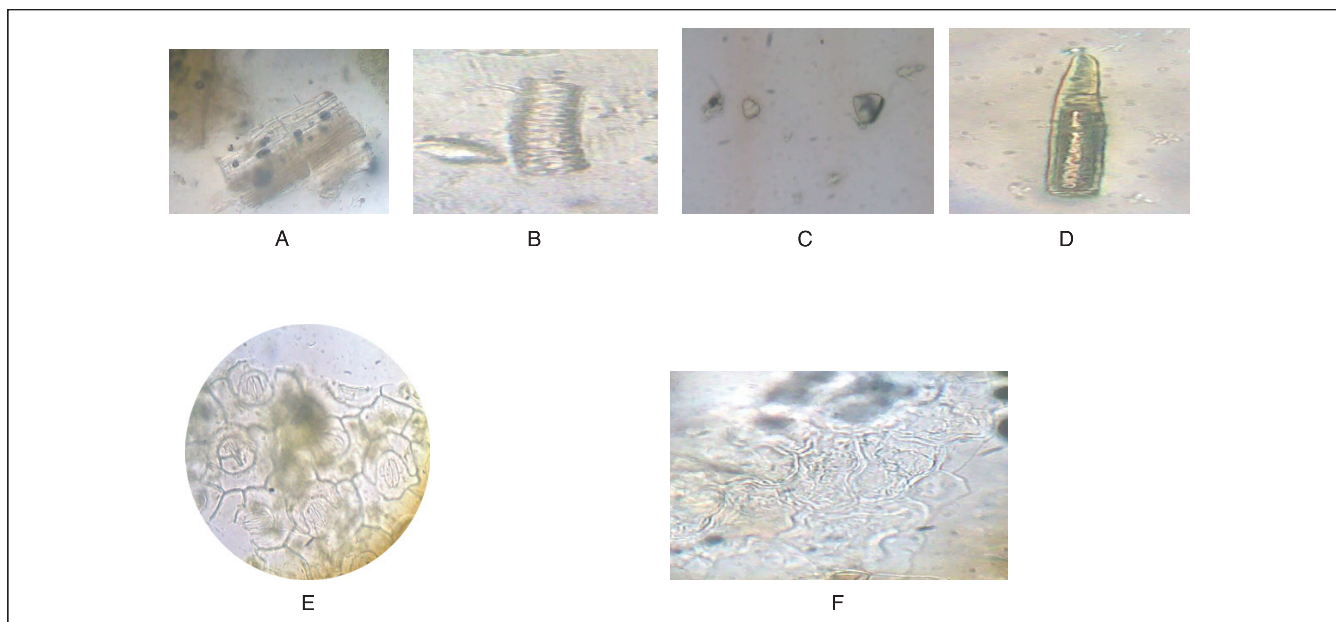

Figure 5: (A) Xylem vessels (pitted); (B) Xylem Vessels (scleriform thikning); (C) Calcium oxalate crystals; (D) Fiber;(E) Stomata (paracytic); (F) Epidermal cells at 100x.

Behavior of powder with chemical reagents

Behavior of leaf powder with different chemical reagents was studied to detect the presence of phytoconstituents with color changes under daylight by reported method.[15] and the results were shown in Table 3.

Physicochemical Parameters

Ash values of a drug give an idea of the earthy matter or the inorganic composition and other impurities present along with the drug. The ash values Table 4 of the powdered *E. adenophorum* Spreng leaf. The total ash value was higher than that of the acid insoluble and water soluble ash value and a decrease in the acid insoluble ash value may be due to presence of smaller quantity of siliceous matters. The weight lose after drying the leaves on 100-105^{0}c in oven are tabulated in Table 4.

The extractive values are primarily useful for the determination of exhausted or adulterated drug. The water soluble extractive Table 5 was higher than the alcoholic extractive value in *E. adenophorum* Spreng revealing presence of larger amount of water soluble constituents in the leaves such as plant acids, carbohydrates and phenolic compounds.

Extractive values

The extracts were prepared according to the polarity and they were concentrated and their values were calculated with reference to air dried drug. The results are tabulated in Table 6.

Quantitative Analysis

The fresh leaf samples were subjected to quantitative analysis for various leaf constants like stomatal number, stomatal

Table 1: Preliminary phytochemical screening of leaf powder of *E. adenophorum* Spreng

Test	Pet. ether	Benzene	Chloroform	Acetone	Ethanol	Aqueous
Alkaloids	+++	++	+++	+	++	+
Carbohydrates	+	–	+	–	+	+
Phytosterols	++	+	+	+	++	+
Fixed oils and fats	+++	++	+	++	+++	–
Saponins	+	+	+	+	+	++
Phenolic compounds and tannins	++	++	++	++	+++	+
Proteins	+	–	+	–	+	+
Gums and mucilages	+	+	+	–	+	++
Flavonoids	+++	++	+	+	++	++

Table 2: Fluorescence characteristics of leaf powder of *E. adenophorum* Spreng

Treatment	Fluorescence Observed
Powder as such	Greenish
Powder treated with methanol	Greenish
Powder treated with nitrocellulose in Amyl acetate	Green
Powder treated with 1N Hcl in methanol	Green
Powder trated with nitrocellulose in amyl acetate after step 4	Dark brown
Powder treated with 1N Sodium hydroxide in methanol	Dark brown
Powder trated with nitrocellulose in amyl acetate after step 6	Yellowish green
Powder treated with 50% sulphuric acid	Dark brown
Powder treated with 50% nitric acid	Reddish brown

Table 3: Behavior of leaf powder *E. adenophorum* Spreng with different reagents observed under ordinary light and UV-radiation

Interaction of powder drug with different reagent	Colour produced under ordinary light	Colour under UV-radiation	
		254nm	366nm
Drug (P) as such	Green	Green	Black
P + Fecl$_3$	Black	Black	Black
P + 1N HCL	Greenish brown	Light green	Black
P + conc. H_2SO_4	Dark brown	Green	Dark brown
P + iodine solution	Brown	Green	Black
P + picric acid	Yellowish	Light green	Dark green
P + alcohol (50%)	Green	Green	Black
P + acetic acid	Light green	Light green	Dark green
P + lead acetate (5%)	Light brown	Green	Dark green
P + HNO_3	Reddish brown	Light green	Black

Table 4: The average percentage ash values of leaf powder of *E. adenophorum* Spreng

Parameters	Average Ash value (%) w/w
Total ash	10.27
Acid insoluble ash	8.3
Water soluble ash	4.3
Loss on drying	0.1567

Table 5: The average percentage Extractive values of leaf powder of *E. adenophorum* Spreng

Parameters	Colour of the extract	Average percentage extractive value w/w
Water soluble extractive	Dark brown	13.71
Alcohol soluble extractive	Green	11.84

index, vein islet number, vein termination number, palisade ratio. The results are shown in Table 7.

DISCUSSION

E. adenophorum Spreng family astrcaceace is an erect, branched or perinial herbs with woody base to 1 meter tall. Stem is dark purple. The leaves are parpinate compound 5.0 × 14.0 cm long, 2.5 × 5.6 cm broad, lanceolate to ovate, characteristics odour and bitter test.

The T. S. of midrib shows dorsiventral structure and distinct biconvex outline in the basal and middle region. The epidermis is covered with striated cuticle, a single layered epidermis cells. Covering trichome are 3-4 cells long in size and show mostly pointed end. The collenchymatous cells are wider towards the ventral side and the collenchymatous cell show distinct angular thickening. The parenchymatous cells mostly round or oval in shape, but the parenchymatous cells on dorsal side have distinctly bigger shape compare to ventral side. The central portion of midrib is occupied by vascular tissues which are arch shape. The T.S. of lamina show single layered epidermis composed of either tangentially or radial elongated cells. The palisade cells followed by 3-5 layers of spongy parenchyma which are usually round to oval in shape showing large space. The T.S. of petiole shows more or less Plano convex out line with slight depression towards the plane side. The epidermal cells are single layered. The covering trichome are 5-7 cells long with tapering end, collenchymatous cells composed of 7-10 layers. Beneath the collenchymatous region, there is continuous layer of parenchyma composed of 7-10 layers, the parenchymatous cells on dorsal side show distinct intracellular space. The average vein-islet number in apex, middle and base region of the leaf is respectively 15-19, 24-30 and 17-22 and the vein-let termination in apex, middle and base region of the leaf is respectively 9-12, 15-20 and 11-15. The palisade ratio of apex, middle and base region respectively 1:5, 1:10 and 1:8. The average number of stomata in upper and lower surface of the leaf is 45-50 and 76-90 to middle region, 9-13 and 43-50 in apex and 16-23 and 23-30 to base region respectively. The stomatal index of apex, middle and base were 42.8-61.9, 69.23-71.42 and 55.17-63.8 respectively. The percentage w/w of the total ash, water soluble ash and acid insoluble ash is 10.27, 4.3, 8.3 and the percent loss on drying at 100-105^{0}C is 0.15675 respectively. The percentage w/w extractive value of water soluble extractive and alcohol soluble extractive are 13.71 and 11.84. The powder drug after treatments with nitrocellulose with amyl acetate give the yellow fluorescence and after the treatments with 50% nitric acid give reddish brown fluorescence. The behaviors of drug powder in different reagents were observed under ordinary light and UV radiation (254 and 365 nm). None of the reagents show the diagnostic colour reaction under either ordinary or UV radiation.

Table 6: Percentage extractives of the successive extracts of *E. adenophorum* Spreng leaves

Extract	Colour of extract	Percentage extractive (w/w)
Petroleum ether	Dark green	2.390
Benzene	Black	1.029
Chloroform	Black	1.179
Acetone	Dark green	2.189
Alcohol	Dark green	3.456
Aqueous	Dark brown	3.968

Table 7: Leaf constants of *E. adenophorum* Spreng (in average)

Parameters	*E .adenophorum* Spreng		
Stomatal number	**Apex**	**Middle**	**Base**
Upper epidermis	9-13	45-50	16-23
Lower epidermis	43-50	76-90	23-30
Stomatal index			
Upper epidermis	42.8-61.9	69.23-71.42	55.17-63.8
Lower epidermis	43.9-62.4	73.40-72.40	57.30-65.10
Vein islet number	15-19	24-30	17-22
Vein termination number	9-12	15-20	11-15
Palisade ratio	1:5	1:10	1:8

CONCLUSION

As there is no pharmacognostic / anatomical work record of this much valued traditional drug, the present work was taken up with a view to lay down standards which could be useful to detect the authenticity of this medicinally useful plant. In other words, the pharmacognostic features examined in the present study may serve as tool for identification of the plant for validation of the raw material and for standardization of its formulations.

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Pharmacognostical and Phytochemical Evaluation of Leaves Extract of *Jatropha curcas* Linn

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ABSTRACT

Jatropha curcas (Euphorbiaceae) is a multipurpose plant with many attributes and considerable potential. Different parts of the *Jatropha curcas* plant are employed in Indian traditional medicine for the treatment of several disorders. The present work was targeted at developing quality parameter on the basis of pharmaceutical parameter leaves are reported to be employed in cancer, as an abortifacient, antiseptic, diuretic, purgative and haemostatic. Pharmacognostical parameters of the leaves were studied with the aim of drawing the pharmacopoeial standards for this species. Macroscopical and microscopical characters, physico-chemical constants, phytochemical study for the presence of various secondary metabolites and HPTLC fingerprint profile of methanolic extract were studied. The determination of these characters will aid future investigators in their Pharmacological analyses of this species.

Key words: *Jatropha curcas*, Euphorbiaceae, HPTLC, Pharmacognostical, Phytochemical analysis

INTRODUCTION

Euphorbiaceae is a diversified family, consisting of approximately 300 genera and 8000 species. Their classification has been studied for 150 years by a number of taxonomists. Webster (1975) divided the family into five subfamilies: Phyllanthoideae, Oldfieldioideae, Acalyphoideae, Crotonoideae and Euphorbioideae.[1] Jatropha L. belongs to the subfamily Crotonoideae and consists of approximately 175 species.[2] Out of which six species are native of India i.e. *Jatropha glandulifera*, *Jatropha tanjorensis* , *Jatropha villosa, Jatropha nana, Jatropha heynei, Jatropha maheswari.*[3] *Jatropha curcas* L. (physic nut or purging nut) is a drought resistant shrub or tree belonging to the family Euphorbiaceae, which is cultivated in Central and South America, South-East Asia, India and Africa. Some of the ethnomedical uses of the extracts of *Jatropha curcas* leaves and roots include use as a remedy for cancer, as an abortifacient antiseptic, diuretic, purgative and haemostatic. The nut of the plant has also been used traditionally for the treatment of many ailments including burns, convulsions, fever and inflammation.[4] The present investigation deals with the study of some Pharmacognostical characteristic of the leaves as a whole and in their powdered form.

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MATERIAL AND METHODS

Collection and authentication of plant material

The plant material was collected from the National Botanical Research Institute Garden, Lucknow, in the month July 2008 and authenticated by a Taxonomist. The voucher specimen No. 95215 was deposited in the departmental herbarium of N.B.R.I. Lucknow, India for future reference. The leaves were separated, dried, coarsely powdered passed through sieve no 40 and stored in a closed container for further use. All reagents used were of analytical grade.

Morphological and microscopical studies

The macroscopical characters (size, shape colour, odour, texture, margin, base, apex and petiole) of the leaves were observed.[5] Then, anatomical study, powder was identified with routine reagents to study the lignified cells, trichomes, stomata, fibres etc. Quantitative microscopy like measurement of vein islet number, vein termination number, Stomatal number, Stomatal index and palisade ratio were determined by methods prescribed by Ayurvedic Pharmacopoeia.[6]

Qualitative microscopy

The required samples of leaves of selected plant were cut and fixed in FAA (formalin-5ml+ 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.[7] Infiltration of the specimens were carried out by gradual addition of paraffin wax (melting point 58-60 ^{0} c) until TBA solution attained super saturation. The specimens were casted into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10- 12 μm. Dew axing of the sections were done by customary procedure.[8] The sections were stained with Toluidine blue as per the method.[9] Since the Toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the protein bodies etc. Where ever necessary sections were also obtained with safranin and fast green and IKI (for starch). Photographs of different magnification were taken with Nikon Labphot 2.

Determination of physicochemical parameters

The ash values, extractive values with various reagents and were determined as per the Indian Pharmacopoeia.[10] The fluorescence characters of the powder with various acids were observed under visible light and UV light (254 & 366 nm) as per the standard procedure.[11-12]

Preliminary phytochemical screening

The freshly collected leaves (4 kg) of *J. curcas* were first air dried and then dried in tray drier under control conditions and powdered. The powdered leaves (1000g) were macerated with petroleum ether to remove fatty substances; the marc was further exhaustively extracted with of 50% ethanol for 3 days (3 × 3L) by cold percolation method and centrifugation at 10,000 rev/min. The extract was separated by filtration and concentrated on rotavapour (Buchi, USA) and then dried in lyophilizer (Labconco, USA) under reduced pressure and thus 95.0 g of solid residue (yield 9.5 % w/w) was obtained. The extract was subjected to following preliminary phytochemical screening for the identification of various active constituents.[13-14]

HPTLC fingerprint profile

Modern High Performance Thin Layer Chromatography (HPTLC) is a powerful and reliable method for qualitative and quantitative analysis. It is also known as planer chromatography or instrumental thin layer chromatography. The “total extract” can only be described by “fingerprint” technique in which a large amount of chromatographic data of “standard” is compared with the data from the samples. This characterization data is obtained from multiwavelength scanning in UV, fluorescence scan, in-situ UV spectra, image comparison and where applicable, data after post chromatographic derivatisation.[15]

Test solution

Powdered plant material (2g) extracted with methanol: water 70:30 (3 × 100 mL, 8 h each) , concentrate at low temperature by rotary evaporation (Buchi, USA), then freeze dried under high vaccum , thus yield 2.36% w/w was obtained.

Standard solution

Dissolve 1 mg of pure compound (marker compound) in 1 ml of pure solvent methanol.

Selection of solvent system

Solvent system	Ratio	Rf value
Ethyl acetate :acetic acid : formic acid : water	10 : 1 : 1 : 1	0.37
Chloroform : methanol : water	9 : 1 : 0.1	0.50
Toluene : ethyl acetate : formic acid	7 : 2 : 1	0.42

Solvent system

Toluene: Ethyl acetate: Formic acid (7 : 2 : 1)

Procedure

High performance thin layer chromatography (HPTLC) can successfully be employed for finger printing of *Jatropha curcas* (JC). HPTLC analysis of JC was performed on pre-activated (100 °C) silica gel G 60 F_{254} HPTLC plates (Merck) as stationary phase and plate was eluted in solvent system toluene : ethyl acetate : formic acid (7 : 2 : 1). After development, the plate was dried and densitometrically scanned at 631nm. (WinCats software, CAMAG, Switzerland).

RESULTS

Macroscopic characters

Macroscopic and sensory evaluation of the leaves revealed that the leaves were green, deciduous, alternate but apically crowded, broadly ovate, cordate, acute, usually palmately, 3 – 5 lobed in out line, 6-40 cm long, 6-35 cmm broad, glabrous, base seven nerved, stipules zero, petiole 2.5-7.5 cm. long. (Figure 1)

Microscopical characteristics

Midrib: T. S. of leaf passing through midrib is pear shaped, covered with thick cuticle. Epidermal cells single layered, meristele centrally located, crescent shape xylem vessels are radially arranged in endarch condition, phloem well developed surrounded by crescent shaped xylem. Phloem

consists of sieve tubes, companion cells, laticifurs. Plenty of cluster, crystal of calcium oxalate surrounds the meristele as well as some are scattered in collenchymatous ground tissue. A number of laticifurs and mucilage canal are also found scattered in the mid rib region (Figure 2). T. S. of leaf passing through lamina shows upper and lower epidermis followed by 1 – 2 layers of palisade cells followed by 2 – 3 layered spongy mesophyll. Cluster crystals of calcium oxalate, mucilage canal and reticulate and annular vessels also found scattered throughout the region.

