Pharmacognostical and Preliminary Phytochemical Studies of Stem Bark of *Ailanthus excelsa* Roxb

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ABSTRACT

Objective: The present study was aimed for pharmacognostic and preliminary phytochemical studies of stem bark of *Ailanthus excelsa* Roxb belonging to the family Simarubaceae. **Materials and Methods:** The pharmacognostic investigation was carried out in terms of macroscopic, microscopic parameters. **Result:** The extract obtained after successive Soxhlet extraction of dried and coarsely powdered stem bark using petroleum ether, chloroform, ethanol and distilled water were subjected to a preliminary phytochemical screening which revealed the presence of carbohydrates, proteins, phytosterols and triterpenenoids. **Conclusion:** The present investigation reflects the structural identity and chemical nature of the crude drug which could be useful in laying down pharmacopoeial standards as standardization is an important aspect in assessing the quality, safety and efficacy of herbal medicines.

Keywords: *Ailanthus excelsa* Roxb, phytochemical, structural identity, the pharmacognostic standardization

INTRODUCTION

Ailanthus excelsa Roxb is popularly known as "Mahaneem" belonging to the family *Simarubaceae*. Its Hindi name is Maharuk. The tree is indigenous to Indian peninsula and is growing wild in the states of Gujarat, Bihar, Andhra Pradesh, and Karnataka. It is a tree of fast growth and propagated in the drier parts of India. It also occurs in parts of Australia. The tree is large and deciduous up to a height of 18-24 m. The leaves are equally pinnate and are 25-75 cm long. The leaflets are alternate or sub opposite with 6-12 pairs, variable in shape, irregularly toothed at the margins, and unequal-sided at the base. The petiolules are 2.5 cm long. Flowers are branched with cream or yellowish in color. Fruit are fiat, papery pod and are 4-6 cm long, 1-1.5 cm broad, lance-shaped, reddish brown, and acute at both ends. Seeds are solitary.[1]

The whole tree is medicinally useful. It is a well-known remedy for the treatment of various types of disorders in the Ayurvedic, homeopathic, and folklore system of medicine in India.[2] Previous investigation revealed that the various part (leaves, bark, stem) of the tree *A. excelsa* Roxb have antitumor, antiviral, antimalarial, antileukemic, antifeedent, hepatoprotective, antiasthmatic, antifertility, and antibacterial activity.[3,4]

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MATERIALS AND METHODS

Plant material and authentication

The stem bark of *A. excelsa* was collected in the month of March from outer hilly areas of Bhopal and was authenticated by Dr. N.K. Pandey (Botanist) in National Research Institute for Ayurveda-Siddha Human Resource Development, Aamkho, Gwalior, Madhya Pradesh.

Pharmacognostic studies

Macroscopic study

Macroscopic characters shape, size, color, odor, texture, etc., of the plant material were studied.

Microscopic study

Preparation of sample (plant part)

The stem bark was cut and removed from the plant and

fixed in formalin-acetic acid (formalin 5 ml + acetic acid 5 ml + 70% ethanol 90 ml). After 24 h of fixing, the sample was dehydrated with tertiary butyl alcohol and in filtered by gradual addition of paraffin wax until tertiary butyl alcohol solution attained super saturation. The sample was kept into paraffin blocks.

Sectioning of sample (plant part)

The paraffin embedded sample was sectioned with the help of rotary micro tome. The thickness of the section was 10-12 μm. After dewaxing, the sections were stained with toluidine blue and observed under microscope. Scanning electron microscope was employed for microscopic evaluation of the internal structure of the stem bark of *A. excelsa*. Roxb.[5]

Powder microscopic characteristics

The powder of the stem bark was viewed under microscope which shows the possible structural details of the sample (Tables 1 and 2).[6]

Preliminary phytochemical studies

The preliminary phytochemical study was carried out to check the presence of phytoconstituents in different extracts (petroleum ether, chloroform, ethanol, distilled water) of stem bark of *A. excelsa* Roxb. The qualitative test gives a general idea regarding the nature of chemical constituents of the crude drugs. The extract was subjected to preliminary phytochemical investigation for the detection of phytoconstituents.

RESULT AND DISCUSSION

Morphological characteristics

The stem bark of *A. excelsa* Roxb exhibits light grey (externally) and yellowish-white (internally) with longitudinal striations on the inner side. It has a disagreeable odor and bitter in taste. It has granular and fibrous texture and irregular fracture.

Histo-morphological characteristics

The stem bark of *A. excelsa* Roxb is 1.6 cm thick and is differentiated into the outer zone of periderm or outer bark and inner zone of secondary phloem or inner bark. The outer bark is 600 μm-1 mm thick. The outer bark consists of broad phellem and a narrow zone of the phelloderm. The inner bark has two zones. The outer zone is called collapsed phloem. The inner zone is called as noncollapsed phloem which contains sieve elements narrow rays and small phloem parenchyma cells (Figures 1-3).[9,10]

Powder microscopic characteristics

The powder of the bark was viewed under microscope. It shows the presence of prismatic crystals and stone cells. It also shows elongated structures like sclereids and long narrow, thick walled fibers (Figure 4 and Table 3).[11]

Phytochemical studies

The preliminary phytochemical analysis of stem bark of *A. excelsa* Roxb reveals the presence of carbohydrates, proteins, phytosterols, triterpenenoids, and saponins

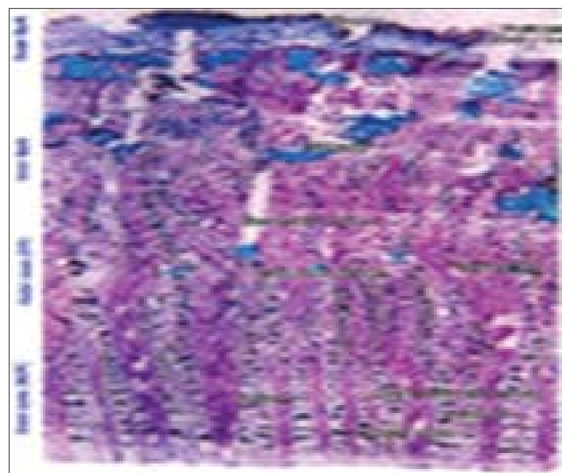

Figure 1: Transverse section of stem bark of *Ailanthus excelsa* Roxb.

Table 1: Ash values of *A. excelsa* Roxb[8]

Type of ash	Percentage of ash value
Total ash	7.20
Water soluble ash	4.36
Acid insoluble ash	2.08
Sulphated ash	5.24

A. excelsa: Ailanthus excelsa

Table 2: Extractive values of *A. excelsa* Roxb

Solvent used	Percentage of extractive value
Petroleum ether	2.52
Chloroform	1.24
Ethanol	6.35
Distilled water	12.15

A. excelsa: Ailanthus excelsa

Table 3: Quantitative parameters of *A. excelsa* Roxb

Parameters	Result
Length of the phloem fiber	250-710.5 μm
Width of the phloem fiber	12.5-35.2 μm
Bitterness value	140 unit/g
Loss on drying	5.10% w/w
Foaming index	<100

A. excelsa: Ailanthus excelsa

Table 4: Preliminary phytochemical screening of stem bark of *A. excelsa* Roxb[7]

Phytoconstituents	Identification tests	Petroleum ether extract	Chloroform extract	Ethanol extract	Distilled water extract
Alkaloids	Dragendroff's test	-	-	-	-
	Wagner's test	-	-	-	-
	Iodine test	-	-	-	-
	Hanger's test	-	-	-	-
Glycosides	Keller Killani test	-	-	-	-
	Conc H_2SO_4	-	-	-	-
	Molisch's test	-	-	-	-
Protein and amino acid	Millon's test	-	+	+	-
	Xantho protein test	+	+	+	-
	Biuret test	-	+	+	-
Sterols	Salkowski test	-	+	+	-
	Liebermann's-Burchard test	-	+	-	-
Lignin	Lignin test	-	-	-	-
	Labat test	-	-	-	-
Phenolic compounds	Ellagic test	-	-	-	-
	Phenol test	-	-	-	-
Flavanoids	NaOH test	-	-	-	-
	Shinoda/Pew test	-	-	-	-
Saponin	Foam test	-	+	+	+
	Haemolysis test	-	+	+	-

+: Present, -: Absent, *A. excelsa: Ailanthus excelsa*

Figure 2: Collapsed phloem.

Figure 3: Noncollapsed phloem.

which gives an idea about the chemical nature of the drug (Table 4).

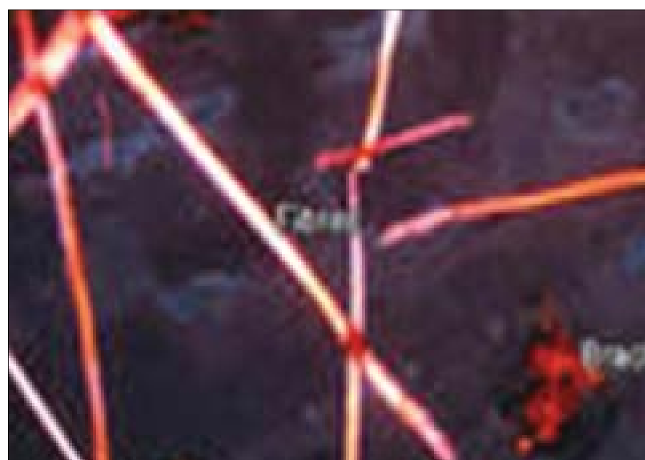

Figure 4: Powder microscopy of *Ailanthus excelsa.*

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Estrogenic Activity of *Bauhinia racemosa* Extract in Female Albino Rats: An Investigational Study