Petiole: T. S. of petiole is almost circular in outline. Cuticle thick followed by dumble shaped thick walled epidermis. Underneath this lies collenchymatous hypodermis followed

Figure 1: Young plant of *J. curcas*, Leaves of *J. curcas*

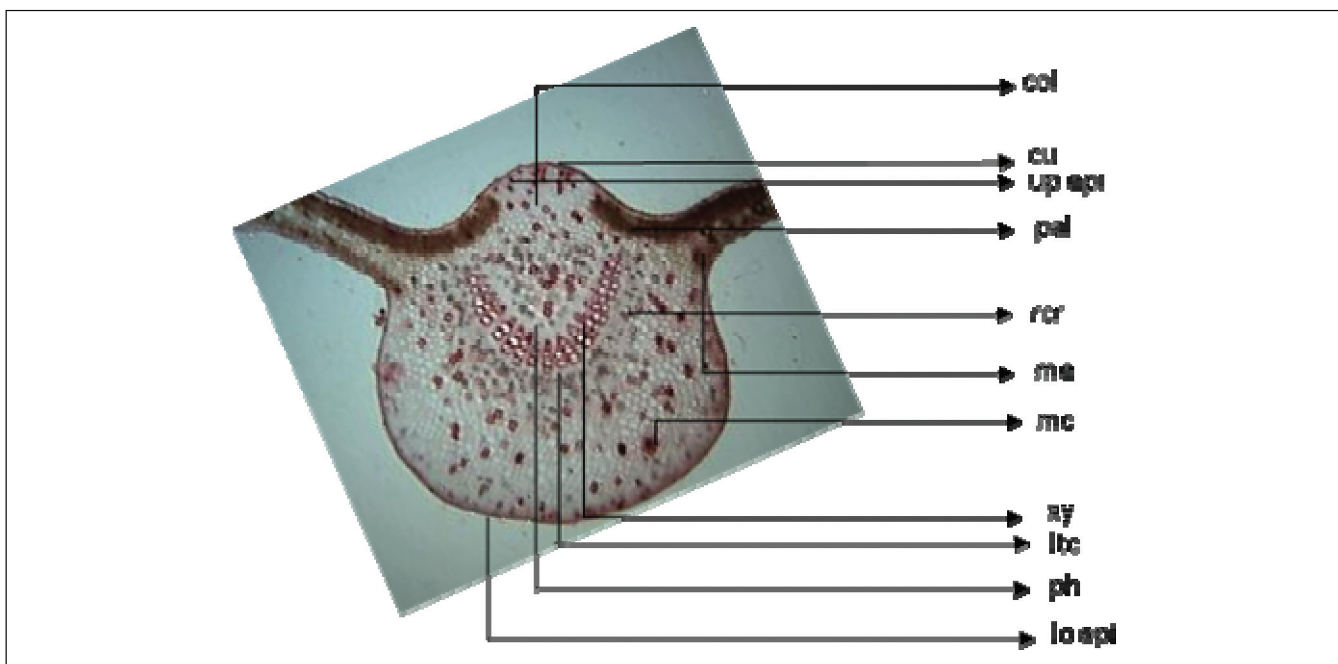

Figure 2: T.S. of leaf of *J. curcas* passing through midrib
Abbreviations: cu, cuticle; up epi, upper epidermis; mc, mucilage canal; ph, phloem; xy, xylem, col, collenchymas; pal, palisade cells; rcr, rosette crystal; me, mesophyll; ltc, laticifurs; lo epi, lower epidermis.

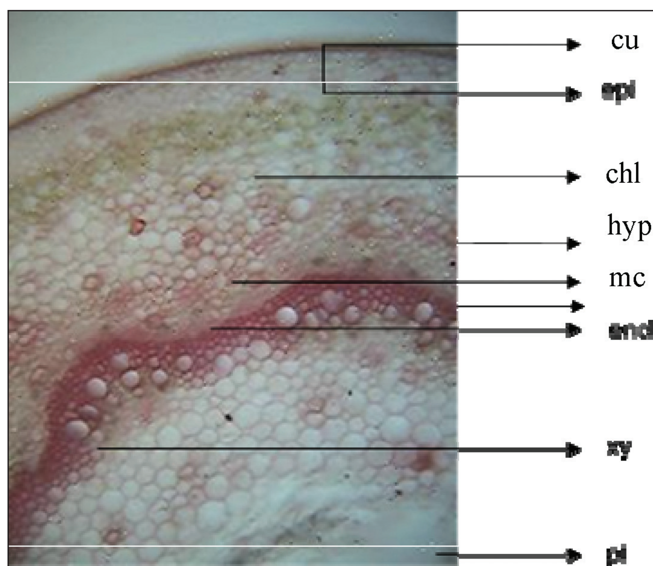

Figure 3: T. S. of Petiole of *J. Curcas*
Abbreviations: cu, cuticle; epi, epidermis; hyp, hypodermis; mc, mucilage canal; chl, chlorenchyma; end, endodermis; xy, xylem, pi, pith.

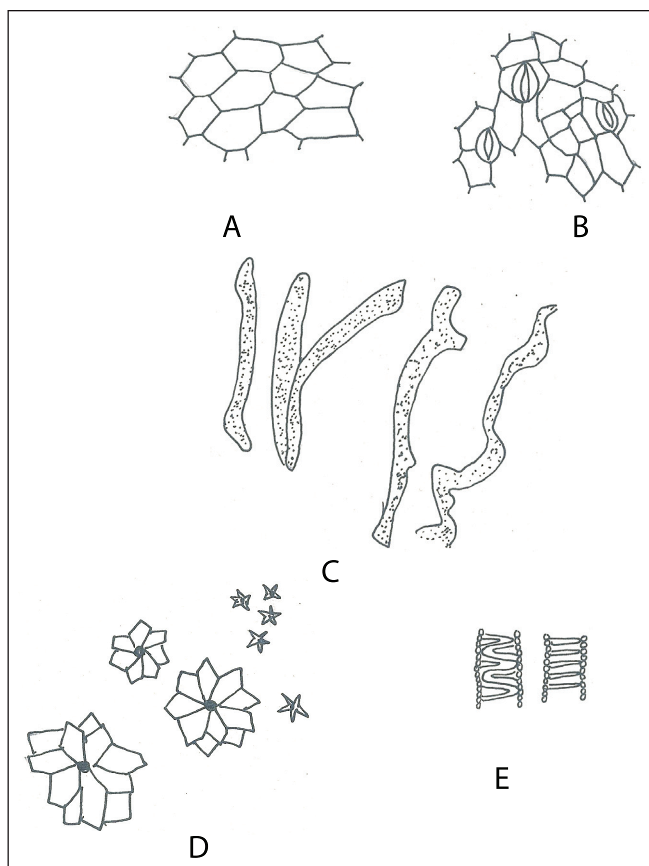

Figure 4: Powder characteristics of *J. curcas* leaf.
*Abbreviation – **A,** upper epidermal cells in surface view; **B,** lower epidermal cells in surface view; **C,** laticefarous vessel; **D,** cluster and rosette crystal of calcium oxalate; **E,** reticulate and annular vessel.*

by 3 – 5 layered chlorenchyma and the remaining cortex being parenchymatous. Mucilage canal and laticifurs are scattered throughout the cortical region. Endodermis discontinuous, phloem well developed, continuous and consists of sieve tubes, companion cells and parenchyma. Xylem wavy continuous, consists of vessels with large lumen, mostly solitary or in the group of 2 – 3 under the ridges. Pith is centrally hollow, embedded with mucilage and latex canal (Figure 3).

Powder microscopy

It is brown in color with no taste and odor. On microscopy it shows upper and lower surface of epidermal cells, laticifers, simple trichomes unicellular and multicellular, rosette and cluster crystals of calcium oxalate of varying size. Vessels with scalariform and spiral secondary wall thickenings (Figure 4).

Quantitative microscopy

Quantitative microscopy of leaves of *J. curcas* revealed the following data were illustrated in Table 1.

Physicochemical parameters

Physicochemical parameters like foreign matter, percentage of moisture content, total ash, acid insoluble ash, water soluble ash, ethanol soluble extractive and water soluble extractive were determined and depicted in Table 2. The results of fluorescence analysis of the powder drug are presented in Table 3.

Table 1: Leaf constants of *Jatropha curcas*

Leaf constant	Range	Average
Stomatal number (upper surface)	2-3	2.2
Stomatal number (lower surface)	19-21	20.2
Stomatal index (upper surface)	6.06-8.57	7.076
Stomatal index (lower surface)	29.41-32.30	30.959
Vein-islet number	2-4	3
Palisade ratio (Base : Middle : Apex)	4.07 : 4.1 : 4.32	4.075 : 4.1 : 4.325

Table 2: Physicochemical parameter of *J. curcas* Linn

Parameter	% w/w*	S.D.
Foreign matter	Nil	
Moisture content	9.5	0.59
Total ash	21.19	0.77
Water soluble ash	14.45	0.47
Acid insoluble ash	1.6325	0.31
Alcohol soluble extractive value	4.825	0.42
Water soluble extractive value	17.125	0.49

**Average of three readings*

Preliminary phytochemical screening

Preliminary phytochemical screening revealed the presence of oils and fats, phytosterol, proteins, amino acids, gum, mucilage and flavonoids in 50% ethanolic extract of leaves. (Table 4)

HPTLC finger print profile

A band (Rf = 0.42) corresponding to marker compound (β-sitosterol) was visible in both reference solution and test solution tracks.

10 μl of the test solution was applicated on precoated HPTLC plate, the plate was then developed in the solvent system [Toluene: Ethyl acetate: Formic acid (7 : 2 : 1)]. The plate was then developed in the solvent system to a height of 8 cm, dried and scanned densitometrically at 631 nm, the peak area was recorded and the calibration curve was prepared by plotting the peak area against concentration of the β-sitosterol applied. The percentage of area of β-sitosterol in the extract was found to be 0.4965% w/w (Figure 5).

Table 3: Fluorescence analysis of *J. curcas* powder

Reagents	Color of the powdered drug		
	Day light	Ultraviolet light	
		Near UV	Far UV
Saturated picric acid	Yellowish brown	Green	Green
Nitric acid	Radish brown	Light brown	Brown
Hydrochloric acid	Brown	Black	Black
Sulphuric acid (80%)	Black	Black	Blank
Glacial acetic acid	Brown	Green	Brown
Iodine solution (N/20)	Brown	Dark green	Black
Ferric solution (5% w/v aq. Solution)	Yellowish brown	Green	Dark brown
Powder as such	Brown	Light green	Dark brown

Table 4: Preliminary phytochemical screening of the 50% ethanolic extract of *J. curcas*

Constituents	Tests	50% EtOH extract
Carbohydrate	Molish's test	–
	Fehling's test	–
Fixed oil & fats	Spot test	+
	Saponification test	+
Proteins & amino acids	Million's test	+
	Ninhydrin test	+
	Biuret test	+
Saponins	Foam test	+
Phenolic compounds	$FeCl_3$ test	–
	Gelatin test	–
	Lead acetate test	–
Phytosterol	Salkowiski test	+
	Libermann burchard test	+
Alkaloids	Dragendroff's test	–
	Mayer's test	–
	Wagner's test	–
	Hager's test	–
Gum & mucilage	Swelling test	+
Flavonoids	Aqueous NaOH test	+
	Con. H_2SO_4 test	+
	Shinoda's test	+

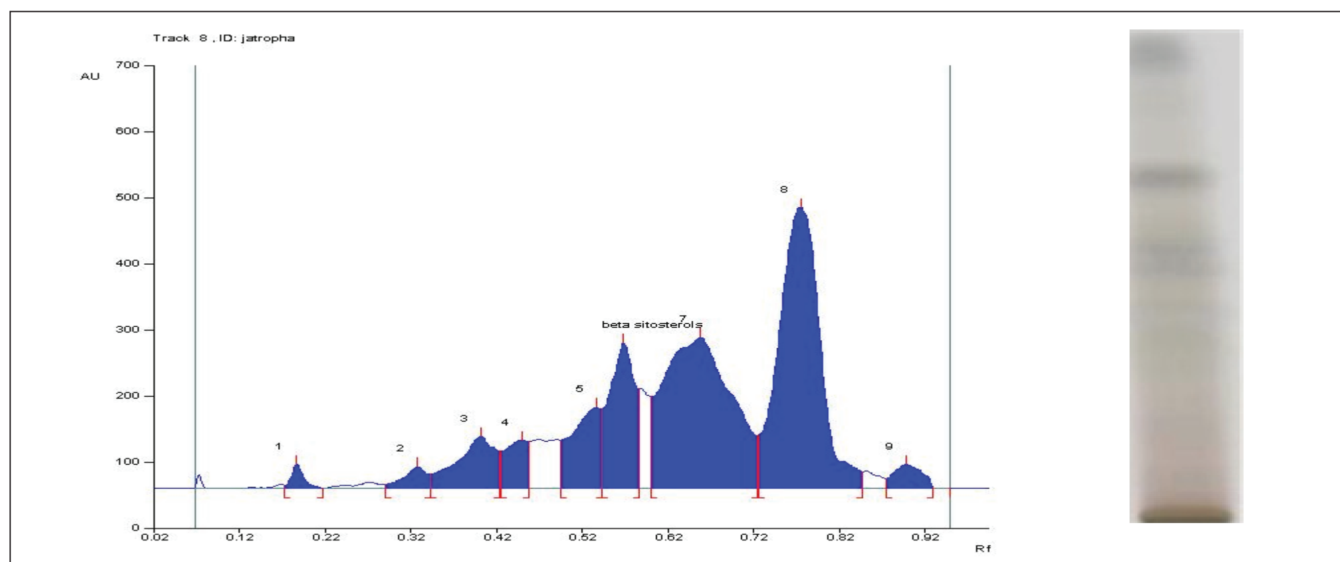

Figure 5: HPTLC finger print profile of 50% ethanolic leaves extract of *J. curcas* Linn in Toluene: Ethyl acetate: Formic acid (7 : 2 : 1) solvent system at wavelength 631 nm.

Table 5: HPTLC details of methanolic extract of *J. curcas* leaves

Under visible light at 631 nm	
Rf values	**Colour**
0.37	Light brown
0.42	Light Blue (β sitosterol)
0.50	Dark blue
0.55	Dark blue
0.60	Dark Brown

DISCUSSION

The characters observed in the leaf like vein islet number, vein termination number, Stomatal number, Stomatal index, palisade ratio, laticifers, simple trichomes unicellular and multicellular, rosette and cluster crystals of calcium oxalate of varying size, vessels with scalariform and spiral secondary wall thickenings are some important diagnostic characters that are useful in determining the authenticity of the drug sample.

The total percentage of ash values, acid insoluble ash, water soluble ash and percentage yield of extractives in different solvents, phytochemical screening and HPTLC are constant features of a part of the plant which may constitute individual drug. These reports would be of much significance in genuineness of the drug sample.

In the present study, Pharmacognostical and phytochemical tests of leaves of *J. curcas* were performed. The pharmacognostical studies comprises of taxonomic characters of the taxon, macro- and microscopical characters and diagnostic characters of the part used. The phytochemical parameters and physical constants were found useful in evaluating the pharmacopoeial standards. The qualitative phytochemical investigation gave valuable information about the different phytoconstituents present in the extracts, which help the future investigators regarding the selection of the particular extract for further investigation of isolating the active principle.

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Pharmacognostic Study and Establishment of Quality Parameters of Leaves of *Ficus racemosa* Linn.

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ABSTRACT

Introduction: *Ficus racemosa* Linn. syn. *Ficus glomerata* Roxb. (Family – Moraceae) is found commonly in India. Recent pharmacological findings indicate that its leaves possess significant anti-diabetic, anti-ulcer, hepatoprotective, anti-diarrheal, analgesic, larvicidal, anti-bacterial and anti-inflammatory activities, which comply with the claims made in the traditional medicinal texts. However, no conclusive pharmacognostic study of its leaves has been performed yet. **Methods:** The present investigation deals with the qualitative and quantitative microscopic evaluation of the leaf material and establishment of its quality parameters, including physicochemical and phytochemical evaluation. **Results:** Chief microscopic characters include vascular bundles having patches of perimedullary phloem and sheath of calcium oxalate cluster crystals. Chief characters of powder include hooked-top trichomes, calcium oxalate prisms, cluster crystals and xylem vessels with reticulate thickening. Various physicochemical parameters were also established. Phytochemical analysis showed the presence of many important classes of phytoconstituents like alkaloids, cardiac glycosides, saponins, flavonoids, tannins and carbohydrates. **Conclusion:** Such a study would serve as a useful gauge in standardization of the leaf material, isolation of medicinally important phytoconstituents, performing pharmacological investigations and ensuring quality formulations.

Key words: Cluster fig, *Ficus glomerata,* Moraceae, Udumbar.

INTRODUCTION

Ficus racemosa Linn. syn. *Ficus glomerata* Roxb. (Family – Moraceae) is also known as (English) Cluster fig, (Hindi) Umrai, (Sanskrit) Udumbar and (Urdu) Gular.[1] Its leaves are used traditionally in several disorders like bronchitis, ulcers, dysentery, menorrhagia, gum diseases, glandular swelling, cervical adenitis, hemoptysis, abscess, chronic wounds and various skin diseases. Galls on leaves are used to treat small pox. The tender leaf buds are applied on the skin, in the form of paste, to improve the complexion. Its latex is used in edema, pain, ulcers, wounds, piles, diarrhea, mumps and diabetes. Along with sesame oil, the latex is used to treat cancer.[2] Recent pharmacological studies of the leaves prove it to have potent anti-diabetic,[3,4] anti-ulcer,[5] hepatoprotective,[6] anti-diarrheal,[7] anti-bacterial[8] and anti-inflammatory,[9,10] analgesic[11] and larvicidal[12] activities. The present investigation deals with the qualitative and quantitative microscopic evaluation of the leaf material and establishment of its quality parameters, including physicochemical and phytochemical evaluation. This thorough evaluation would be useful in standardization of the leaf material, bioactivity-guided fractionation of therapeutic phytoconstituents, performing further pre-clinical or clinical investigations and manufacturing quality formulations.

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MATERIALS AND METHODS

Collection and authentication of leaves

Leaves of *F. racemosa* were collected from the herbal garden of R. K. College of Pharmacy, Rajkot in March, 2010. Herbariums and voucher sample were prepared and deposited in Department of Pharmacognosy, R. K. College of Pharmacy (Voucher no. RKCP/COG/01/2010). Authentication was done by Dr. A. N. Pandey, Department of Biosciences, Saurashtra University.

Pharmacognostic studies

Morphology of fresh leaves of *F. racemosa* was studied. Photomicrography of stained and unstained transverse sections of fresh leaves was performed using Win DVR software. Leaf constants were established using camera lucida. The leaves were dried under shade, powdered to 60#, stored in airtight containers and used for powder study and quantitative microscopy (Table 1).[13]

Table 1: Quantitative microscopy

Parameter	Mean value ± SD
Stomatal Index	
Upper surface	5.5 ± 0.5
Lower surface	16.5 ± 0.5
Palisade ratio	6 ± 1
Vein islet number	13 ± 0.5
Vein termination number	16 ± 1
Cluster crystal diameter	12.88μ – 23.39μ – 37.2μ
Length of covering trichome	22.55μ – 44.85μ – 88.15μ

Number of observations = 10
SD = Standard Deviation

Table 2: Physicochemical evaluation

Parameter	% w/w ± SD
Loss on drying	12.3 ± 0.2
Ash values	
Total ash	12.7 ± 0.5
Acid insoluble ash	0.7 ± 0.1
Water soluble ash	3.1 ± 0.2
Extractive values	
Water soluble extractive	16.3 ± 0.3
Alcohol soluble extractive	4.3 ± 0.2
Petroleum ether extractive	1.7 ± 0.2

Number of observations =10
SD = Standard Deviation

Physico-chemical evaluation

Various physico-chemical parameters like loss on drying, ash values (total ash, water soluble ash and acid-insoluble ash) and extractive values (water soluble, alcohol soluble and petroleum ether soluble extractives) were established using the powdered drug (Table 2).[14]

Phytochemical study

Fluorescence of the powder with various reagents was observed under visible and UV light (Table 3). The powder was extracted with 500ml of different solvents at 70°C for two hours each. Various phytoconstituents present in the leaves were detected by their respective chemical tests using the appropriate extracts (Table 4).[15-21]

Table 3: Fluorescence of powder with various reagents

Reagent Added	Visible light	Short wave U.V. light	Long wave U.V. light
None	Light Green	Dark Green	–
Water	Light Green	Dark Green	–
Conc. H_2SO_4	Light Green	Dark Green	Green
Conc. HNO_3	Light Brown	Dark Green	Green
Conc. HCl	Light Green	Dark Green	Green
10% HCl	Light Green	Dark Green	Yellow
10% H_2SO_4	Light Brown	Dark Green	Yellow
10% HNO_3	Light Green	Dark Green	Yellow
Glacial Acetic Acid	Light Brown	–	Yellow
Aq. NaOH	Light Green	Dark Green	–
Alc. NaOH	Light Green	Dark Green	Reddish Yellow

Table 4: Phytochemical screening

Phytoconstituent	Test	Result
Alkaloids	Dragendorff's test	+ve
	Wagner's test	+ve
	Mayer's test	+ve
Flavonoids	Shinoda test	+ve
	Lead acetate test	+ve
Sterols	Salkowski test	+ve
	Libermann Buchardt test	+ve
Cardiac glycosides	Legal's test	+ve
	Baljet test	+ve
	Keller Killiani test	+ve
	Kedde's test	+ve
Saponin glycosides	Foam test	+ve
	Lead acetate test	+ve
Tannins	Folin ciocalteu test	+ve
Carbohydrates	Fehling's test	+ve
	Molisch test	+ve
Gums	Ruthenium red test	+ve

RESULTS AND DISCUSSION

Pharmacognostic study

Macroscopical characteristics

Leaves are simple, 7.5-15 cm × 3.2-6.3 cm, ovate-oblong or elliptic-lanceolate, apex acute, margin wavy, surface glabrous, texture membranous, base symmetric, venation reticulate, color of upper surface dark green and lower surface light green. Three prominent veins seem to be arising from the base of the lamina. Petiole is angular and reddish-brown (Figure 1).