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ABSTARCT

Objective: The objective was to evaluate the effect of petroleum ether, ethanol, and water extract of *Bauhinia racemosa* in female albino rats. **Materials and Methods:** Plant extracts were tested for their estrogenic activity using ovariectomized method at two dose level: 200 mg/kg and 400 mg/kg, respectively. Further, plant extracts and standard drug ethinyl estradiol (1 μg/kg) combination were tested for synergistic estrogenic activity. **Results:** *In-vivo* investigation revealed that ethanol extract at dose of 200 mg/kg and 400 mg/kg shows significance increase 325.23 ± 5.23, 328.84 ± 4.56 and 3.252 ± 0.47, 3.288 ± 0.27 in uterine wet weight and uterine weight ratio, respectively, in female albino rat as compared to control and petroleum ether and water extracts. Simultaneous administration of standard drug ethinyl estradiol (1 μg/kg) with ethanol extract at dose of 200 mg/kg and 400 mg/kg potentiates (327.36 ± 5.78, 330.95 ± 6.21 and 3.273 ± 0.64, 3.309 ± 0.49 uterine wet weight and uterine weight ratio respectively in female albino rat) the estrogenic activity as compared to individual administration of ethanol extract as a synergistic effect. **Conclusion:** It was observed that ethanol extract of *B. racemosa* produced significance estrogenic activity.

Keywords: *Bauhinia racemosa,* ethinyl estradiol, estrogenic activity, ovariectomy

INTRODUCTION

The plant *Bauhinia racemosa* Lam. (*Caesalpiniaceae*) commonly known as APTA in Indian system of medicines widely distributed throughout India, Ceylon, China, and Timor. The genus *Bauhinia* L. is often called as "*Orchid tree*" ornamental value.[1] *B. racemosa* Lam. *Fabaceae Caesalpinioideae* is a small deciduous tree with a dark brown scabrous bark, distributed in Asian countries like India, Pakistan, and Sri Lanka.[2] It is one of the sacred plants of Hindus, worshipped during the great festival of Dusehra. Leaves are exchanged along with *Acacia ferruginea* as a token of love and respect. The bark is astringent and is used for inflammation, chronic dysentery, diarrhea, glandular inflammations, ulcer also effective for goiter, and some skin diseases.[3,4] Decoction of leaves is given to relieve headache in a malarial fever, flowers are used to treat hemorrhage, piles, cough, and as laxative.[2] A new tetracyclic phenol and other constituents reported from the roots[5] and structural determination of seed polysaccharide by mythylation studies.[6] Methanol extract of stem bark has anti-inflammatory, analgesic and antipyretic,[7] antitumor and antioxidant,[8] antimicrobial,[9] and hepatorenal functions in mice.[10] The roots of the plant have found to consists of new tetracyclic 2,2-dimethylchroman derivative, de-omethylracemosol compound (Compound-3).[11] Furthermore, the various extracts of *B. racemosa* lam. roots belonging to family *Caesalpiniaceae* were found to contain 1,7,8,12b-tetrahydro-2,2,4-trimethyl-2H-benzo[6,7] cyclohepta[1,2,3-de][1] benzopyran-5,10,11-triol.[12] Earlier investigations on this species have resulted in isolation of two compounds from the heartwood, a new dibenzoxepin derivative, pacharin (Copound-1), and a novel tetracyclic phenol, racemosol (Copound-2). Besides this, Stillene (resveratrol) was also isolated from the heartwood of this plant.[13] Many plants have proved to possess significant estrogenic activity, for example, *Azadirachta indica, Citrus medica, Ruta graveolens, Achyranthes aspera, Jasminum officinale, Mansonia gagei, Cissampelos pareira, Carica papaya, Moringa oleifera, Mimosa pudica, Nelumbo nucifera, Crataeva nurvala, Leonotis ocymifolia.*[14-26] In the present investigation, estrogenic activity of *B. racemosa* was investigated using experimental *in vivo* models.

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MATERIALS AND METHODS

Plant material

The stem bark of fully matured plant was identified with the help of local botanist and then collected in the month of June from Toranmal, Dist. Nandurbar near to Satpuda Hills, Maharashtra, India. The plant material further authenticated by Botanical Survey of India (B.S.I.) vide voucher specimen (No. BADIB5) Pune, Maharashtra, India.

Experimental animals

Healthy colony breed immature female Wistar rats weighing 100 g were housed in groups of six, under standard laboratory conditions of temperature (25 ± 2°C) and 12/12 h light/dark cycle. Animals had free access to standard pellet diet and water ad libitum. All animal experimentation was carried out after approval of the protocol by the Institutional Animal Ethical Committee vid no. NIB/IAEC/09-10/86 dated 15/01/2010.