Microscopy: Transverse section

Lamina of the transverse section shows a prominent hypodermis beneath the upper epidermis. Underlying the

Figure 1: Leaves of *Ficus racemosa*

hypodermis are bi-layered, compact, radially elongated palisade cells followed by spongy mesophyll composed of 3-4 layers of loosely arranged parenchymatous cells with scattered calcium oxalate cluster crystals. Midrib consists of a well-developed collenchyma below upper epidermis and above lower epidermis. Ground tissue consists of loosely arranged polygonal parenchymatous cells having calcium oxalate prisms and cluster crystals. Vascular bundles are bicollateral, crescent shaped, having patches of perimedullary phloem and 3-4 secondary vascular bundles above the primary vascular bundle. Sheath of calcium oxalate cluster crystals are present below the primary vascular bundle. Starch grains are scattered throughout the ground tissue. Trichomes are covering, long, unicellular or bicellular, few having a hooked-top (Figure 2, 3).

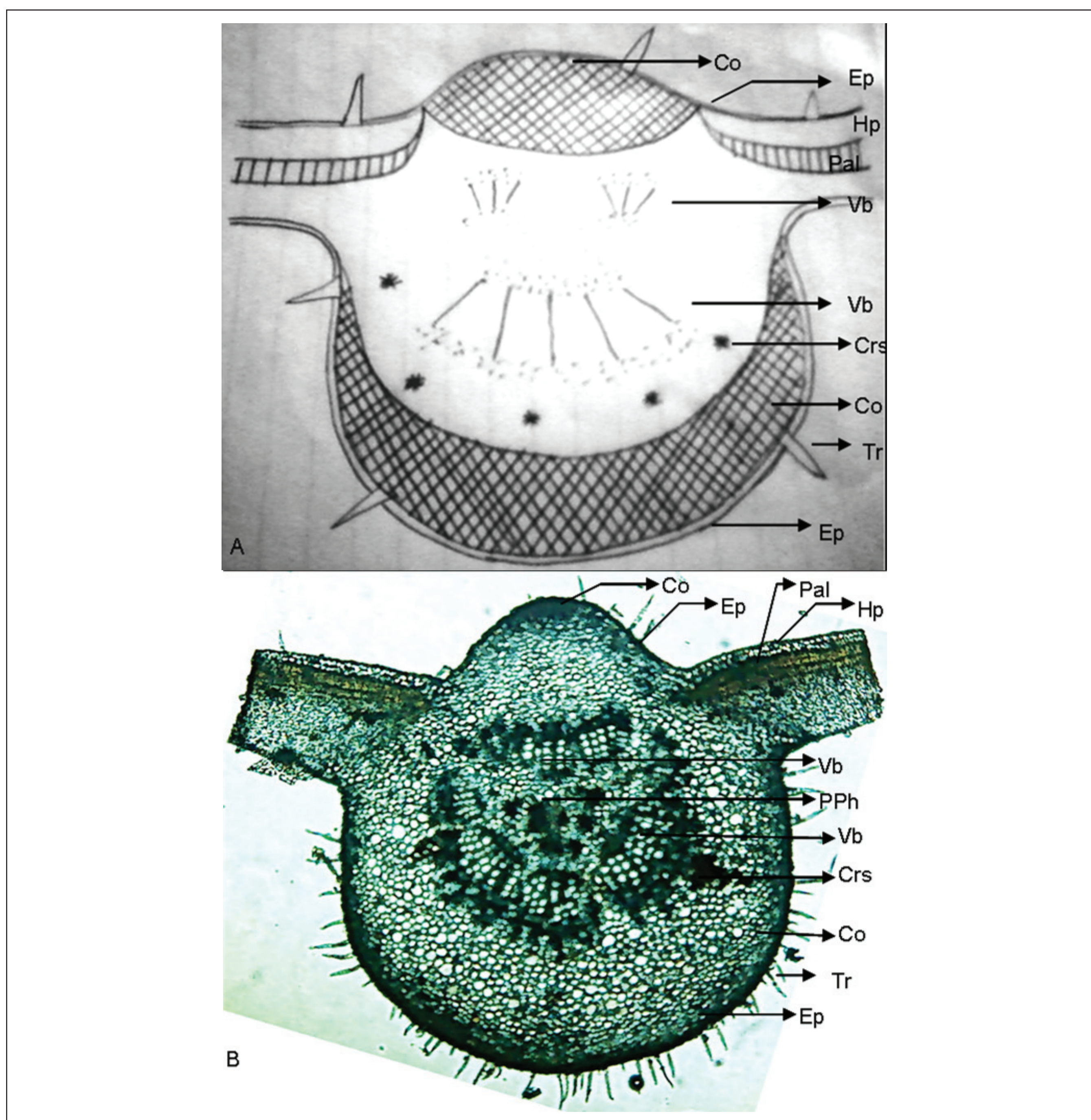

Figure 2: A - Diagrammatic T. S. of leaf. B - Detailed T. S. of leaf (X40)
(Co, Collenchyma; Ep, Epidermis; Hp, Hypodermis; Pal, Palisade; Vb, Vascular bundles; Crs, Calcium oxalate cluster crystal sheath; Tr, Trichomes; PPh, Perimedullary phloem)

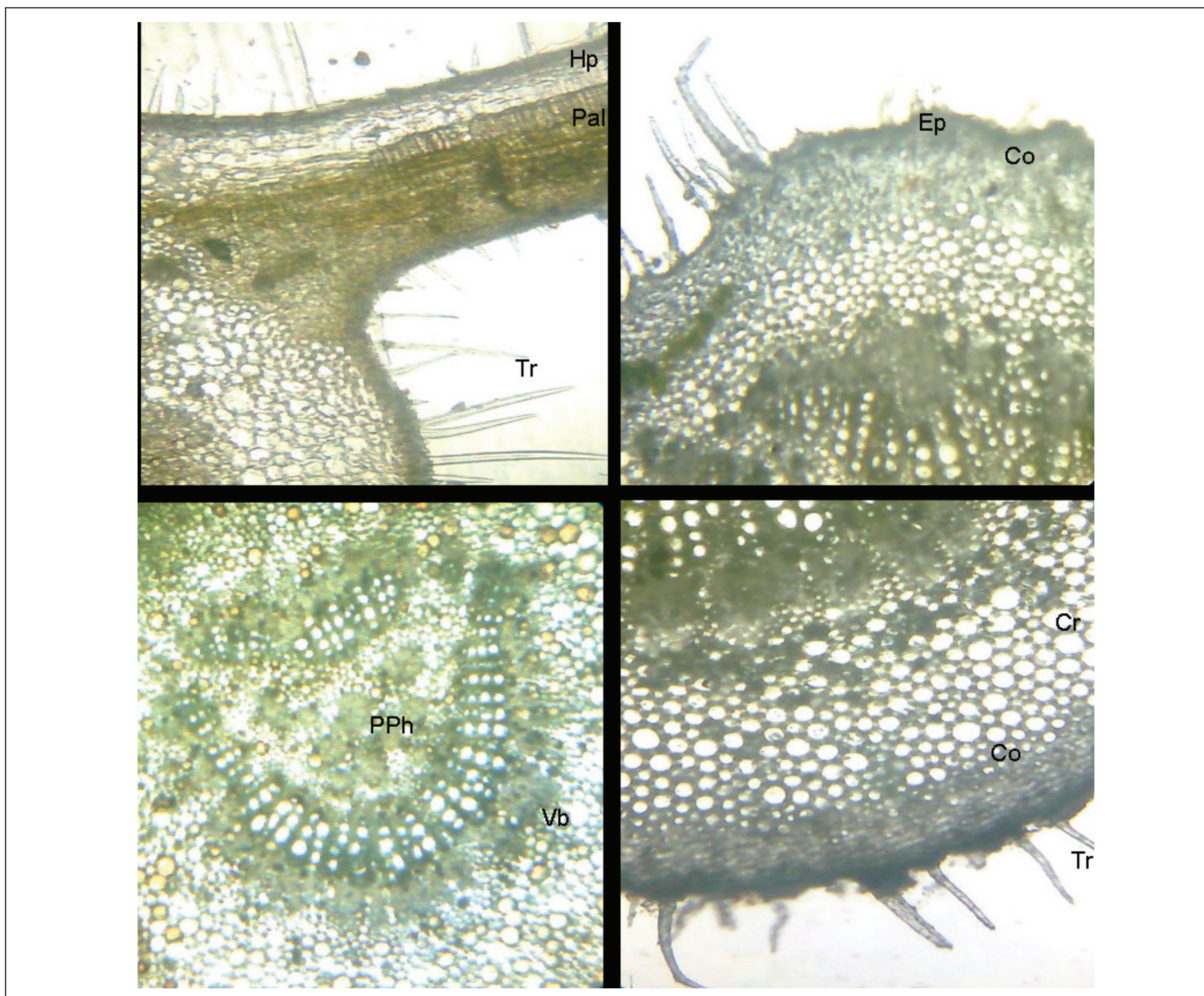

Figure 3: T. S. of leaf showing single enlarged portions (X400)
(Co, Collenchyma; Ep, Epidermis; Hp, Hypodermis; Pal, Palisade; Vb, Vascular bundles; Cr, Calcium oxalate cluster crystals; Tr, Trichomes; PPh, Perimedullary phloem)

Microscopy: Powder characteristics

It is a dark green powder with no distinct odor or taste. The important diagnostic features of the powder include parts of epidermis in surface view showing straight walled epidermal cells and anomocytic stomata, xylem vessels with reticulate or annular thickening, calcium oxalate cluster crystal sheath, calcium oxalate prisms, starch grains and numerous covering trichomes, unicellular or bicellular, narrow, conical, pointed, few having a hooked-top (Figure 4).

CONCLUSION

Leaves of *Ficus racemosa* have been proven to possess a wide spectrum of pharmacological activity. The present work deals with the microscopic, physicochemical and phytochemical evaluation of the leaves. Main microscopic characters include cluster crystal sheath of calcium oxalate and vascular bundles having patches of perimedullary phloem. Diagnostic characters of powder include hooked-top trichomes, calcium oxalate cluster crystals, calcium oxalate prisms, starch grains, anomocytic stomata and xylem vessels with reticulate thickening. Such a pharmacognostic study is useful for standardizing crude drugs and can be used to differentiate closely related species. Various physicochemical parameters were established which can be important in detecting adulteration and mishandling of the crude drug. Phytochemical analysis showed the presence of many important classes of phytoconstituents like alkaloids, cardiac glycosides, saponins, flavonoids, tannins and carbohydrates. This indicates that the plant can be useful for treating different diseases because the therapeutic activity of a plant is due

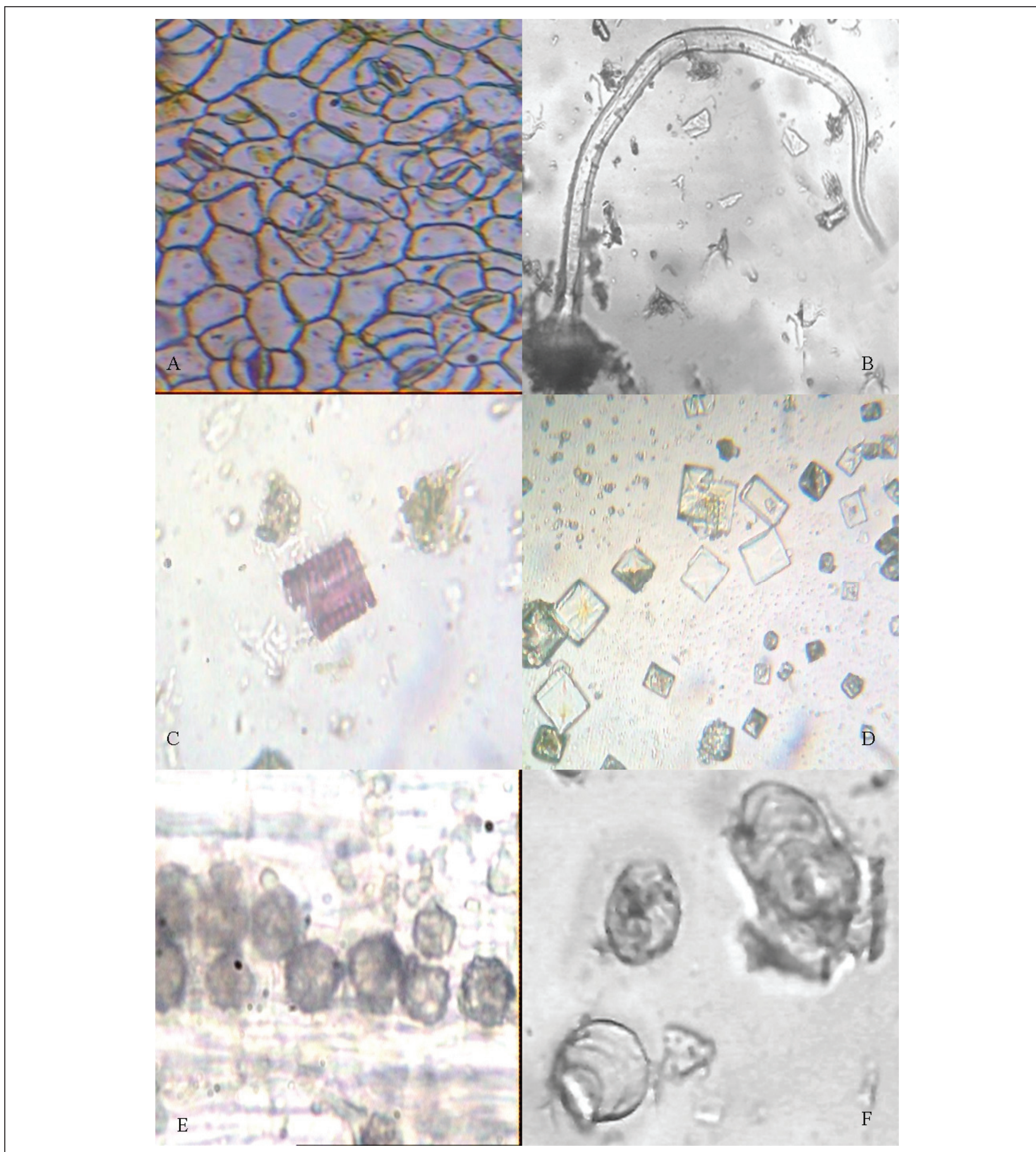

Figure 4: Powder study (X400)
A, Epidermis in surface view having anomocytic stomata; B, Hooked-top covering trichome; C, Xylem vessels with reticulate thickening; D, Calcium oxalate prism crystals; E, Ground tissue having calcium oxalate cluster crystal sheath; F, Starch grains.

to the presence of particular class of compounds. Such a detailed study would be decisive in performing standardization of the leaf material, preparation of its monograph, isolation of phytoconstituents, performing further pre-clinical and clinical investigations and manufacturing of its formulations.

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Pharmacognostic and Phytochemical Investigations of Stems of *Hibiscus micranthus Linn*.

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ABSTRACT

The present investigation deals with Pharmacognostical, physicochemical and phytochemical analysis of *Hibiscus micranthus* Linn.,. The macroscopic and microscopic characters, physical constant values, extractive values, ash values, micro chemical analysis and fluorescence analysis were performed. The presence of lignified, thick wall, libiform type of fibres with pointed tips & vessel elements with oblique perforations plates having short, pointed tails as seen in the powder of stem were the distinguishing microscopic features and can be used as anatomical markers. Chemomicroscopic characters present included lignin, starch, suberin, mucilage, cellulose, protein bodies and calcium oxalate crystals. Physical constants performed were loss on drying, ash content, acid insoluble ash and water soluble ash. Extractive values in pet. ether (60-80°C), chloroform, alcohol and hydroalcoholic were determined. Fluorescence studies of the powder were carried in ordinary light and UV light with various solvents. Phytochemical screening of successive extracts showed positive reactions for steroids, flavonoids, carbohydrates, phenols and tannins. The fingerprints of the hydroalcoholic extract were obtained by HPTLC technique in three best mobile phase solvent systems. The flavonoid content of hydroalcoholic extract was determined by colorimetric method. Chemical profiling of hydroalcoholic extract was also performed by GC-MS analysis. Further a HPLC method with photodiode array detector was followed to quantify rutin in hydroalcoholic extract of *Hibiscus micranthus* (HEHM). The present study provides details to characterize the Pharmacognostical, physicochemical and phytochemical parameters. An accurate and rapid HPLC quantification method has also been developed for quality control determination of rutin from *Hibiscus micranthus* stem.

Key words: *Hibiscus micranthus,* Pharmacognostical analysis, Chemical profiling, HPTLC, HPLC, GC-MS.

INTRODUCTION

Human population in countries around the world has been using plants from thousands of years for treating/ameliorating various ailments of humans and animals. This traditional knowledge about the plants can be transferred to several generations only by proper documentation of their botanical, physicochemical, phytochemical characters and along with their medicinal uses in the form of monographs as per the WHO guidelines and presented as herbal Pharmacopoeia. These monographs enable to identify, authenticate, detect adulterants and standardize and use the plant material for therapeutic purposes.

Hibiscus micranthus Linn, is a shrubby, erect, branched, slender and stellately hairy plant. It is widely distributed in hotter parts of India, Ceylon, Saudi Arabia and tropical Africa. In India, the plant is known by different vernacular names in different regions as Chalabharate in telugu, sittamutti in tamil, chanakbhindo in gujrati and as okder in Sanskrit. Traditionally the plant is considered a valuable febrifuge in india, Ceylon, Saudi Arabia and tropical Africa.[1] In India certain parts of Gujarat, the fruits and flowers of this plant is used as hypoglycemic agent.[2] The plant has been scientifically validated for its antipyretic, anti-inflammatory, hematological effects[3] antimicrobial, antiviral, antitumor,[4] female antifertility, viralizing[5] and anabolizing[6] activities. Few compounds like Phenolic acids, flavonoids, β-sitosterol, alkanes, fatty alcohols and acids have been reported on carrying out conventional column chromatographic analysis. Upon literature survey, it was revealed that, no work as been reported on its pharmacognostic diagnostic features and chemical analysis by modern analytic tool like GC-MS, HPLC which reveals more details of its chemical composition. The present study deals with complete pharmacognostical and chemical profiling by using HPLC, HPTLC, GC-MS analysis.

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MATERIALS

Plant material

The whole plant parts were collected in bharat institute of technology, mangalpally, Ibrahimpatnam & were authenticated by Taxonomist Jayaraman at the National Institute of Herbal science, Chennai, India. In order to ensure the sample used was from the same source throughout the experiment, the sample was collected in sufficient quantities at a time.

The plant *Hibiscus micranthus Linn.,* was washed thoroughly with running tap water, followed by rinsing with distilled water and then leaves, stem & roots were separated and cut into small pieces. The leaves and stems were shade dried at room temperature, while roots were dried in oven at 45° C for two weeks. The dried parts of the plant were powdered in mill to a mesh size of 150 and stored in an air tight container till further use.

Chemicals and equipments

All the chemicals used in the study were of analytical grade (SD fine chemicals pvt ltd. Mumbai) obtained from the central store house of the institution. Rutin was obtained from lobei chem. Pvt ltd Mumbai. Microtome (secor, India) UV spectrophotometer 1801 shizadzu, Muffle furnace (Biotechnics, India) , Nikon camera, HPLC (waters), HPTLC (Camag, Switzerland), GCMS shimadzu.

METHODS

Pharmacognostic studies

Macroscopic: The following macroscopic characters for the fresh stems were noted with the help of organs of senses: size and shape, color, odor and taste whether herbaceous or woody, upright or creeping, smooth or ridged, hairs present or not if so whether of the glandular or covering form.

Microscopy

Plant Collection and preparation for anatomical studies

The plant specimens for the anatomical study were collected from Bharat institute of technology, mangalpally, Ibrahimpatnam. Care was taken to select healthy plants and normal organs. The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin-5 ml + Acetic acid-5 ml + 70% ethyl alcohol- 90 ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60 C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.[7]

Sectioning

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10-12 µm. dewaxing of the sections was by customary procedure.[8] The sections were stained with Toluidine blue. Glycerin mounted temporary preparations were made for acerated/cleared materials.[9]

Powder microscopy

Powdered material of stem part was cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell component were studied and measured.[10]

Histo chemical tests

Examination of the powder for starch grains, lignin, mucilage, calcium oxalate crystals, cutin and suberin were carried out using standard techniques.[11]

Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 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. Descriptive terms of the anatomical features are as given in the standard anatomy books.[12]

Physicochemical studies

The Loss on drying, total ash, acid insoluble, water soluble ash and successive soxhlet extractives values were assayed according to standard Indian pharmaco poeia methods. For fluorescence analysis of the powder sample it was treated with different chemical reagents to observe various colour reactions in ordinary and UV light.[13]

Phytochemical investigation

Chemical tests were employed in the preliminary phytochemical screening for various secondary metabolites such as tannins, phenols, steroids, carbohydrates, proteins, alkaloids, saponins, anthracene derivatives, flavonoid glycosides, and cyanogenetic glycosides.[14]

EXTRACTION

The powdered stems were exhaustively extracted with 70% Hydroalcoholic for 1 week in a soxhlet extractor. The collected extracts were filtered and evaporated under

vacuum, yielded thick green residue. The residues was dried and stored in air tight container for further use.

HPTLC FINGERPRINTING ANALYSIS

The TLC fingerprint profile of Hydroalcoholic Extract of Hibiscus micranthus (HEHM) was carried out by HPTLC technique.

Preparation of sample

5 gms of *H. micranthus* stem powder sample was extracted with 25 ml 70% ethanol for 8 h under reflux, filtered the extract and repeated the process thrice. Pooled the filtered extracts and evaporated to dryness. Dissolve the residue in 50 ml 70% methanol. Aliquot of the extract was taken for TLC analysis.

Chromatographic development

Apply 10 μl to the chromatographic plate using a suitable applicator and place the plate in twin trough chamber, add mobile phase in one trough and plate in another. Allow the plate to equilibrate for about 20 minutes, and develop the plate to 8 cm. Remove the plate from the chamber and dry in air.

Colour development[15]

Plates were derivatized with anisaldehyde-sulphuric acid and Lieberman burchard reagent.

Thin Layer Chromatographic conditions

Stationary phase- Silica Gel 60 F254, **Solvent front:** 7 cm, **Detection:** 254 nm and 366 nm. **Instrument used:** High Performance Thin Layer Chromatography (CAMAG, Switzerland). **Applicator:** Linomat V, **Derivatisation: 1. Anisaldehyde-sulphuric acid reagent, 2. Liebermann-Burchard reagent, Mobile Phase – I**: Ethyl acetate: methanol – glacial acetic Acid (10:1.35:1), **Mobile phase – II**: Chloroform – methanol (9.9:0.1), **Mobile phase – III**: Ethyl acetate: formic acid – glacial acetic acid – water (10:1.1:1.1:2.6).

SPECTROPHOTOMETRIC ANALYSIS OF TOTAL FLAVONOIDAL CONTENT OF HEHM

The total flavonoidal content of HEHM was determined by aluminum chloride colorimetric method.[16]

Preparation of Standard

The standard curve was prepared using rutin with methanol as solvent. The total flavonoidal content of the extract was obtained using the standard curve.

Preparation of Sample solution

The sample solution of HEHM extract 1 mg/ml was prepared using methanol as solvent. The solution is passed through a vacuum filter containing whatman filter paper of pore size 0.45 μ to get particulate free sample.

Procedure

HEHM extract/standard rutin (1 ml) was mixed with 2 ml of methanol, 1 ml of 10% aluminum chloride, 1 ml of 1M potassium acetate and 1 ml of distilled water. The mixture was incubated at room temperature for 30 min. Blank sample was prepared by omitting the standard/HEHM extract. The absorbance of the mixture was measured at 415 nm with a Shimadzu UV-1801 spectrophotometer**.**

HPLC ANALYSIS OF HEHM

Preparation of Sample solution

100 mg of the HEHM extract was dissolved in methanol and suitably diluted to get a concentration of 10 μg/ml. The solution was subjected to sonication for degassing and later passed through a vacuum filter containing whatman filter paper of pore size 0.45 μ to get clear sample solution.

Preparation of standard solution

The procedure followed was same as that of sample solution, except the standard used was rutin 10 μg/ml.

Testing procedure

Test solution and standard solution are subjected to HPLC separately.

HPLC operating conditions

Shimadzu chromatographic system with two LC-10AT VP pumps, variable wavelength programmable UV–vis detector SPD-10A, VP CTO, -10 AS VP column oven (Shimadzu) A reversed phase C18 column (25 cm × 4.6 mm i.d., particle size 5 _m; YMC, IMC, Wilmington, NC, 28403, U.S.A.) and the HPLC system was monitored by software "Class-VP series version 5.03 (Shimadzu)". Mobile Phase: Methanol: 2% acetic acid in water (70:30), Flow rate: 1 ml/minute, Injection volume: 20 μl, Detection: 264 nm

CHARACTERIZATION OF HEHM BY GCMS ANALYSIS

Sample Preparation

200 mg of the sample was dissolved in 1 ml of the n-hexane. The mixture was sonicated for 15 minutes. 3 μl of the test solution was directly injected into the system.

Chromatographic analysis

The sample was analyzed using Shimadzu GC-MS-QP2010 Plus apparatus equipped with quadrapole detector and split injection system. The GC was fitted with a ZP-624 capillary column (30 mm × 1.4 mm, film thickness 0.25 μm). The temperature programmed was as follows: injector temperature 220° C, initial oven temperature at 120° C for 2 minutes, then rises to 250° at the rate of 10° C per minute at 250° C for 25 minutes, transfer line temperature 220° C. Helium was used as carrier gas at 35.6 Kpa pressure with flow 2.5 ml/min and electronic pressure control on. The EM voltage was 952.9 V with lower and upper mass limits set at 30 & 350 m/z. Samples were solved in n-hexane and injected automatically. MS spectra of separated compounds were compared with one from Wiley 7 Nist 05 mass spectral database. The identity of the spectra above 95% was needed for the identification of compounds.

RESULTS

Pharmacognostic Studies

Macroscopical study revealed the dried stems are generally cylindrical, up to 1 cm thick, woody, upright, outer surface smooth in young stems and rough in old stems, greenish externally, yellowish internally, fracture splintery, taste astringent and slightly bitter, odour without any characteristic aroma, agreeable.[17]

Microscopical characters are the stem has epidermal layer of squarish cells with thick cuticle; it is broken at certain places due to growth in thickness of the stem (Fig No. 1). The epidermis is followed by a narrow zone of chlorenchymatous cortex and four or five layers of parenchymatous inner cortex. Secondary phloem is wide and continuous all around the stem. It has wide dilated funnel shaped rays at certain places (Fig No. 2). In other regions, the secondary phloem has tangential blocks of phloem fibres alternating with narrow segments of phloem elements. Secondary xylem is a thick hollow cylinder and consists dense xylem fibres and radial files of vessels which are separated by wide gaps. The vessels are circular, thin walled and diffuse in distribution; they include both wide and narrow vessels, the wide vessels are 40 μm in diameter; the narrow vessels are 20 μm wide. The pith is wide and parenchymatous. It consists of angular, thick walled parenchymatous cells.

Stem powder analysis (Fig No. 3 & 4) revealed the presence of Fibres and vessel elements abundant in the powder. The fibres are libriform type with lignified thick walls and pointed tips. They are 500-650 μm long. The vessel elements are cylindrical and elongated. They have perforations plate which may be horizontal or oblique. The vessel elements with oblique perforations plates have short, pointed tails. The lateral wall pits elliptical, multiseriate and alternate. The vessel elements are 200 μm long and 40 μm wide.

Physicochemical studies

Physical constants like Ash values, Extractive values and Loss on Drying at 110° C were determined and results are shown in **Table No. 1.** The behavior of powdered drug in different solutions towards ordinary and UV light were observed and the results are recorded in **Table No. 2.** The Preliminary Phytochemical tests of different extracts were performed, identified with using specific reagents and results

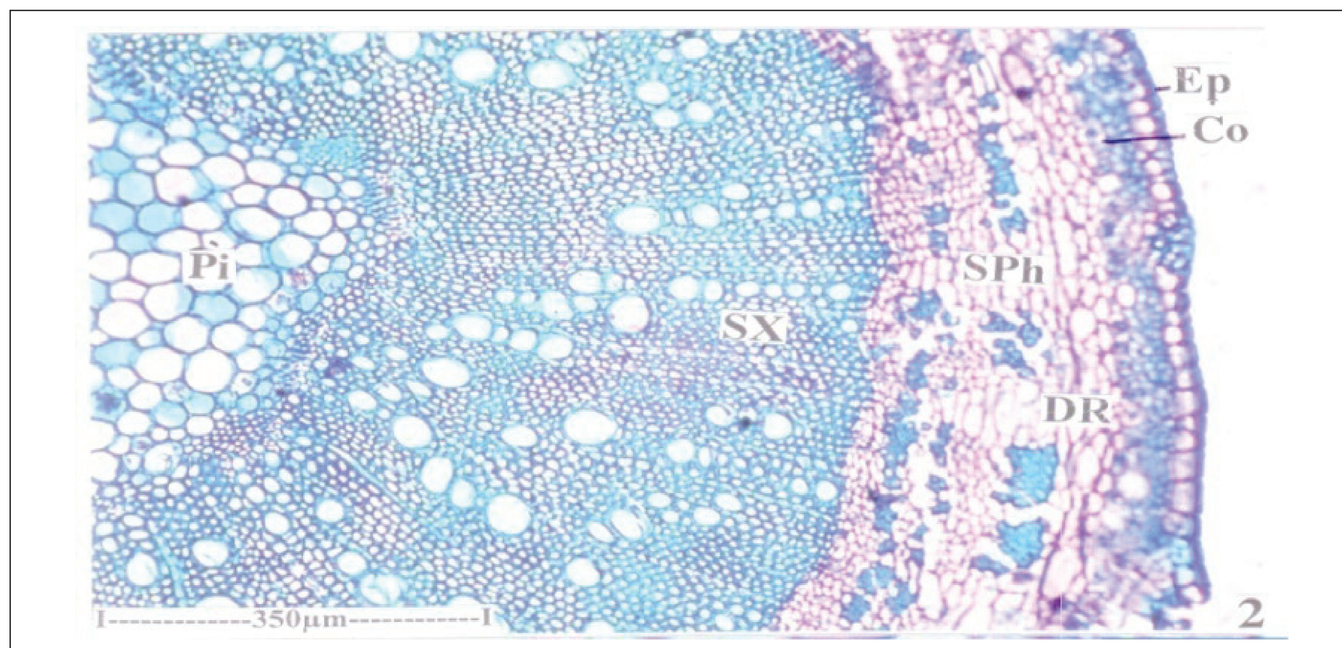

Figure 1: T.S of stem – a sector enlarged

are shown in **Table No. 3.** HPTLC fingerprinting studies was carried out & R_f values are measured and tabulated in **Table No. 4.**

Histo-chemical tests

Histo-chemical reactions were obtained using toluidine blue a polychromatic stain. Th dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. whereas IKI (blue for starch)

Extraction

The hydroalcoholic extract obtained was thick green and yield was found to be 7.6%

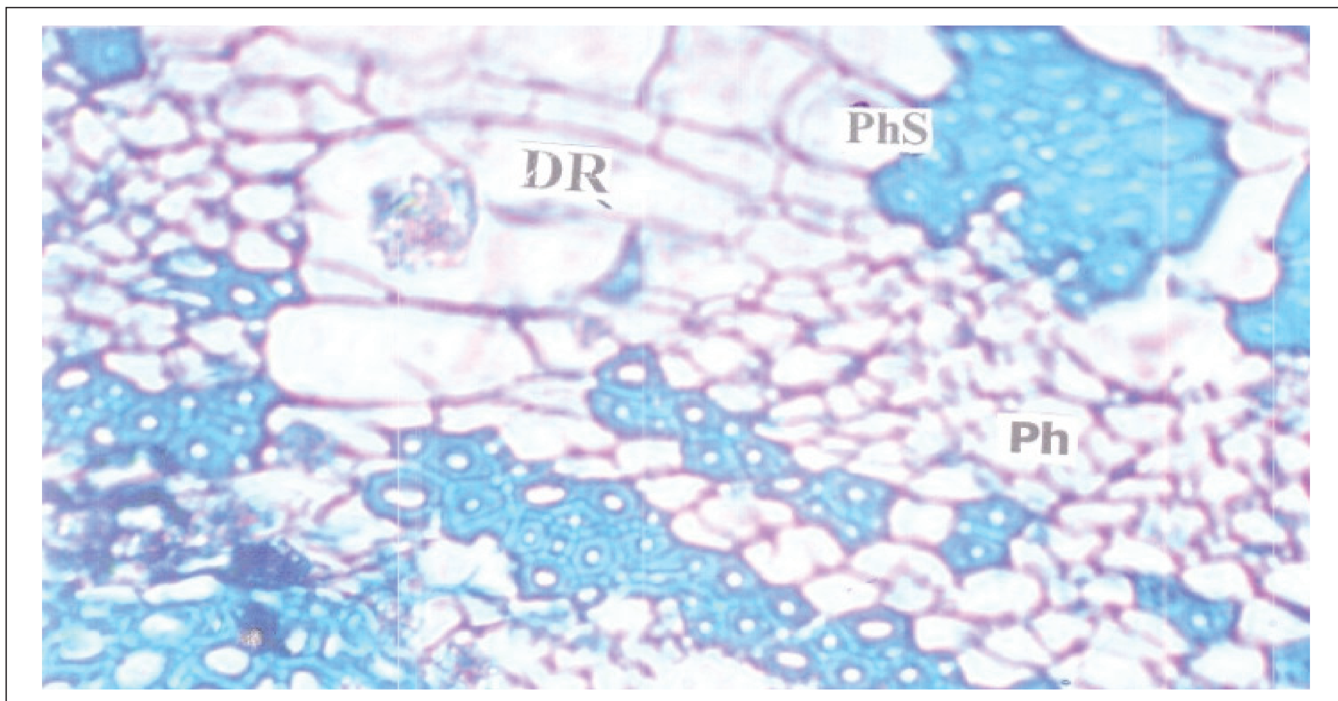

Figure 2: T.S of stem – secondary phloem

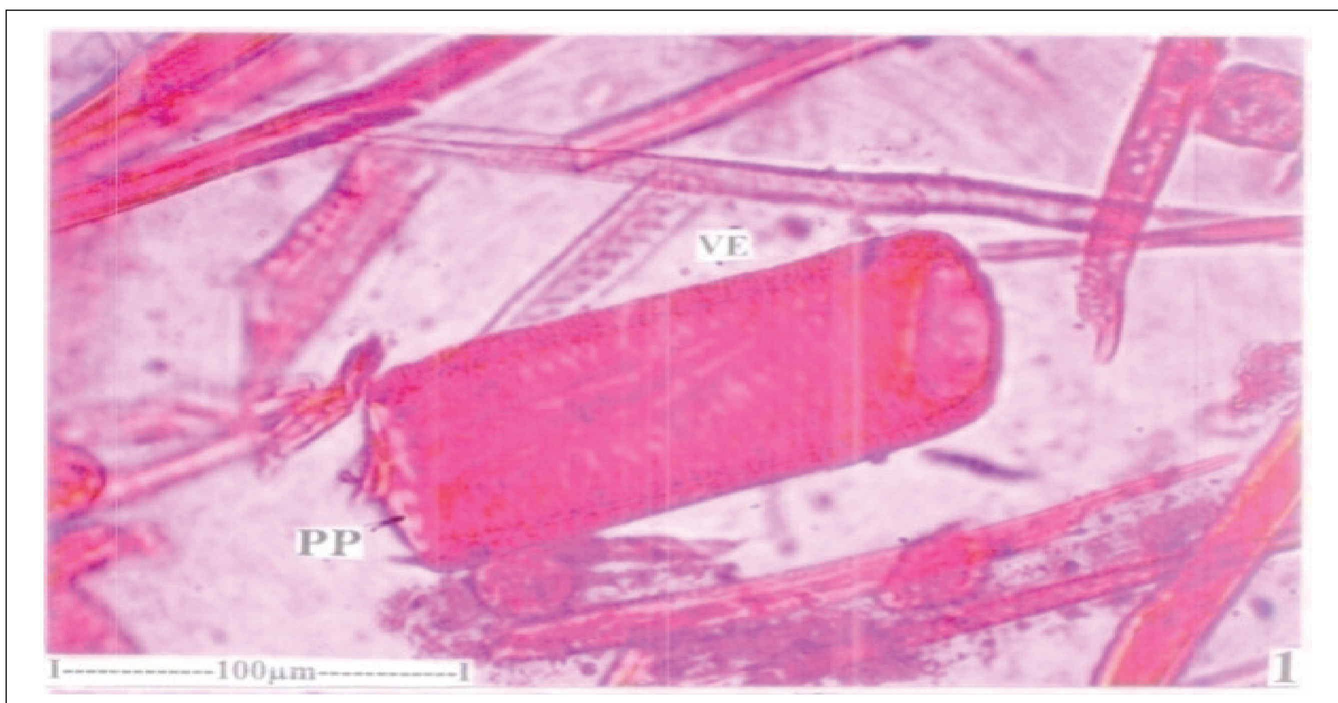

Figure 3: Vessel element and fibre

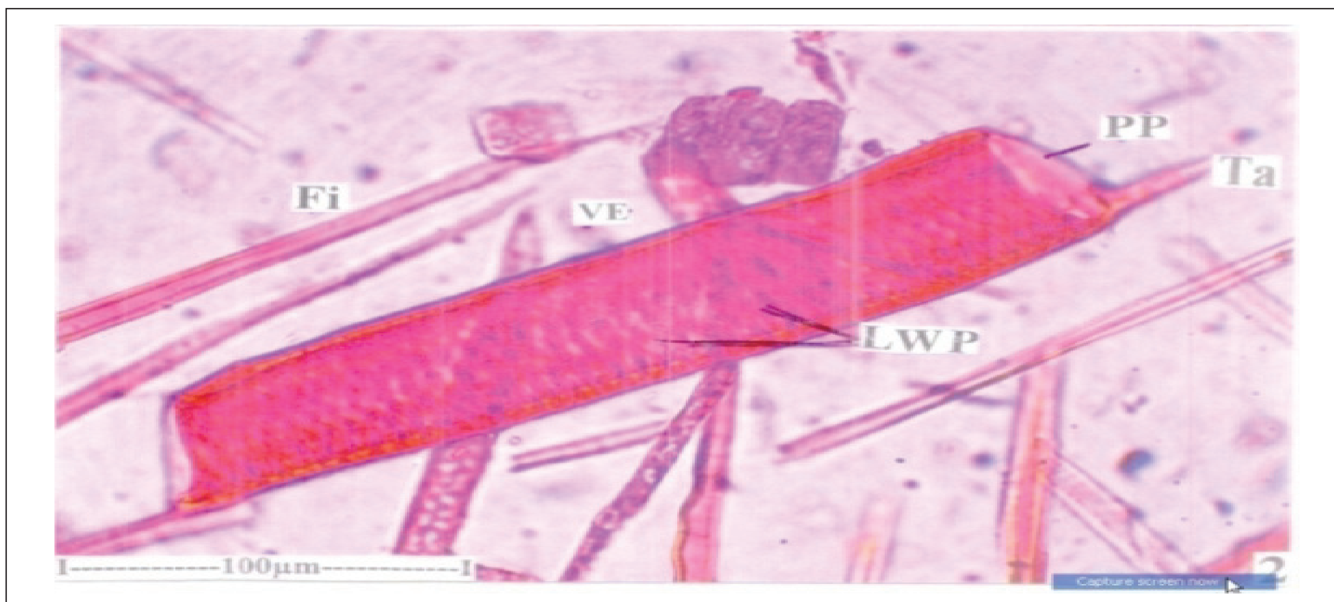

Figure 4: Tailed vessel element showing lateral wall pits and fibres
Abbreviations: VE- vessel, PP-perforation plate, LWP-Lateral wall pits, Fi-Fibre, Ta-Tail, Ph: Phloem, PhS: Secondary phloem, SX: Secondary xylem, DR: Druses, Pi: Pith, Co: Cortex, Ep: Epidermis