Chemical and reagents

5% polyethylene glycol (PEG-400), ethinyl estradiol (German Remidies, India), petroleum ether (60-80°C), ethanol (95%). All Chemical and reagents were of analytical grade.

Statistical analysis

The data were presented as mean ± Standard error mean. The statistical significance between the groups has been tested by analysis of variance followed by Dunnett's test. A $P < 0.05$ were considered as significant.

Acute toxicity study

Acute oral toxicity study was performed as per OECD 423 guideline. Extract was administered up to the maximum dose of 2000 mg/kg and animals were observed for mortality.

In vivo assessment of estrogenic activity in female albino rats

Colony-bred immature female albino rats Wistar strain, weighing 100 g were maintained in rat cages in barrier rooms under 12:12-light: Dark cycle, with a temperature of 22 ± 1°C. The animals were divided into six groups consisting six rats in each group.

Group-I: Control, received 0.2 ml 5% (PEG-400) orally.

Group-II: Received 1 μg/kg ethinyl estradiol/rat/day in olive oil s.c.

Group-III: Received test extract of *B. racemosa* stem bark 200 mg/kg orally.

Group-IV: Received test extract of *B. racemosa* stem bark 400 mg/kg orally.

Group-V: Received test extract of *B. racemosa* stem bark 200 mg/kg orally and ethinyl estradiol 1 μg/kg/rat/day in olive oil s.c.

Group-VI: Received test extract of *B. racemosa* stem bark 400 mg/kg orally and ethinyl estradiol 1 μg/kg/rat/day in olive oil s.c.

All the above treatments were given for 7 days. On the 8th day of the experiment, the animals were sacrificed by cervical decapitation and bilaterally ovariectomized by the dorsolateral approach under light ether anesthesia. The uteri and adrenals were dissected out and separated from the adherent tissues and weighed up to the nearest mg on an electronic balance. The uterine ratio was calculated by dividing uterine wet weight into milligrams by body weight in grams. The increase in the uterine ratio was associated with the estrogenic effect of the extract as described by Vogel.[27]

RESULTS AND DISCUSSION

Acute toxicity studies for dose selection

Acute oral toxicity studies of petroleum, ethanol, and water extract revealed that the extract was toxic at dose level of 2000 mg/kg. Two of three animals at both test level of 2000 mg/kg died. Thus, as per OECD 423 guideline it was concluded that LD 50 value for above extract was 1000 mg/kg.

Estrogenic activity in female albino rats

The administration of standard drug ethinyl estradiol to the bilaterally ovariectomized immature female albino rats (1 μg/kg s.c.) caused increased in uterine wet weight and weight ratio to 331.52 ± 6.94, and 3.315 ± 0.25*, respectively, when compared with the control group. Ethanol extract of *B. racemosa* Lam. stem bark on administrating orally at the doses of 200 and 400 mg/kg body weight caused a significant increase in the uterine wet weight and weight ratio to 325.23 ± 5.23*, 328.84 ± 4.56* and 3.252 ± 0.47*, 3.288 ± 0.27*, respectively, when

compared with control. Furthermore, the simultaneous administration of above doses of test extract with that of standard drug have shown a significant increase in uterine wet weight and weight ratio when compared to control the group. The value of significance was found to (*$P < 0.05$) which indicates almost significant activity for ethanol extract as shown in Table 1 and Figure 1. Results thus obtained for a test and simultaneous administration of standard and test indicates significant dose dependent estrogenic activity in female albino rats. The present investigation for pharmacological studies of ethanol crude extract confirms the role of plant in controlling the fertility in female albino rats as estrogenic substance may cause the expulsion of ova from the tube, disruption of leuteotrophic activity the blastocyst, disrupt the functional equilibrium which may result in failure in fertility (Figures 2 and 3). In the case of petroleum ether and water extracts, the data obtained were statistically nonsignificant as extracts did not cause increase in uterine wet weight and weight ratio shown in Tables 2 and 3, respectively.

In several animal species, including rats and mice have shown to possess uterotropic effects on administration of estrogen, and such effects are, therefore, may responsible for the growth and proliferation of endometrial cell number, vaginal cornification. There are various phytoestrogens such as isoflavones, lignans, and resveratrol responsible for anti-fertility activity. Furthermore, flavonoids have found to possess anti-fertility activity. Besides this, experimental evidenced has also shown that, β-sitosterol, a phytosterol

Table 1: Effect of ethanol extract of *B. racemosa* stem bark on uterine weight in bilaterally ovariectomized immature female albino rats