Table 1: Physico-chemical parameters of *Hibiscus micranthus*

Parameters	% constituents
% LOD at 110° C	1-2.0%
% Ash content	2.5%
% Acid insoluble ash	0.5%
% water soluble ash	2.0%
% Extractive values	
Pet. Ether (60-80° C)	1.5
Chloroform	3.4
Alcohol	6.6
Water	4.10

Table 2: Fluorescence studies of *Hibiscus micranthus*

Treatment	Ordinary light	UV 254 nm
Powder as such	Pale Yellowish	Yellowish fluorescence
Powder + concentrated Hydrochloric acid	Light Brown	–
Powder + concentrated sulphuric acid	Deep reddish brown	–
Powder + concentrated nitric acid	Orange	–
Powder + 10% sodium hydroxide	Orange red	Dark green colour
Powder + glacial acetic acid	Yellowish green	Pale yellow
Powder + chloroform	Greenish yellow	Greenish fluorescence
Powder + Distilled water	Pale yellow	Pale yellow fluorescence

HPTLC fingerprinting analysis

HPTLC fingerprinting studies was carried out & R_f values are recorded and tabulated in **Table No. 4.**

Table 3: Results of phytochemical screenings of successive extracts of stem of *H. micranthus*

Constituents	Pet ether	chloroform	alcohol	water
Steroid	+++	+++	-------	-------
Triterpenoid	-------	-------	-------	-------
Flavonoid	-------	-------	+++	-------
Phenols	-------	-------	+++	-------
Tannins	-------	-------	+++	-------
Alkaloids	-------	-------	-------	-------
Saponins	-------	-------	-------	-------
Sugars	-------	-------	-------	+++
prteins	-------	-------	-------	+++

+++ Present
------- Absent

SPECTROPHOTOMETRIC ANALYSIS TOTAL FLAVONOIDAL CONTENT OF HEHM

Spectrophotometric analysis of HEHM for total flavonol content was determined by using standard curve prepared by using rutin. The linearity was found in the range of 10-to200 µg/ml. The total flavonol content was expressed as rutin equivalent in % w/w of the extract. The flavonoidal content was found to be 3.86 mg/100 gm of the extract.

HPLC analysis of HEHM

The rutin content of the HEHM of stem was determined by HPLC method. The analysis was performed by the injection of 20 µl of extract on a lichrospher 100RP-18(5 µm) column (250 × 4 mm), elution using mobile phase as methanol and 2% acetic acid in water (70:30) with runtime of 10min and detection by UV detector at 355 nm. Rutin

Table 4: Rf values of the HPTLC fingerprint of *Hibiscus micranthus* in mobile phase-I/II/III

Rf values of the HPTLC fingerprint of *Hibiscus micranthus* in mobile phase-I

Extract	Amount applied (µg/spot)	Rf values (*H. micranthus* stem extract)			
		UV 254 nm	UV 366 nm	After derivatisation with anisaldehyde sulfuric Acid reagent under visible light	After derivatisation with Anisaldehyde sulfuric acid reagent under UV 366 nm
Alcohol: water (70:30)	1000	0.63, 0.71 (dark)	0.64 (yellowish green)	0.67 (pink)	0.08 (blue quenching), 0.66 (pink)

Rf values of the HPTLC fingerprint of *Hibiscus micranthus* in mobile phase-II

Extract	Amount applied (µg/spot)	Rf values (*H. micranthus* stem extract)			
		UV 254 nm	UV 366 nm	After derivatisation with anisaldehyde sulfuric Acid reagent under visible light	After derivatisation with Anisaldehyde sulfuric acid reagent under UV 366 nm
Alcohol: water (70:30)	1000	0.28, 0.78 (dark)	0.31, 0.35, 0.44 (light red)	--------	0.17 (light blue), 0.38 (blue), 0.43 (red), 0.54 (light blue)

Rf values of the HPTLC fingerprint of *Hibiscus micranthus* in mobile phase-III

Extract	Amount applied (µg/spot)	Rf values (*H. micranthus* stem extract)			
		UV 254 nm	UV 366 nm	After derivatisation with Libermann burchard reagent under visible light	After derivatisation with Libermann burchard reagent under UV 366 nm
Alcohol: water (70:30)	1000	0.11 (dark)	0.02, 0.11, 0.16, 0.55, 0.64 (blue)	0.11, 0.77 (yellow)	0.12 (light blue), 0.16 (blue), 0.20 (green), 0.26 (blue)

eluted at 3.691 min and the peak area was compared to the standard. The rutin content of the stem was found to be 0.12% w/w of air dried extract.

GC-MS analysis

The results of GC-MS analysis is presented in Table No. -5

DISCUSSION AND CONCLUSION

In India, people have been using the plants from their surroundings to treat various ailments apart from drugs mentioned in traditional system of medicine such as ayurveda and siddha. Now it has become necessary to lay standards even to such ethanobotanical drugs in similar lines with that of traditional drugs as knowledge about the identity and uses of these plants are being lost. Further, any pharmacological or pharmaceutical investigations on these plants first require proper authentication.

According to World health organization guidelines[18] (WHO) the macroscopy, microscopy, physiochemical and phytochemical description of a medicinal plant are the important steps towards establishing its identity and purity and should be carried out before any test/project are undertaken.

Hibiscus micranthus has scientifically proven for multifaceted biological functions. However, there are no detailed pharmacognostic and phytochemical investigations on this plant to help in proper identification. Hence, to provide key diagnostic tools of identification the present study is undertaken.

To diagnose this plant crude drug the following pharmacognostic and phytochemical characters of the drug are the important.

Macroscopy- The macroscopical characters of the stem can serve as diagnostic parameters.

Table 5: Volatile compounds from methanolic extract of stem of *H. micranthus Linn.* as detected by GC-MS

peak	Retention Time	Compound	% matching with Wiley library
1	0.94	Ethyl-D5 ethyl ether	94
2	1.86	Hexane, 1-chloro	96
3	4.53	2-Methylpropane-1, 2-diol	94
4	5.20	Octane, 4-ethyl	98
5	5.42	Alpha-D-Galactopyranoside, methyl	72
6	6.38	Methyl ethane-2, 2, 2-D3-Sulfonate	93
7	6.61	Butanedioic acid monomethyl ester	99
8	7.12	2-n-propylthiane	99
9	7.51	Benzoic acid	99
10	7.79	Formic acid, pentyl ester	99
11	8.38	Nonanoic acid	98
12	8.52	Benzofuran, 2,3-dihydro-	97
13	8.67	1, 3, 5-cycloheptatriene	97
14	8.76	3-pyridinecarboxylic acid	98
15	8.95	3, 3-Dimethylthietane	97
16	9.19	2-Methoxy-4-vinylphenol	93
17	9.29	Methyl-beta-D-arabinopyranoside	88
18	9.39	2, 6-Dimethyl-3-trans-propenylpyrazine	99
19	9.61	1-Di(t-butyl)silyloxypropane	95
20	9.82	1, 10-Decane-1, 1, 10, 10 d4-diol	97
21	9.97	Propanoic acid, 2-methyl-, methyl ester	95
22	10.11	2-cyclopenten-1-one, 2-methyl	97
23	10.25	Benzamide	98
24	10.55	2-ethyl-2’, 2’, 2’-D3-Cyclopentanone	87
25	10.80	(1, 1’-Bicyclopropyl)-2-octanoic acid, 2’-hexyl-, methyl ester	93
26	10.97	Benzene, methyl-	96
27	11.38	Suberic acid monomethyl ester	95
28	11.47	Thiacyclohexan-4-ol	97
29	11.56	2-allylpent-4-enoic acid, methyl ester	41
30	11.68	L-Menthol	97
31	11.82	Dodecanoic acid	99
32	11.95	Ethanone, 1-(4-hydroxyphenyl)-	98
33	12.09	2-Octenal	98
34	12.20	3’, 5’-Dimethoxyacetophenone	33
35	12.27	2-Butynedioic acid, diethyl ester	97
36	12.37	Octanedioic acid	98
37	12.37	Octanedioic acid	95
38	12.52	1, 6-anhydro-beta-D-Glucopyranose	84
39	12.68	Alpha-Methyl-alpha-propylsuccinimide	99
40	12.76	1, 2.4-Trimethoxybenzene	91
41	12.83	Acetohydrazide, 2-hydroxy-2-phenyl-N2-but-2-enylideno	98
42	13.04	2-cyclohexyldimethylsilyloxybut-3-yne	94
43	12.12	4-pyridinecarboxylic acid, 3-hydroxy-5-(hydroxymethyl)-2-methyl-	100
44	13.19	Alpha-Methyl-D-mannopyranoside	92
45	13.40	Octahydro-Naphthalene-1,8A-diol	99
46	13.57	1-Methyl-4-Phenyl-1, 2, 3, 6-tetrahydropyridine	87
47	13.74	4-Methyl-5-imidazolemethanol	97
48	13.86	Dihydrojasmone	99
49	13.98	Eicosanoic acid	98
50	14.13	3-Fluorobenzoic acid, dodec-9-ynyl ester	99
51	14.76	Oxirane, hexadecyl	97
52	14.93	Eicosanoic acid	90
53	15.01	Mome inositol	98
54	15.29	Heptadecanoic acid	97
55	15.55	Hexadecanoic acid, methyl ester	99
56	16.02	2-Butenal, 2-methyl-4-(2, 6, 6-trimethyl-1-cyclohexen-1-yl)	99
57	16.37	2H-Pyran-2-one, 5-ethylidenetetrahydro-4-(2-hydroxyethyl)-	96
58	16.50	Pluchidiol	99
59	16.84	1,10-Dimethyl-2-methylene-trans-decalin	100
60	16.92	1-Methyl-1-n-decyloxy-1-silacyclobutane	96
61	18.97	Cyclopropanebutyric acid-2[(2-nonylcyclopropyl)methyl]-, methyl ester	99
62	19.24	9, 12, 15-Octadecatrienoic acid, methyl ester	99
63	19.65	1-Docosanol	99

peak	Retention Time	Compound	% matching with Wiley library
64	21.20	7-Hexadecyn-1-ol	98
65	21.56	9, 12, 15-Octadecatrienoic acid, methyl ester	99
66	24.36	Capsaicin	96
67	26.54	Triacontanoic acid, methyl ester	99
68	30.56	Octadecane	99
69	31.18	4, 8, 12, 16-Tetramethylheptadecan-4-olide	98
70	31.80	Cycloheptanon, 3-butyl-	97
71	32.01	Tetratriacontane	97
72	36.03	Cyclopentanetridecanoic acid, methyl ester	97

Microscopy- The microscopical studies of the transverse section and powder showed presence of abundant lignified, thick wall, libiform type of fibres with pointed tips & vessel elements with oblique perforations plates having short, pointed tails, which are distinguishing microscopic features and serve as anatomical markers.[19]

Histochemical- plant metabolites are generally located in vegetative or reproductive organs. These chemicals have several uses. In Pharmacognosy discipline, these are also been utilized for identification and detection of purity of the crude drug.[14]

Ash values- are measure of inorganic content of the drug. These values are constant for pure drugs and increased/ decreased when contaminated with soil and adulterants.[14]

Extractive values- Based on extractive values the correct time of collection of drug, type and conditions of extraction process and nature of chemical constituents present in the drug can be determined. These are also useful for the evaluation especially when the constituents of drug cannot be readily estimated by any other means. The drug under investigation found to contain more of polar constituents.[14]

Loss on drying- is determination of moisture content of the drug. Presence of moisture in the crude drugs serves has suitable media for bacterial growth, causes degradation of moisture sensitive chemical constituents and gives information about moisture absorbing chemical constituents of plant drugs. The crude drug powder contains less than 5%.[14]

Fluorescence studies- The drug emits visible radiations of different wavelength when observed in various solvents at 254nm. The stem powder exhibited Yellowish fluorescence (powder as such), Dark green colour (10% sodium hydroxide solution), pale yellow (glacial acetic acid), Greenish fluorescence (chloroform), yellow fluorescence (methanol), yellow fluorescence (distilled water) and green colour (10% potassium hydroxide). These tests may consider as one of the parameters for characterization of the genuine drug samples.[20]

Secondary metabolites- The phytochemical screening of successive extracts revealed the presence of phenols, tannins, steroids, carbohydrates and flavonoids. Thus, the preliminary phytochemical tests are helpful in finding chemical constituents in the plant material that may lead to their quantitative estimation and in locating the source of pharmacologically active chemical compounds.[14,21-23]

HPTLC fingerprinting- helps in quantification, identification and checking the purity of the crude drug. The hydroalcholic extract of *Hibiscus micranthus* produces three different patterns of bands in **Mobile Phase – I**: Ethyl acetate: methanol – glacial acetic Acid (10:1.35:1), **Mobile phase – II**: Chloroform – methanol (9.9:0.1), **Mobile phase – III**: Ethyl acetate: formic acid – glacial acetic acid – water (10:1.1:1.1:2.6). The separation efficiency of mobile phase-III was maximum and total of five spots with Rf values 0.02, 0.11, 0.16, 0.55, 0.64 (blue) where detected under UV 366 nm. For identification of the drug, fingerprints in all three mobile phases will be helpful.

Estimation of rutin by HPLC- The Hydroalcoholic extract was quantified by HPLC studies which showed the presence of selected marker compound rutin and its retention time and λ max were similar to standard. The Photo diode array detector was used and set in the range of 200-780 nm. The rutin content was found to cotain 0.12% w/w of air dried extract. Thus, the proposed method is rapid, selective, requires a simple sample preparation procedure, and represents a good procedure for their quantification in plant material and in routine quality control of herbal drugs.

GC-MS analysis of HEHM- Further HEHM was analyzed by GC-MS to detect for volatile components. A total of 56 compounds were identified from the methanolic extract of the stem parts. The identified compounds represented 89.41% of the extract. The main components of the methanolic extract of stem parts were 7-Hexdecyn-1-ol(11.32%), 9,12,15-Octadecatrienoic acid methyl ester (7.88%), triacontanoic acid methyl ester (5.21%), octadecane (3.74%), 1-docosanol (3.61%), cyclopropanebutyric acid 2-((2-nonylcyclopropyl) methyl)-methyl ester (3.95%),, 1-methyl-1-n-decyloxy-1-silacyclobutane (5.82%), Hexadecanoic acid methyl ester

(2.16%), oxirane hexadecyl-(2.60%), octahydro-naphthalene-1,8A-diol(2.19%), and octanedioic acid(4.13%). The GC-MS analysis revealed that the methanolic extract is mainly composed of fatty acid esters.

The pharmacognostic and phyto-chemical investigations of the *Hibiscus micranthus L.* stem has been carried out for the first time. Chemo profiling by Spectophotometric, HPLC, HPTLC and GC-MS analysis can be utilized for identification, quantification and characterization of chemical markers present in *Hibiscus micranthus* Linn. This could also serve in the establishing data for preparation of monograph of this plant.

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Molluscicidal Activity of *Spilanthus acemella* Murr. on the Snail *Lymnaea acuminate*

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ABSTRACT

Spilanthus acemella Murr. also known as toothache plant is investigated for its preliminary phytochemical analysis and molluscicidal activity. The water extract found maximum extractive value (3.730%) than other solvents used. All the tested metabolites were qualitatively present in ethanolic extract and aqueous extract. Different concentrations of plant parts (root stem, leaf) were used for molluscicidal activity against snail *Lymnaea acuminate.* The maximum activity was found in stem LC_{50} at 364 mg/liter and ethanolic extract LC_{50} at 20 mg/liter.

Key words: *Lymnaea acuminate*; LC_{50}; Metabolites; Phytochemical;

INTRODUCTION

The snail *Lymnaea acuminata* is the intermediate host of the liver flukes *Fasciola hepatica* Linnaeus and *Fasciola gigantica* Cobbold. It causes 94% of fascioliasis in cattle and other livestock in the northern part of India.[1-2] This disease ranks as a major cause of mortality and morbidity among livestock.[1,3] A number of synthetic molluscicides have been used to control the population of vector snails but their continued use for several decades has disrupted the ecological system and led to a resurgence of mollusk populations.[1] Therefore, much effort has been focused on plant materials for potential use as commercial pesticides. Plant containing bioactive compounds can be more interesting than the synthetic chemical molluscicides due to their inherent combination of chemical substances.

Spilanthes acmella is an acutely threatened plant species of Asteraceae family.[4] In view of the medicinal properties of *S. acmella*, the increased demand for it in the pharmaceutical industry. It has been well documented for its uses as spices, antiseptic, anti-bacterial, anti-fungal, anti-malarial and as remedy for toothache, flu, cough, rabies diseases and tuberculosis.[5-9] Prasad et al.[10] reported that *S. acmella* also possessed excellent anti-microbial activities against red *Halophillic cocci* from salt cured fish. There are reports that *S. acmella* contains alkaloids that have the potential to act as an insecticide[11-12] and were found to be able to control *Aedes aegypti* in Kenya.[13] However the molluscicidal activity of *Spilanthes acmella* has not been reported so far. So in present work we attempt to evaluate molluscicidal activity against *Lymnaea acuminate*.

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MATERIALS AND METHODS

Collection of plant material and Lymnaea acuminata

Plant material collected from pot cultivated plants in Rajasthan University campus. Plant material was authenticated by Herbarium, Department of Botany, Rajasthan University, Jaipur, Rajasthan, India. Colleted plant parts (Root, stem and leaf) shade dried and grinded with pestle mortar. The crude powder and ethanolic extract was used for molluscicidal activity.