Groups	Treatment	Doses	Uterine wet weight (mg/100 g)	Uterine weight ratio
			Mean±SEM	Mean±SEM
I	Control	0.2 ml of 5% polyethylene glycol orally	240.84±5.32	2.408±0.12
II	Ethinyl estradiol	1 μg/kg s.c.	331.52±6.94*	3.315±0.25*
III	Ethanol extract	200 mg/kg orally	325.23±5.23*	3.252±0.47*
IV	Ethanol extract	400 mg/kg orally	328.84±4.56*	3.288±0.27*
V	Ethinyl estradiol+ethanol extract	1 μg+200 mg/kg	327.36±5.78*	3.273±0.64*
VI	Ethinyl estradiol+ethanol extract	1 μg+400 mg/kg	330.95±6.21*	3.309±0.49*

n=6, Values are in mean±SEM, *$P<0.05$ when compared with control, SEM: Standard error of the mean, *B. racemosa: Bauhinia racemosa*

Table 2: Effect of petroleum ether extract of *B. racemosa* stem bark on uterine weight in bilaterally ovariectomised immature female albino rats

Groups	Treatment	Doses	Uterine wet weight (mg/100g)	Uterine weight ratio
			Mean±SEM	Mean±SEM
I	Control	0.2 ml of 5% polyethylene glycol orally	240.84±5.32	2.408±0.12
II	Ethinyl estradiol	1 μg/kg s.c.	331.52±6.94*	3.315±0.25*
III	Petroleum ether extract	200 mg/kg orally	245.12±5.94	2.451±0.47
IV	Petroleum ether extract	400 mg/kg orally	240.85±6.23	2.408±0.59
V	Ethinyl estradiol+petroleum ether extract	1 μg+200 mg/kg	246.74±5.98	2.467±0.45
VI	Ethinyl estradiol+petroleum ether extract	1 μg+400 mg/kg	248.41±7.64	2.484±0.26

n=6, Values are in mean±SEM, Statistically non-significant data. SEM: Standard error of the mean, *B. racemosa: Bauhinia racemosa*

Table 3: Effect of water extract of *B. racemosa* stem bark on uterine weight in bilaterally ovariectomized immature female albino rats

Groups	Treatment	Doses	Uterine wet weight (mg/100g)	Uterine weight ratio
			Mean±SEM	Mean±SEM
I	Control	0.2 ml of 5% Polyethylene glycol orally	240.84±5.32	2.408±0.12
II	Ethinyl estradiol	1 μg/kg s.c.	331.52±6.94*	3.315±0.25*
III	Water extract	200 mg/kg orally	231.54±3.98	2.315±0.45
IV	Water extract	400 mg/kg orally	239.25±4.52	2.392±0.58
V	Ethinyl estradiol+water extract	1 μg+200 mg/kg	240.14±3.54	2.401±0.47
VI	Ethinyl estradiol+water extract	1 μg+400 mg/kg	237.95±4.67	2.379±0.67

n=6, Values are in mean±SEM, Statistically non-significant data, SEM: Standard error of the mean, *B. racemosa: Bauhinia racemosa*

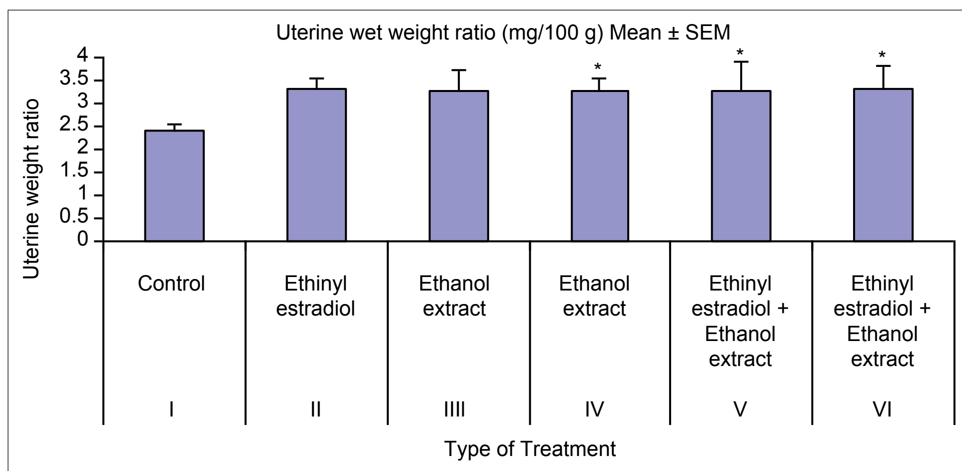

Figure 1: Effect of ethanol extract of *Bauhinia racemosa* stem bark on uterine weight in bilaterally ovariectomized immature female albino rats.

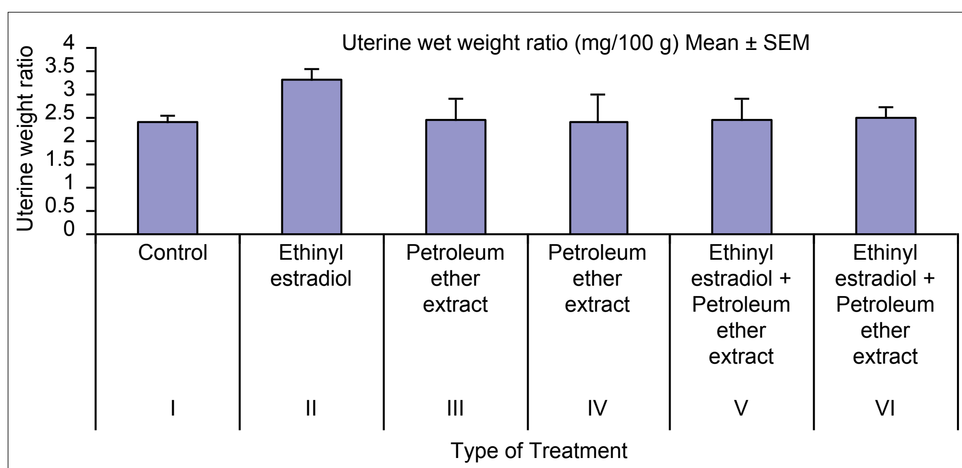

Figure 2: Effect of petroleum ether extract of *Bauhinia racemosa* stem bark on uterine weight in bilaterally ovariectomized immature female albino rats.

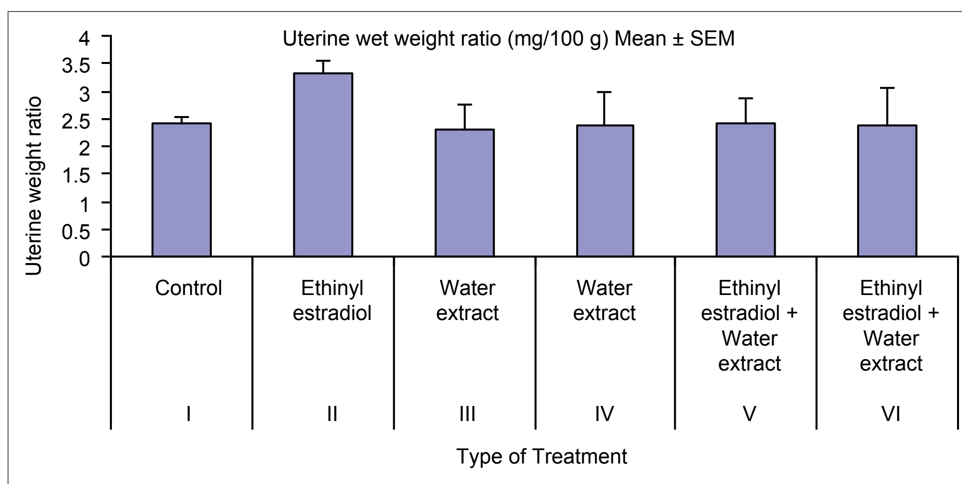

Figure 3: Effect of water extract of *Bauhinia racemosa* stem bark on uterine weight in bilaterally ovariectomized immature female albino rats.

can exert estrogenic effects in females. The ethanolic extract of stem bark of plant *B. racemosa* lam. has shown content of phytosterol moiety, β-sitosterol as confirmed by high performance thin layer chromatograph analysis which may be the reason responsible for inducing estogenic effect in female albino rats.

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High-Performance Thin-Layer Chromatography Fingerprinting of Ethnopharmacological Important Seeds of *Wrightia tinctoria*

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ABSTRACT

Introduction: *Wrightia tinctoria* R. Br. belongs to family *Apocynaceae* commonly called as sweet Indrajao, Pala indigo plant, Dyer's Oleander. "Jaundice curative tree" in south India. **Materials and Methods:** A method has been developed for different extracts of *W. tinctoria* for high-performance thin-layer chromatography (HPTLC) fingerprinting analysis for identification and quantification of the marker compound. **Result:** The satisfactory resolution was obtained in the solvent system toluene:ethyl acetate v/v (8:2) for petroleum ether extract, toluene:ethyl acetate v/v (7:3) for chloroform extract and toluene:ethyl acetate:formic acid v/v (7:3:0.1) for alcoholic extract. **Conclusion:** The HPTLC fingerprinting profile developed for different extracts of *W. tinctoria* will help in proper identification and quantification of the marker compound.