Adult *L. acuminata* (2.60 ± 0.30 cm long) were collected from University aquarium containing dechlorinated tap water and authenticated from Department of Zoology, University of Rajasthan, Jaipur, Rajasthan, India

Preparation of extracts[14]

The stem, leaf and roots of *Spilanthes acmella* was cut into small pieces, dried and powdered. The resultant was then

subjected for successive extraction with petroleum ether, benzene, chloroform, ethanol and water with soxhlet apparatus. The extracts were then concentrated in vacuum under reduced pressure using rotary flash evaporator and dried in desiccators. These extracts were then subjected to preliminary phytochemical screening for the detection of various plant constituents. Each of these extracts was processed further to evaluate the presence of carbohydrates, proteins, tannins, flavonoids, steroids and alkaloids following the established protocols.[15] The crude powder was treated with acids like 1N HCl, H_2SO_4, HNO_3, Acetic acid and alkaline solutions like 1N NaOH and Ammonia for analysis.

Molluscicidal activity

Toxicity experiments were done using the method described by Singh and Agarwal.[2] For each test group, ten test organisms were maintained in each of five glass aquaria containing three liters of dechlorinated tap water at 25°C. The test organisms were exposed to different concentrations of crude powder of root, stem, leaf and ethanolic extract by adding the test materials to aquariums (Table 1).

Snail mortality, established by the contraction of body within the cell and a failure of response to a needle probe, was recorded every 24 hrs for 72hrs.

RESULTS

The shade dried plant material subjected to sequential extraction in petroleum ether, benzene, chloroform, ethanol and water. Maximum yield was found in ethanol extract (3.134%) Total extractive values in different solvents are shown in table2.

Preliminary phytochemical investigation revealed that petroleum ether extract contains protein and tanins, benzene extract contains tanins, chloroform extract contains tanin, flavonoids, alkaloids and steroids, ethanol extract and aqueous extract contains alkaloids, steroids, tannins, flavonoids, carbohydrates and proteins (Table 3).

The powdered material of *Spilanthes acmella* treated with different acids, bases and other chemicals. After treatment powder was observed and fluorescence were tabulated and shown in Table 4.

The toxicity of *Spilanthes acmella* crude powders and ethanolic extract was time and dose dependent. After 72 hrs the toxicity of stem (LC_{50} = 364 mg/L) was higher than root (LC_{50} = 374 mg/L) and leaf (LC_{50} = 492 mg/L) (Table 5). The ethanolic extract was exceptionally toxic to *Lymnaea acuminate* (LC_{50} = 20 mg/L at 72 hrs).

DISCUSSION

The present study indicates the molluscicidal potential of leaf, stem, root and ethanolic extract. Toxicity of ethanolic extract against the snail *Lymnaea acuminate* in the present study was comparable (LC_{50} = 20 mg/L) to synthetic pesticide Phorate (LC_{50} = 15.50 mg/L). Preliminary phytochemical screening was showed that the ethanolic extract contain all the bioactive metabolites so it has higher activity.

The present study, though not conclusive, gives ample evidence that the mortality of snails caused by the etanolic extract of

Table 1: Plant material used for toxicity trials

S. No.	Plant material used	Test concentrations* (mg/L)
1	Root powder	100,200,300,400,500
2	Stem powder	100,200,300,400,500
3	Leaf powder	100,200,300,400,500
4	Ethanolic extract	10,20,30,40,50
5	Phorate**	10,20,30,40,50

*Concentration of test material in dechlorinated tap water.
** Standard synthetic molluscicidal

Table 2: Successive solvent extraction of air dried plant material of root, stem and leaves of *Spilanthus acemella*

Solvent	Color and Consistency	Extractive value (%w/w)
Petroleum ether	Yellowish green sticky	0.736
Benzene	Yellowish green sticky	0.735
Chloroform	Yellowish green viscous	0.825
Ethanol	Yellowish green sticky	2.305
Aqueous	Brownish Red powder	3.730

Table 3: Preliminary Phytochemical Test for Different Extracts of stem, root and leaves of *Spilanthus acemella* (Obtained by Successive Solvent Extraction)

Test	Petroleum ether	Benzene	Chloroform	Ethyl alcohol	Aqueous
Proteins	–	–	–	++	++
Carbohydrates	–	–	–	+	++
Tannins	–	+	++	+	++
Flavonoids	–	–	+	++	++
Alkaloids	–	–	+	++	+

Table 4: Fluorescence Analysis of Drug Powder of Bark & Wood of *Spilanthus acemella*

Test	Color
Powder + HNO_3	GY-YW
Powder + Acetic Acid	GY-YW
Powder + 5% Iodine solution	YW-RD
Powder + 5% $FeCl_3$	YW-OR
Powder + Diluted NH_4 + $K_4Fe(CN)_4$	DA-BL
Powder + 40% NaOH + few drops of Lead Acetate	GN-OR
Powder + 10% NaOH + $CuSO_4$	GN-YW
Powder + Acetic Acid + H_2SO_4	GY-BN
Powder + conc. HNO_3 + excess NH_3	YW-RD
Powder + Acetic Acid + H_2SO_4	GY-OR

GY- gray, YW- yellow, GN- green, OR- orange, RD- red, BL- blue, BN- brown

Table 5: Toxicity of root, stem, leaf and ethanolic extract of *Spilanthus acemella* on the snail *Lymnaea acuminate*

Plant parts used	Treatment period in hrs* (LC 50 in mg/liter)**		
	24hrs	48 hrs	72hrs
Leaf	475	426	392
Stem	459	437	364
Root	466	441	374
Ethanolic extract	55	42.34	20
Phorate	33.05	21.25	15.50

* Batches of 10 snails were exposed to test material listed in table 4 for the indicated time period. Mortality was measured after every 24 hrs with cumulative count of dead snails. Each set of trials repeated three times.
** LC50 – lethal concentration for 50 % of snails.

Spilanthes acmella may be because of its alkaloids. To confirm this, further purification and identification of the active compound is needed. Screening of plant extracts represents a continuous effort to find new antiparasitic drugs. These herbal formulations in harmonious integration with other safe methods of pest control can serve as ecofriendly and economically viable means of pest control in the near future.

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Detection of Antimicrobial Activity of *Solanum melogena* L. (Egg plant) Against Pathogenic Microorganisms

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ABSTRACT

Introduction: *Solanum melogena* L. or egg plant is one of few cultivated Solanaceous species originating from the old world. **Methods:** To determine antimicrobial effects of egg plant, well agar diffusion assay for bacteria and colony diameter method for fungi were used. Crude extracts of different parts of *Solanum melogena* L. (egg plant) were tested for their antimicrobial activities against seven pathogenic microorganisms that include two strains of fungi (dermatophytes) and five strains of bacteria. **Results:** Root and fruit extracts showed significant effects on tested microorganisms. *Epidermophyton floccosum* considered the most susceptible fungus for extract of these parts with MIC value of 15 and 28 mg/ml respectively. Meanwhile, the bacterium *Proteus vulgaris* exhibited susceptibility to all tested extracts with less MIC value. **Conclusions:** Crude extracts of different parts of *S. melogena* showed variable potential effects on various types of pathogenic microorganisms.

Key words: Bacteria, Dermatophytes, Egg plant, Antimicrobial action

INTRODUCTION

Solanum melogena L. (known as egg plant in the United States and aubergine in France and England) is one of few cultivated Solanaceous species originating from the old world. It has been also a common crop in the Middle East and around the Mediterranean basin and is now cultivated worldwide.[1] The plant is a very good source of dietary fiber, potassium, manganese, copper and thiamin (Vitamin B_1). It is also a good source of vitamin B6, folate, magnesium and niacin.[2]

Like other plants, *S. melogena* exposures to a wide rang of pathogenic organisms during its growth periods. The fungus *Verticillium* which causes wilting disease is one of such pathogenic organisms that infect egg plant.[3] From five plant seeds, however, *S. melogena* seeds found to have least number of fungal species. The isolated fungi from such seeds were *Aspergillus restichus* and *Phoma sp.*[4] Generally, the genus *Solanum* has immense natural diversity and natural host plant resistance. It includes many species of plants resistant to attack by pathogenic organisms such as insects.[5] This resistance is attributed to the presence of leptine glycoalkaloids (solanine and chaconine).[6]

Although antimicrobial activities of other species belong to the genus *Solanum* were studied, there is little information about antimicrobial activity of *S. melogena*. Thus, whole plant parts were tested each alone for their antimicrobial activity on different types of pathogenic microorganisms.

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MATERIALS AND METHODS

Plant preparation

Seeds of *Solanum melogena* L. (Solanaceae) were obtained from institute of agriculture in Karbala province (Iraq). Cultivation was performed in prepared field in the same institute from June to August 2009. Mature whole plants were harvested and washed under running tap water. Then, roots, stems, leaves, flowers and fruits were separated from each other and allowed to air-dry at room temperature after washing once again with distilled water.

Plant extracts

Dried parts were grounded by electrically blender until obtaining approximately fine powder. To prepare aqueous extract, 20 g of dried parts was extracted with 100 ml sterilize distilled water in electrically blender for 5 min and left for 1 h. the mixture was filtered through sterilized gauze and the extracts were concentrated to dryness at room temperature.

Test organisms

All tested pathogenic organisms were clinically isolated from patients at AL-Hussein general hospital of Karbala-Iraq in August 2009. Two strains of dermatophytes (skin pathogenic fungi) and five strains of bacteria were isolated. Isolated bacteria were diagnosed using API 20 system (Biomérieux, Netherlands-France), while fungal strains were diagnosed based on criteria recorded by Rippon[7] and Emmons et al.[8] The isolated bacteria were: *E. coli, Staphylococcus aureus, Klebisella pneumoniae, Proteus vulgaris,* and *Bacillus subtilis.*

The isolated fungi were: *Trichophyton mentagrophytes* and *Epidermophyton floccosum.*

Antibacterial assay

Standard culture of bacteria for antibacterial assay was prepared from isolated strains in Mueller-Hinton broth (HiMedia, Mumbai-India) equivalent to a 0.5 MacFarland standard (reading to 1×10^8 cfu/ml) and diluted 1:10.

Well agar diffusion method recommended by NCCLS[9] was used. A well of 6 mm was performed in plate with Mueller-Hinton agar (HiMedia, Mumbai-India) inoculated with isolated bacterial strains. Various concentrations (6.25, 12.5, 25, 50 mg/ml) of dried extracts were prepared in sterilize distilled water. Each well filled with 50 µl of specific concentration of extract. Cefotaxime sodium (30 µg) as standard antibacterial supplied by KonTam pharmaceuticals co. Zhongshan-China and distilled water were used as controls.

Antifungal Assay

Colony diameter method recorded by Kücüc and Kivan[10] was used. Different concentrations (1.25, 2.5, 5, 10 and 20 mg/ml) of dried extract were mingled with melting Sabouraoud's glucose agar of the following components: glucose 20 g, peptone 10 g, agar 15 g, chloramphenicol 0.05 g and 1000 ml of distilled water. Then, poured in sterile Petri dishes. A disk (9 mm) of old grown fungi (at 28 °C for I week) was inoculated on the center of culture media. Plates were incubated at 28 °C for I week. Griseofulvin (10 µg), as standard antifungal, supplied by Arabic Drug Industry manufacture (Bagdad-Iraq) and distilled water were used as controls. Perpendicular colony diameters (mm) of grown strains were measured.

Determination of Minimum Inhibitory Concentration (MIC)

MICs in bacteria were determined as described by NCCLS[9] and in fungi as recorded by Santos and Hamdan[11]. Crude

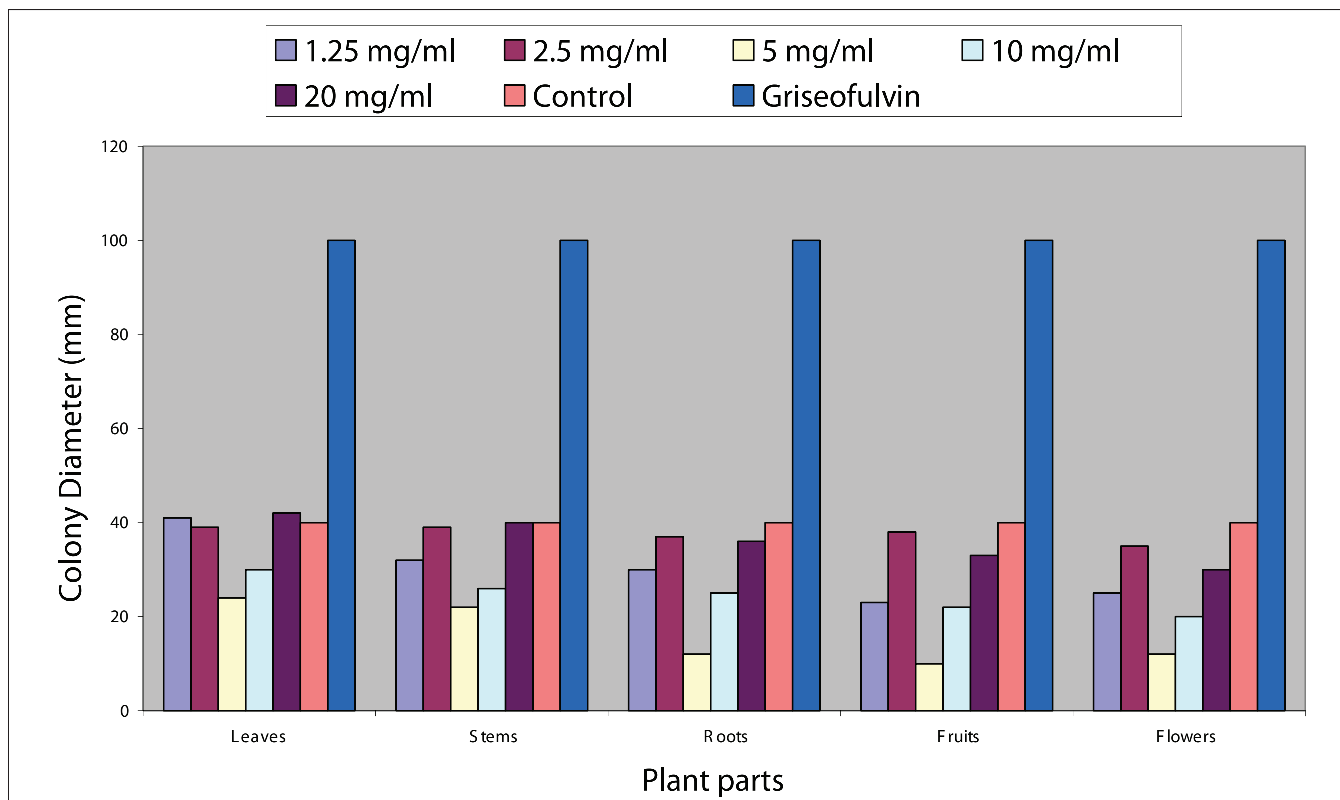

Figure 1: Colony diameter of *T. mentagrophytes* grown on media containing plant extracts

extracts were twofold diluted in Mueller-Hinton broth for bacteria and in Sabouraoud's glucose broth for fungi. A 100 µl of each dilution was dispensed in well of microdilution plates (96-wells). Well was inoculated with 50 µl of previously prepared standard culture of bacteria and fungi. The inoculated plates were incubated at 35º C and examined for visible growth in order to determine MIC. The previous controls were also included.

Statistical analysis

Data were statistically analyzed by using two-way variance of analysis (ANOVA) with less significant difference (L.S.D.) at $P < 0.05$.

RESULTS

Fresh collection of *S. melogena* after cultivated for two months were tested for their antimicrobial activities against seven pathogenic microorganisms. Antifungal activity of crude extracts of egg plant was examined against two strains of dermatophytes. Root and fruit extracts showed significant effects on those dermatophytes (Fig. 1 and 2). *E. floccosum* revealed more susceptibility to these extracts with MIC value of 15 and 28 mg/ml respectively (Table 2).

The antibacterial effects of all extracts varied depending on strain and plant parts. Greatest diameter of zones of inhibition resulting from root extract could be considered a good indicator for root activity on tested bacteria compared with other parts. From all of bacteria strains, *K. pneumoniae* revealed more resistance to all *S. melogena* extracts even to cefotaxime. Meanwhile, *P. vulgaris* exhibited susceptibility to all tested extracts with less MIC value. Moreover, *B. subtilis* could also be considered more susceptible to crude plant extracts (Table 1).

DISCUSSION

In addition to nutritional values of *S. melogena* (egg plant), the medical value of this plant are being revived once again in the beginning of the twenty-one century based on phenolic and alkaloid contents. Antioxidant activities are the main function of such phenolic compounds that include caffeic and chlorogenic acids and flavonoids such as nasunin.[2] Naturally, egg plant produces such compounds to protect them against oxidative stress resulting from exposure to harmful elements, as well as from infection by bacteria and fungi.

S. melogena roots and fruits showed high efficiency to inhibit growth of most tested microorganisms. Nasunin, a potent antioxidant and free radical scavenger, considers the main compounds in fruit of egg plant,[2] while glycoalkaloides are the important alkaloids found in root at various concentrations[6]. This alkaloid has many activities including antibacterial and diuretic properties.[12]

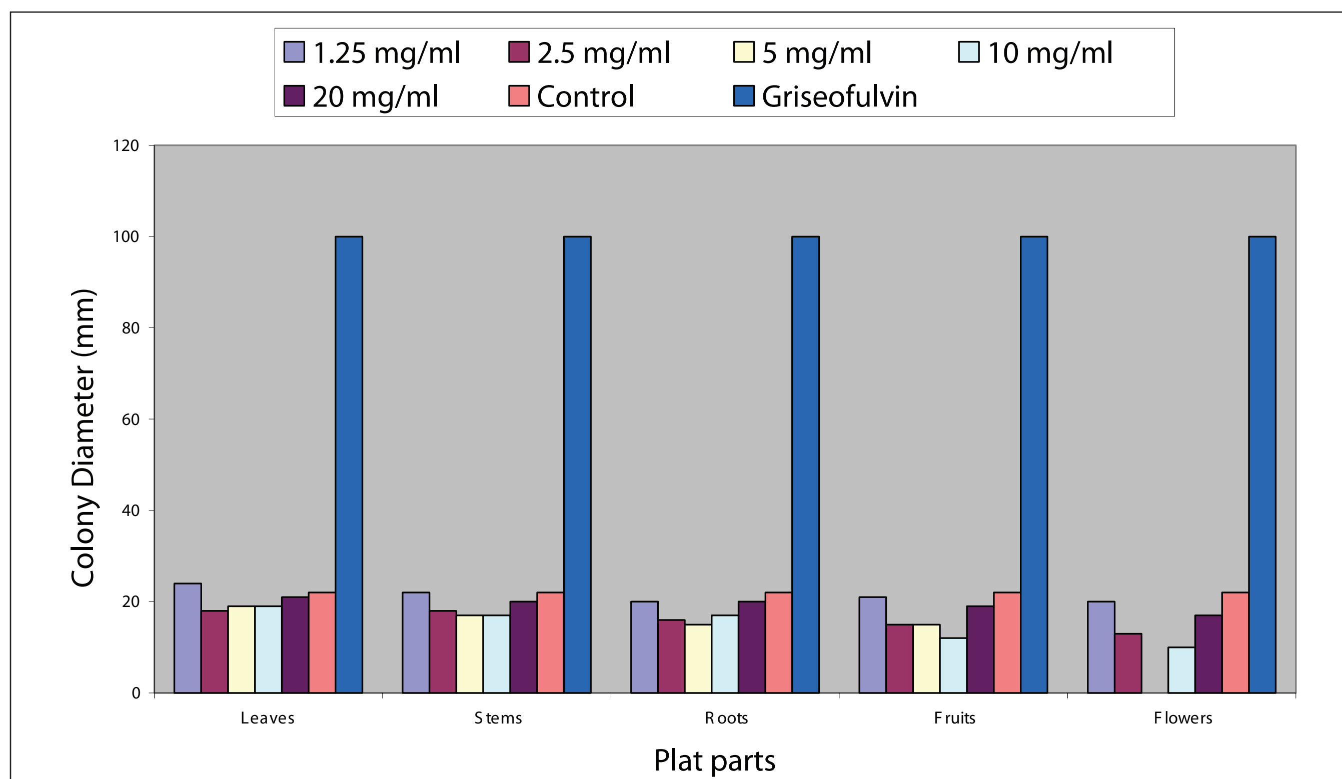

Figure 2: Colony diameter of *E. floccosum* grown on media containing plant extracts

Table 1: Antibacterial activity of different parts of *S. melogena* using well agar diffusion method

Plant parts	Zone of inhibition (mm)					
	Concen. (mg/ml)	*S. aureus*	*E. coli*	*K. pneumoniae*	*B. subtilis*	*P. vulgaris*
leaves	50	12	16	-	20	17
	25	-	-	-	16	14
	12.5	-	-	-	-	-
	6.25	-	-	-	-	-
Stems	50	-	13	-	17	16
	25	-	-	-	11	13
	12.5	-	-	-	-	-
	6.25	-	-	-	-	-
Roots	50	13	15	-	21	16
	25	-	13	-	17	15
	12.5	-	-	-	15	14
	6.25	-	-	-	-	11
Fruits	50	15	15	-	23	18
	25	-	-	-	15	14
	12.5	-	-	-	-	11
	6.25	-	-	-	-	-
Flowers	50	-	11	-	20	14
	25	-	-	-	-	11
	12.5	-	-	-	-	-
	6.25	-	-	-	-	-
Cefotaxime	30 μg/ml	27	22	-	34	21

Table 2: MICs (mg/ml) of *S. melogena* extracts in pathogenic microorganisms

Strain	Leaves	Stem	Root	Flower	Fruit
S. aureus	47	>50	44	>50	48
E. coli	45	38	20	30	48
K. pneumoniae	>50	>50	>50	>50	>50
B. subtilis	20	6	10	48	20
P. vulgaris	20	20	8	20	8
T. mentagrophytes	>50	>50	25	50	32
E. floccosum	>50	40	15	50	28

Antibacterial effects of crude extracts showed non specific action on either of gram positive or gram negative bacteria. Whereas, Saraf and Suva[13] found that leaves of egg plant had antibacterial activity on gram negative only. Furthermore, *K. pneumoniae* exhibited much more resistance to all extracts of all parts. This resistance was also noted toward standard antibacterial agent (cefotaxime) that used as control for comparable study. *K. pneumoniae* has a thick polysaccharides capsule that serves as protective structure against most antibiotics[14].

Many studies illustrated that different plant extracts may have antidermatophytic effects against *T. mentagrophytes* and *E. floccosum*. From these studies, most of plants showed the ability to inhibit the growth of both species, such as *Erigeron floribundus,*[15] *Acacia concinna,*[16] coffee and tea plants.[17]

In recent study, growth of *T. mentagrophytes* and *E. floccosum* were also inhibited in the presence of egg plant extracts in culture media, especially root extract, through suppressive the growth of fungal colonies and decreasing of MIC value. *E. floccosum* showed susceptibility to most plant extracts than those of *T. mentagrophytes*. This result was also noted with the extract of *Erigeron floribundus*.[15]

From our data, results indicated that the inhibition activity of egg plant is dependent on the concentrations. The higher the concentration of egg plant, the greater the inhibitory action of the related plant extracts to the test organism.

Recently, soluble defense molecules are discovered in leaves of *S. melogena* that contribute to its antimicrobial activities.[18] While, acceptable explanation for the antimicrobial activities of *S. melogena* after dried and extracted is based on the presence of phenolic and alkaloid compounds, especially chlorogenic acid that considers the predominant phenolic compound found in all varieties of egg plants.[2]

CONCLUSION

Crude extracts of different parts of *S. melogena* showed variable potential effects on various types of pathogenic microorganisms. Root and fruit extracts had more inhibitory effects on isolated microorganisms than other plant parts. The fungus *E. floccosum* and the bacterium *P. vulgaris* considered the most susceptible to tested plant extracts. However, the present results may add another benefit to egg plant to be becomes a favorable plant to eat.

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Comparative antibacterial activities of extracts of dried ginger and processed ginger

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ABSTRACT

Ginger (*Zingiber officinale* Rosc.) is a well-known spice and herbal medicine to treat such symptoms as fever, cough, etc, especially its antibacterial activity, which made it widely used in clinic or as preservatives in food industry. The paper compared the antibacterial activities in solvents of dried ginger and processed ginger. The results showed that each organic solvent had antibacterial activity against gram-positive bacteria (two strains) and gram-negative bacteria (four strains). However, no significant differences appeared between dried ginger and processed ginger and among of ginger organic extracts.

Key words: Dried ginger, Processed ginger, Ginger extract, Antibacterial activity.

INTRODUCTION

Ginger (*Zingiber officinale* (Willd) Rosc.), a member of Zingiberaceae family, is a well-known spice applied to people's daily diet in Asian and Indian. In China, ginger rhizoma has also been used as a commonly non-prescribed traditional Chinese medicine to treat various diseases including common cold, cough and gastrointestinal upsets.[1] Nowdays, it was reported that ginger had good activities in antioxidant, antibacterial,[2,3] anti-tumor[4] activities, etc,[5-7] especially its antibacterial, which made it widely used in clinic or as preservatives in food industry. The aim of this study was analyzed antibacterial activities of different ginger extracts and further determined its minimal inhibitive concentration (MIC), which would provide a theoretical basis for its clinical application of Chinese medicine or as a food additive.

MATERIALS AND METHODS

Materials

Dried rhizome and processed ginger were obtained from the Shuyu pharmacy of Jinan, China, in March 2010. Chemical reagents in the experiment were purchased from the Tianjin Chemical reagent factory.

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Aqueous extract of dried and processed ginger

Dried gingers or processed gingers were powdered, and macerated in water for 2 h. The aqueous solution was boiled twice, each 1.5 h and then concentrated to final concentration of 500 μg/mL.

Ethanol extraction of dried and processed ginger

The powder dried ginger or processed ginger (5.0 kg) was macerated in ethanol (70%, v/v) and mixed well, in water bath for 3 h at 50 ˚C. The solvent was filtered and concentrated to final concentration of 500 μg/mL.

Acetone extraction of dried and processed ginger

The powder dried gingers or processed ginger (5.0 kg) were soaked in actone (solid/ liquid, s/l=1/6) for 72 h at room temperature. The solvent was filtered usig six layers gauze and concentrated to final concentration of 500 μg/mL.

Methanol extraction of dried and processed ginger

The powder dried gingers or processed ginger were soaked in methanol for 72 h at room temperature. The solvent was filtered and repeated this step twice, and adjusted to final concentration of 500 μg/mL.

Hexane extraction of dried and processed ginger

The powder dried gingers or processed ginger were soaked in water reflux for 8 h at 70 ˚C and extracted at room

temperature. The residues were dissolved with hexane and filtered. Filtration solution was concentrated to final concentration of 500 µg/mL.

Chloroform extraction of dried and processed ginger

The dried gingers were powdered and soaked in ethanol (95%) reflux and filtered. The solvent was collected and concentrated, and extracted with chloroform. The extraction was adjusted to final concentration of 500 µg/mL. Chloroform extraction of processed ginger was prepared using the same method.

Antibacterial assay

Selected test microorganisms were gram-positive bacteria, that was *Staphylococcus aureus* (ATCC25923) and its isolated strain from air; Gram-positive bacteria included *Escherichia coli* (ATCC25922), *Shigella fiexneri* (ATCC12022), *Proteus vulgaris* (ATCC13315), *Pseudomonas aeruginosa* (ATCC27853). Antibacterial tests were carried out by the disc diffusion method.[8] Sterile paper discs (6 mm in diameter) prepared from whatman were impregnated with drug-containing solution placed on the inoculated agar. Negative control and positive control was using ethanol and appropriate antibiotics respectively (table 1). The inoculated plates were incubated at appropriate temperature for 24 h. The antibacterial activity was evaluated by measuring the diameter of inhibition zone against the test microorganisms. Each assay in this experiment was replicated thrice.

Minimal inhibitive concentration (MIC) assay

MIC was studied for the microorganisms that were determined as sensitive in the disc diffusion method.[9] The aqueous extract and ethanol extract were first diluttted to 250 µg/mL, and then serial two-fold dilutions were made with MH broth in the concentration rang from 10 to 250 µg/ mL. The last tube containing 2 mL of MH broth without extract and 50 µL of the inocula was used as a negative control.

Table 1: Antibiotics used in different strains

Bacteria types	Strains	Antibiotics
Gram-positive bacteria	*Staphyloccocus aureus*	Erythromycin
	Staphyloccocus aureus (isolated)	Erythromycin
Gram-negative bacteria	*Escherichia coli*	Ciprofloxacin
	Proteusbacillus vulgaris	Ciprofloxacin
	Pseudomonas aeruginosa	Ciprofloxacin
	Shigella castellani	Gentamicin

RESULTS

The effect of ginger extracts on gram-positive bacteria

The results indicated that organic extracts of dried ginger and processed ginger had an inhibitory effect on *Staphyloccocus aureus* (ATCC25923) and isolated strain from air, compared with negative control. Antibacterial activities showed no significant difference among the groups, while aqueous extracts had no inhibitory activity (Fig. 1 and Fig. 2).

The effect of ginger extracts on gram-negative bacteria

The results displayed that except aqueous extract, each group had antibacterial actitivity of four gram-negative bacteria, including of *Escherichia coli* (ATCC25922), *Shigella fiexneri* (ATCC12022), *Proteus vulgaris* (ATCC13315), *Pseudomonas aeruginosa* (ATCC27853), and no significance appeared between each organic extract. However, their antimicrobial activities were very lower, compared to positive control. Antibacterial activity of hexane extract was the smallest in all the organic extracts. For each extract, the antibacterial activity of processed ginger was better than that of dried ginger (Fig. 3 - Fig. 6).

MIC of gingers extract

The results showed that MIC of each extracts were ranged from 50 to 125 µg/mL. For the same strain, each extract had different MIC, such as MIC against *Escherichia coli* (ATCC25922), which was the least, only 50 µg/mL, while

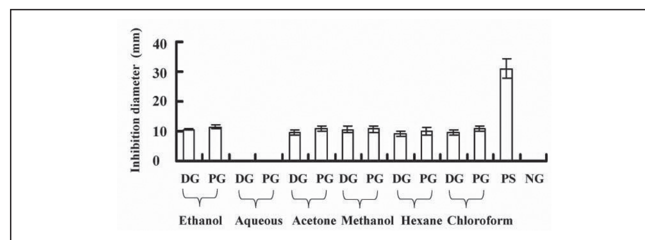

Figure 1: The effect of extracts of dried ginger and processed ginger on *Proteusbacillus vulgaris*. DG means dried ginger extract; PG means processed ginger extract; their concentrations in the experiments were 500 µg/mL (This part was the same as following figures).

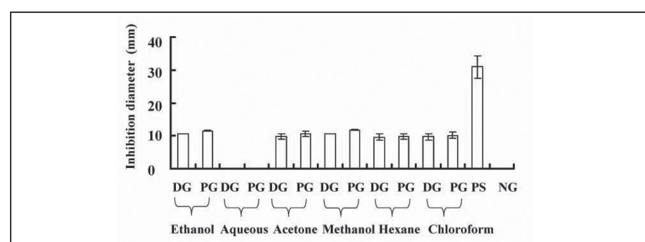

Figure 2: The effect of extracts of dried ginger and processed ginger on *Proteusbacillus vulgaris* (isolated).

its hexane extract was 62.5 μg/ mL. Hexane extract against other gram-negative bacteria, as the highest inhibitory concentration, was 125 μg/mL (Table 2).

DISCUSSION

It was reported there were contradictions in antimicrobial activity of ginger, which mainly reflected two aspects.[10-12] One was that its active ingredients - essential oils and flavonoids, their antibacterial effects varied greatly in literature. The other was that the same extract had contrary antimicrobial activity.

The study showed that no significance appeared in antibacterial activity of various ginger extracts, which explained the fact that ginger volatile oil and its flavonoids, as the antimicrobial active substances, were all dissolved in organic solvent, and so there was no significant difference. However, The different polarity and solubility of organic solvents, resulted in different antibacterial activities for various strains. Ginger extracts could be used as preservatives, if solvents were not toxic.

Although no significant difference, antibacterial activity in processed ginger extract was slightly higher than that in ginger extract. During the dried ginger were processed, gingerol was dehydrated and formed into flavonoids, which resulted in the higher content of flavonoids in processed ginger than that in dried ginger.[13] Correspondingly, its antibacterial activity was higher than that of dried ginger.

In summary, antibacterial activities existed in organic ginger extracts, but no significance appeared among the extracts.

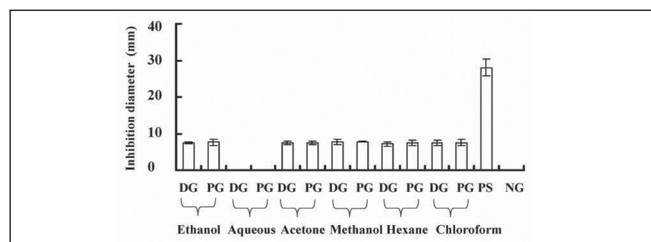

Figure 3: The effect of extracts of dried ginger and processed ginger on *Escherichia coli*.

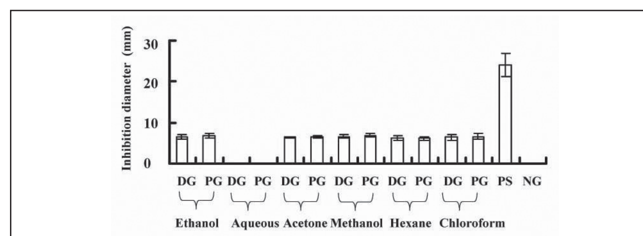

Figure 4: The effect of extracts of dried ginger and processed ginger on *Pseudomonas aeruginosa*.

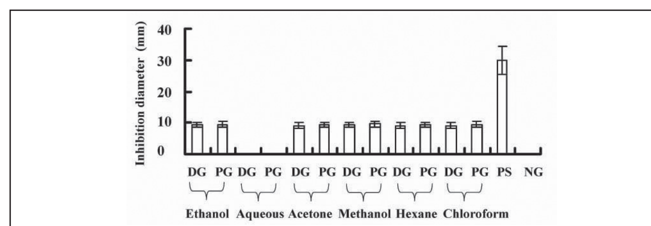

Figure 5: The effect of extracts of dried ginger and processed ginger on *Shigella castellani*.

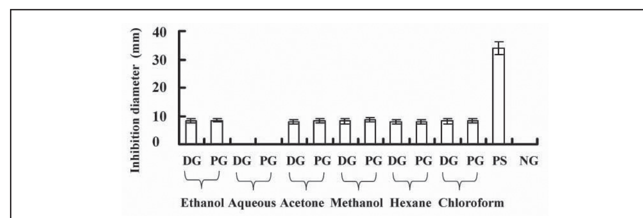

Figure 6: The effect of extracts of dried ginger and processed ginger on *Proteusbacillus vulgaris*.

Table 2: MIC of extracts of dried and processed ginger

Extract	MIC					
	Staphyloccocus aureus	Staphyloccocus aureus (isolated)	Escherichia coli	Pseudomonas aeruginosa	Shigella castellani	Proteusbacillus vulgaris
EE of DG	100	100	50	100	100	100
EE of PG	100	100	50	100	100	100
ME of DG	100	100	50	100	100	100
ME of PG	100	100	50	100	100	100
HE of DG	125	125	62.5	125	125	125
HE of PG	125	125	62.5	125	125	125
AC of DG	100	100	50	100	100	100
AC of PG	100	100	50	100	100	100
CE of DG	100	100	50	125	100	100
CE of PG	100	100	50	125	100	100

Notes: EE means ethanol extract; AE means aqueous extraction; DG means dried ginger; PG means processed ginger; DIE means dichloromethane extract; ME means methanol extract; HE means hexane extract; CE means chloroform extract; AC means acetone.

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Anti-anxiety Activity of *Cymbopogon citratus* (dc.) stapf Leaves Extracts on the Elevated Plus-Maze Model of Anxiety in Mice

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ABSTRACT

The Methanolic extract of *Cymbopogon citratus* leaves at the dose of 200 mg/kg increased the percentage of time-spent and the percentage of arm entries in the open arms of the elevated plus-maze (EPM) and decreased the percentage of time-spent in the closed arms of EPM. Moreover, it prolonged the ketamine-induced latency to sleep but had no significant effects on total sleeping time induced by ketamine. Also, the locomotor activity was affected but not to the same extent as observed for diazepam. The anxiolytic effects of methanol extract *Cymbopogon citratus* leaves may be related to their content of flavonoids. This study validates the traditional use of the plant in management of anxiety.

Key words: *Cymbopogon citrates*, Diazepam, Elevated plus maze.

INTRODUCTION

Anxiety disorders are the most common type of psychiatric disorders, with an incidence of 18.1% in total world population and a lifetime prevalence of 28.8%.[1-2] Anxiety is an important component of many other psychiatric or medical conditions.[3] Anxiety disorders are the most common mental illness in the world and became a very important area of research interest in psychopharmacology. Interest in alternative medicine and plant-derived medications that affect the 'mind' is growing. Self administration of herbal medicines was the most popular alternative therapies to the official medicine. The use of herbal medications by physicians in Europe and Asia is becoming very common and researchers are exploring the traditional remedies to find a suitable cure for these 'mind affecting diseases.[4]

Lemon grass (*Cymbopogon citratus*, Stapf) family Graminae is a widely used herb in tropical countries, especially in Southeast Asia. The essential oil of the plant is used in flavour, fragrancing and aromatherapy, medicinal tea, culinary herb,[5] and treatment for skin diseases.[6] It is known as a source of ethnomedicines.[7] *C. citratus* is used in different parts of the world in the treatment of digestive disorders, fevers, menstrual disorder, rheumatism and other joint pains.[8] Essential oil of Lemon grass was evaluated for sedative/hypnotic activity through pentobarbital sleeping time, anxiolytic activity by elevated plus maze and light/dark box procedures and anticonvulsant activity through seizures induced by pentylenetetrazole and maximal electroshock. Essential oil was effective in increasing the sleeping time, the percentage of entries and time spent in the open arms of the elevated plus maze as well as the time spent in the light compartment of light/dark box. In addition, essential oil delayed clonic seizures induced by pentylenetetrazole and blocked tonic extensions induced by maximal electroshock, indicating the elevation of the seizure threshold and/or blockage of seizures spread.[9]

A review of literature revealed that *Cymbopogon citratus* is highly reputed plant, and has been widely employed in herbal medicine and aromatherapy but no significant work has been carried out on the anxiolytic effects of the plant extracts. So, the present study was designed to evaluate effect of the methanolic extract of *Cymbopogon citratus* on CNS in mice.

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MATERIAL AND METHODS

Plant material

Cymbopogon citratus (DC.) stapf. leaves were collected in the month of February 2007, from Panjab University, Chandigarh, India. The taxonomic identity of the plant was confirmed by Dr. H.B. Singh, Head, Raw Materials Herbarium & Museum, National Institute of Science Communication and Information Resources (CSIR), New Delhi 110067.

Extraction

The dried leaves of *Cymbopogon citratus* was minced (200 g) and were macerated in 600 ml of methanol and water. The extract was concentrated in a rotating evaporator under reduced pressure to give a residue (10%, w/w). The residue was dissolved in normal saline for final suitable concentrations.