Keywords: High-performance thin-layer chromatography fingerprinting, *Wrightia tinctoria,* petether extract, chloroform extract, alcoholic extract

INTRODUCTION

Herbal drugs play an important role in health care programs, especially in developing countries. Ancient Indian literature incorporates a remarkably broad definition of medicinal plants and considers all plant or parts of plants to be potential sources of medicinal substances. However, a key obstacle, which has hindered the acceptance of the alternative medicines in the developed countries, is the lack of documentation and stringent quality control. There is a need for documentation of research work carried out on traditional medicines. With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as medicine. The process of standardization can be achieved by stepwise pharmacognostic studies, include morphological, anatomical study and biochemical characterization by qualitatively as well as quantitatively. These studies help in identification and authentication of the plant material. Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine that will contribute to its safety and efficacy.[1]

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Wrightia tinctoria R. Br. (Family: *Apocynaceae*) commonly called "Indrajau" is distributed throughout the world and occurs abundantly in India. It is a deciduous tree with white fragrant flowers. The seeds and bark of this plant be used in Indian traditional medicine as antidiarrheal and antidysenteric.[2]

W. tinctoria R. Br. belongs to family *Apocynaceae* commonly called as sweet Indrajao, Pala Indigo plant, Dyer's Oleander. "Jaundice curative tree" in south India. Sweet Indrajao is a small, deciduous tree with a light gray, scaly smooth bark. Native to India and Burma, *Wrightia* is named after a Scottish physician and botanist William Wright (1740-1827). Sweet Indrajao is called dhudi (Hindi) due to its preservative nature. The juice of the tender leaves is used efficaciously in jaundice. Crushed fresh leaves when filled in the cavity of decayed tooth relieve toothache. In Siddha system of medicine, it is used for psoriasis and other skin diseases. Oil 777 prepared out of the fresh leaves of the plant has been assigned to analgesic, anti-inflammatory, and anti-pyretic activities and to be effective in the treatment of psoriasis. The plant is reported to contain the presence of flavanoid, glycoflavones-iso-orientin, and phenolic acids. The various chemical constituents

isolated from various parts of the plant are reported as 3, 4-Seco-lup-20 (29)-en-3-oic acid, *lupeol*, *stigmasterol* and *campesterol*, *indigotin*, *indirubin*, *tryptanthrin*, *isatin*, *anthranilate* and rutin *triacontanol*, *wrightial*, *cycloartenol*, cycloeucalenol, *β-amyrin*, *alpha-amyrin*, and *β-sitosterol*, 14α-methylzymosterol. Four uncommon sterols, desmosterol, clerosterol, 24-methylene-25-methylcholesterol, and 24-dehydropollinastanol, were isolated and identified in addition to several more common phytosterols. The triterpinoids components of the leaves and pods of *W. tinctoria* also isolated.[3]

The present study attempts to highlights the high-performance thin-layer chromatography (HPTLC) profile of petroleum ether, chloroform and alcoholic extract of seed of *W. tinctoria* for the contribution in standardization and increasing the knowledge on this plant.

MATERIALS AND METHODS

Plant material and extraction

The *W. tinctoria* seed was collected from its natural habitat in Jharkhand, identified by Botanist at National Botanical Research Institute National Botanical Research Institute, Lucknow, Voucher specimens were preserved at the Herbarium of the institute. The seeds of *W. tinctoria* washed dried and powered, extracted with per ether, chloroform, ethanol and water in successive session in soxhlet percolator. Extractable value calculated by evaporating the solvent in a vacuum evaporator. In petroleum ether, no crystalline component obtained instead get rubbery material whereas chloroform and ethanolic extracts are semisolid in nature.

HPTLC fingerprinting

HPTLC study of different extracts was carried out by the method of Harborne[4] and Wagner *et al.*[5]

Development of solvent system

A number of solvent were tried individually as well as in combination for separation of different components of extract, but the satisfactory resolution was obtained in the solvent system toluene:ethyl acetate v/v (8:2) for petroleum ether extract, toluene:ethyl acetate v/v (7:3) for chloroform extract and toluene:ethyl acetate:formic acid v/v (7:3:0.1) for alcoholic extract.

Sample application and development of chromatogram

The samples (volume applied 6.0 µl) were spotted in the form of bands with a Hamilton syringe on precoated HPTLC aluminum silica gel GF-254 plates (20 cm × 10 cm) with the help of Linomat 5 applicator (CAMAG). Prepared plates were developed in previously saturated CAMAG twin trough chamber (20 cm ×10 cm) in linear ascending direction.

Detection of spots

The developed plates were dried by hot air to evaporate solvents from the plate. The developed plate was sprayed with 5% sulfuric acid in methanol as spray reagent and dried at 100°C in hot air oven for 3 min. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images under ultra violet light at 254 nm and 366 nm, scanning wavelength respectively. The retention factor (Rf) values and finger print data were recorded by WIN CATS software.