Animals

Swiss albino mice (20-30 gm) of either sex were housed in standard environmental conditions. Food and water were available ad libitum. All experiments were carried out between 09.00 and 13.00 h.

Elevated plus-maze (EPM)

The EPM is apparatus comprised of two open arms (35 cm × 5 cm) and two closed arms (30 cm × 5 cm × 15 cm) that extended from a common central platform (5 cm × 5 cm). That was elevated to a height of 50 cm above floor level.[10] Mice were given a single i.p. dose of the plant extract 30 min before their placement on the EPM. The number of entries and the time spent in the open and closed arms were recorded during a 5-min test period. The percentage of open arm entries (100 × open/total entries) was calculated for each animal. Diazepam at dose of 0.5 mg/kg i.p. was used as standard.

Locomotor activity

The actions of *Cymbopogon citratus* leaves methanolic extract on spontaneous locomotor activity were measured automatically by breaking of infrared beams as described by Rabbani et al.[11] The units of the activity counts were arbitrary and based on the beam breaks by movement of mice. Each mouse was i.p. injected with the plant extract (100 and 200 mg/kg) and after 30 min placed in a novel cage in the infrared apparatus. The locomotor activity was measured at 5-min interval for 15 min. Six mice were used for each treatment group. Diazepam at dose of 0.5 mg/kg i.p. was used as reference drug.

Ketamine-induced sleeping time

The effect of the studied extract on ketamine-induced sleeping time was measured as described by Mimura et al.[12] After 30 min pretreatment with the plant extracts (200 mg/kg i.p.) or vehicle, animals (six for each group) were injected with ketamine (100 mg/kg, i.p.). The interval between the administrations of ketamine until the loss of the righting reflex was recorded as onset of sleep. The time from the loss to regaining of the righting reflex was considered as duration of sleep.[13] Diazepam (0.5 mg/kg) was used as standard drug.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with post hoc Tukey test. $P < 0.05$ was considered significant. All data are expressed as mean ± S.E.M

RESULTS

Effect of *E. sphaericus* fruits extract on the elevated plus-maze

In the elevated plus-maze, the behavior observed confirmed the anxiolytic activity of diazepam as reported previously.[14] The methalonic extract of *Cymbopogon citratus* leaves at a dose of 200 mg/kg increased the percentage of time spent and percentage of arm entries in the open arms ($P < 0.05$, Fig. 1a,b) and decreased the percentage of time spent and percentage of arm entries in the closed arms ($P < 0.05$, Fig. 2a,b). The extract at 100 mg/kg had no significant effects on any of the measured parameters (Figs. 1a,b and 2a,b). In a similar fashion to the studied extract, diazepam increased the percentage of time spent and percentage of arm entries in the open arms ($P < 0.05$, Fig. 1a, b). The magnitude of the anxiolytic effects of 200mg/kg mg/kg of *Cymbopogon citrates* leaves of methanolic extract were very close to that observed with 0.5 mg/kg of diazepam.

Effects of of Cymbopogon citratus leaves extract on spontaneous locomotor activity

Fig. 3 shows the cumulative locomotor activity during 15 min of test. In animals pretreated with *of Cymbopogon citratus* leaves methanolic extract (200 mg/kg), the locomotor activity was decreased by 22% compared with vehicle treated controls. Administration of diazepam at 0.5 mg/kg suppressed the locomotor activity to a greater extent (83%).

Effects of of Cymbopogon citratus leaves extract on ketamine-induced sleeping time

Results are reported in Fig. 4. In saline treated animals the righting reflex was lost after 118"2s of ketamine injection. Injection of *Cymbopogon citratus* leaves extract (200 mg/kg) and diazepam significantly suppressed the latency to sleep by 34% and 57%, respectively ($P < 0.05$, Fig. 4). On the contrary, the total duration of the sleep was not affected by *Cymbopogon citratus* leaves (+23%, $P > 0.05$) In animals treated with diazepam, however, the duration of sleep was significantly increased by 116%.

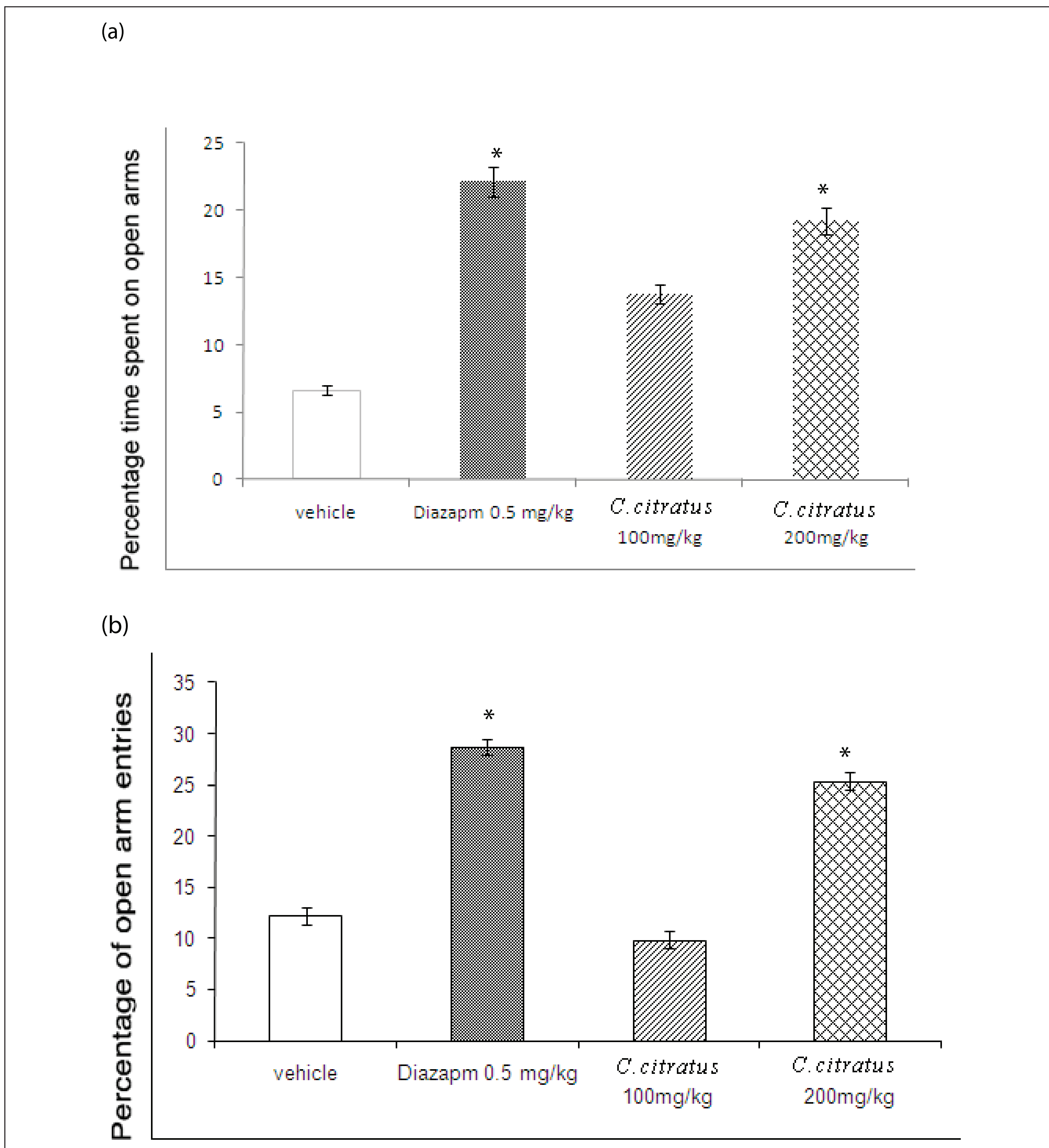

Figure 1: Effects of *Cymbopogon citratus* leaves methanolic extract on: (a) the percentage of time spent in the open arms; and (b) the percentage of open arm entries of the elevated plus-maze (EPM). Data are presented as means" ± S.E.M from a group of six mice each. $*P < 0.05$ compared with vehicle-treated control.

DISCUSSION AND CONCLUSION

The aim of the present study was to evaluate the anxiolytic effect of Methanolic extract of *Cymbopogon citratus* leaves. Various doses of the plant extract were tested on the EPM. Only at 200 mg/kg, the plant extract produced anxiolytic effect with a mild sedative action, at doses lower than 200 mg/kg, there was no significant changes in the behavior parameter that was measured on the EPM. Doses higher than 200 mg/kg produce severe sedative effects (data not shown) and were not considered suitable for further evaluation. As expected, diazepam

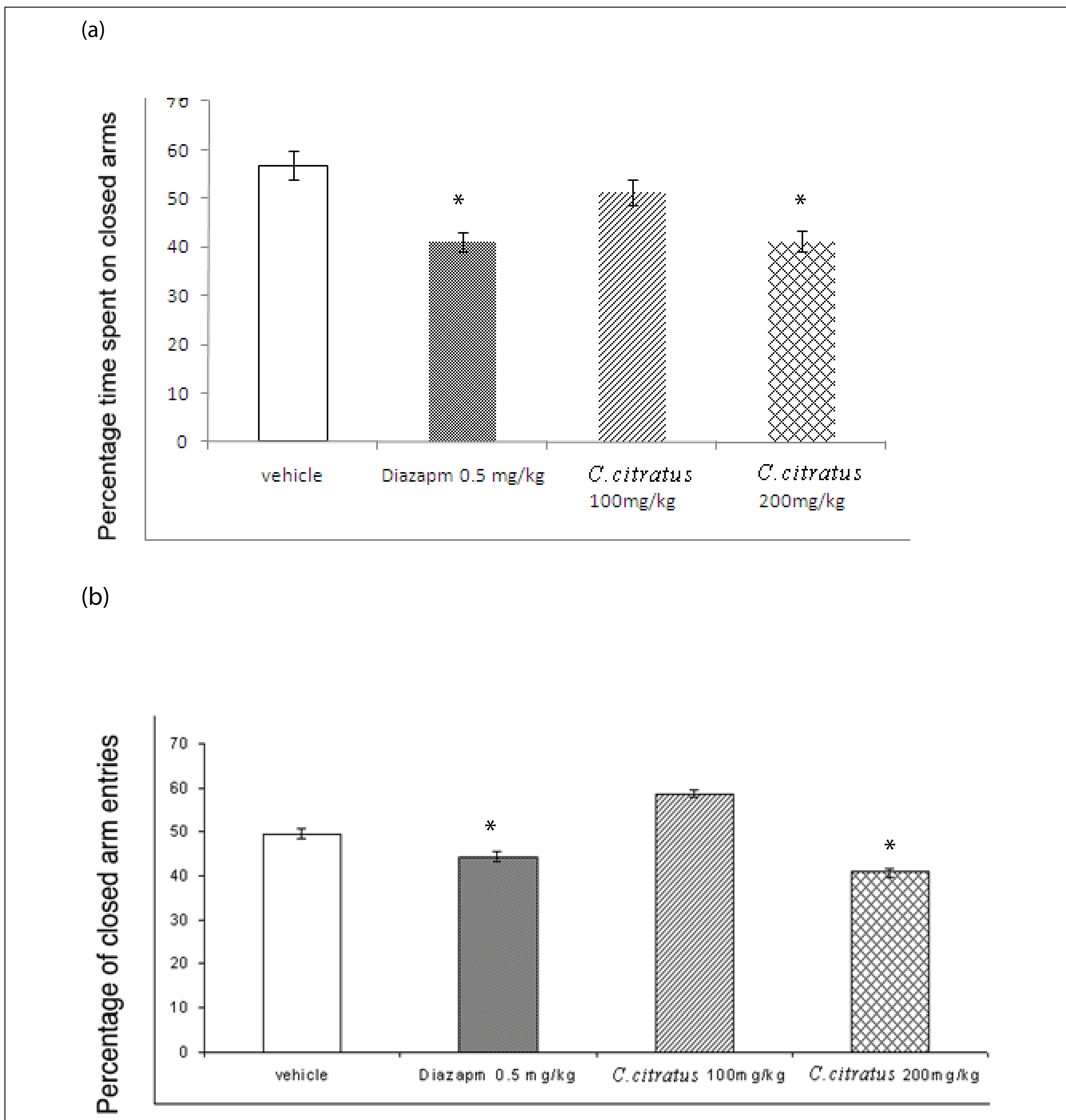

Figure 2: Effects of *Cymbopogon citratus* leaves methanolic extract (a) the percentage of time spent in the closed arms; and (b) the percentage of closed arm entries of the elevated plus-maze (EPM). Data are presented as means" ± S.E.M from a group of six mice each. *$P < 0.05$ compared with vehicle-treated control.

produced significant increases in open arm time and in number of entries into the open arms. These data are consistent with the results of numerous previous studies, which have shown that diazepam and other benzodiazepines produce significant anxiolytic effects in a variety of anxiolytic screening procedures, including elevated plus-maze test procedures.[15-19] The decrease aversion to the open arms is a result of an anxiolytic effect expressed by an increased number of open arm entries and time spent in the EPM. This primary index of anxiety is spatiotemporal in nature: it is reduced by anxiolytic drugs and can be increased by anxiogenic compounds.[20] The decreased time spent on the central platform is another indication of a reduced 'decision-making' behavior. Both parameters are accepted as reliable indicators of anxiety and fearfulness.[21]

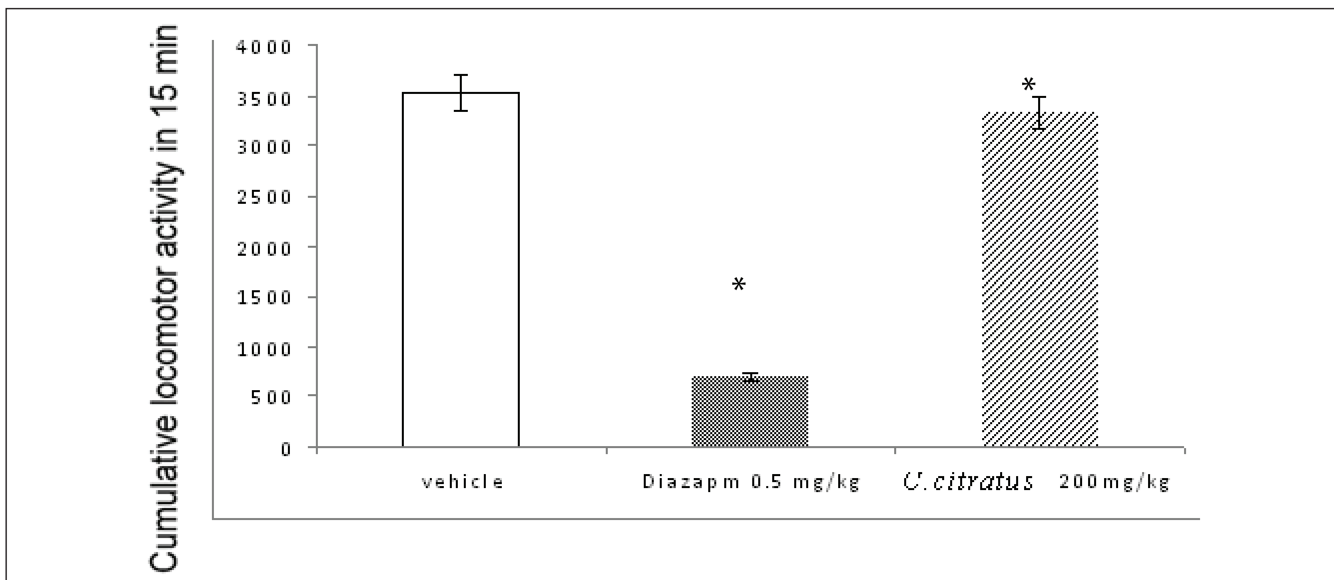

Figure 3: Effects of *Cymbopogon citratus* leaves methanolic extract on locomotor activity in mice. Data are presented as means" ± S.E.M from a group of six mice each. $^*P < 0.05$ compared with vehicle-treated control.

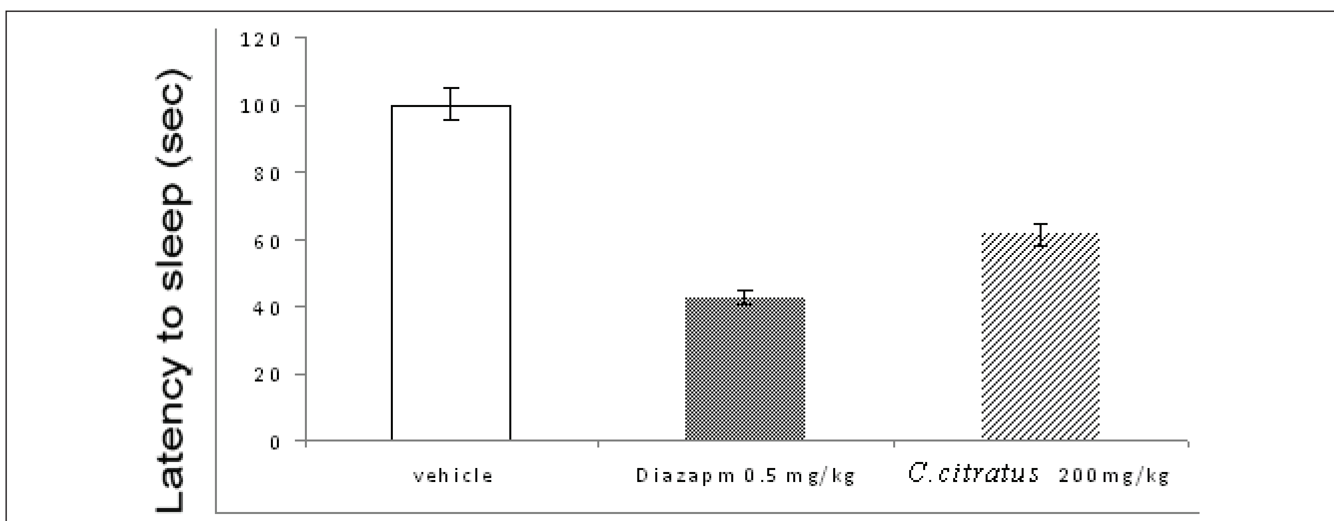

Figure 4: Effects of *Cymbopogon citratus* leaves methanolic extract on the latency to loss of righting reflex. After 30 min pretreatment with the plant extracts or vehicle, animals were injected with ketamine (100 mg/kg, i.p.). The interval between the administrations of ketamine until the loss of the righting reflex was recorded as onset of sleep. Results represent means"± S.E.M. from six mice. $^*P < 0.05$, compared with vehicle-treated control.

The other behavioral parameter that was altered in the plus-maze was the number of entries into the enclosed arms. The fact that the number of entries into enclosed arms was lower in animals treated with the plant extract or diazepam indicates that these compounds could have sedative properties. Number of entries into the enclosed arms has been taken as a measure of locomotor activity by a number of researchers.[20-22] In the locomotor study, both the plant extract (at anxiolytic dose) produced a significant reduction in locomotor activity count suggesting a sedative property, although the diazepam sedative effect was more severe.

In addition to the locomotor study, the data from the interaction of the plant extract with ketamine also showed a different profile of activity from diazepam. The extract of *Cymbopogon citratus*, shortened the latency to sleep induced by ketamine but did not significantly change the duration of sleep. Diazepam reduced the latency to sleep and increase significantly the sleeping time. Further phytochemical screening showed the presence of flavonoids, saponins and triterpenoids in methanolic extract.

In summary, the present results demonstrated an anxiolytic-like effect from *Cymbopogon citratus* leaves methanolic extract

with a mild sedative action. Further pharmacological investigations are underway to identify the active constituents of the plant extract responsible for the showed activities.

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