RESULTS

HPTLC fingerprinting of different extracts of *W. tinctoria* had been carried out by using various types of the solvent system for separation of as many as phytochemicals. Results revealed that the presence of several constituents in the extracts. The number of constituent (peaks) in the extract and their (Rf) are summarized in Tables 1-3 and chromatographic profile had been shown by Figures 1-4

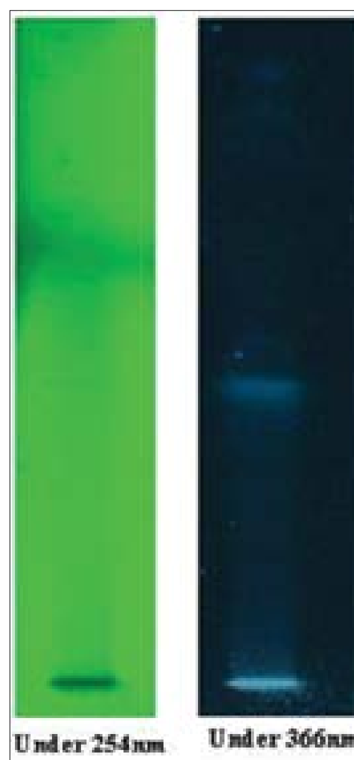

Figure 1: Chromatographic profiles of petroleum ether extract under 254 and 366 nm.

Table 1: Number of constituents (peaks) in the petroleum ether extract and their Rf

Track 2, ID: W (PET)										
Peak	Start position Rf	Start height AU	Max position Rf	Max height AU	Max %	End position Rf	End height AU	Area AU	Area %	Assigned substance
1	0.01	0.1	0.02	11.2	1.84	0.04	1.3	127.5	0.43	Unknown*
2	0.17	5.1	0.18	13.6	2.25	0.21	3.4	227.8	0.77	Unknown*
3	0.34	11.7	0.43	183.2	30.26	0.48	50.2	10878.7	36.94	Unknown*
4	0.55	24.7	0.58	41.0	6.78	0.58	37.0	1094.2	3.72	Unknown*
5	0.60	43.7	0.62	55.0	9.09	0.64	19.7	1737.1	5.90	Unknown*
6	0.69	54.0	0.71	62.3	10.30	0.72	59.8	1745.6	5.93	Unknown*
7	0.77	66.6	0.84	209.6	34.62	0.92	26.2	13148.0	44.64	Unknown*
8	0.97	27.5	0.97	29.3	4.85	1.00	2.8	492.3	1.67	Unknown*

Rf: Retention factor

Table 2: Number of constituents (peaks) in the chloroform extract and their Rf

Track 5, ID: W (CHLORO)										
Peak	Start position Rf	Start height AU	Max position Rf	Max height AU	Max	End position Rf	End height AU	Area AU	Area	Assigned substance
1	0.05	2.3	0.07	24.4	1.90	0.08	1.7	325.6	0.58	Unknown*
2	0.16	0.4	0.17	14.7	1.15	0.18	8.2	160.8	0.28	Unknown*
3	0.21	7.4	0.24	26.2	2.04	0.26	20.0	861.7	1.53	Unknown*
4	0.28	22.2	0.30	31.9	2.49	0.31	30.5	737.5	1.31	Unknown*
5	0.33	34.2	0.35	54.3	4.23	0.36	47.1	1319.5	2.34	Unknown*
6	0.40	65.2	0.49	171.4	13.34	0.51	37.1	10347.2	18.34	Unknown*
7	0.51	137.5	0.52	148.8	11.59	0.55	95.1	4009.8	7.11	Unknown*
8	0.55	95.4	0.59	177.9	13.85	0.62	36.8	8578.9	15.21	Unknown*
9	0.62	67.1	0.71	174.1	13.55	0.73	59.8	11529.2	20.44	Unknown*
10	0.73	159.9	0.73	165.9	12.92	0.76	92.6	4113.1	7.29	Unknown*
11	0.78	95.4	0.85	274.9	21.40	0.88	0.7	13946.6	24.72	Unknown*
12	0.89	1.8	0.93	20.0	1.55	0.94	7.2	484.2	0.86	Unknown*

Rf: Retention factor, CHLORO: Chloroform

Table 3: Number of constituents (peaks) in the alcoholic extract and their Rf

Track 4, ID: W (ALCO)										
Peak	Start position Rf	Start height AU	Max position Rf	Max height AU	Max %	End position Rf	End height AU	Area AU	Area %	Assigned substance
1	0.16	3.6	0.20	20.5	2.23	0.23	7.0	832.4	2.19	Unknown*
2	0.30	33.8	0.34	71.5	7.75	0.38	30.9	3457.6	9.09	Unknown*
3	0.39	30.0	0.41	43.9	4.77	0.42	40.2	1120.5	2.95	Unknown*
4	0.42	40.9	0.49	113.6	12.32	0.51	79.9	6424.8	16.89	Unknown*
5	0.52	80.1	0.52	87.3	9.47	0.55	32.2	2013.7	5.30	Unknown*
6	0.55	32.3	0.60	75.4	8.17	0.63	26.0	3536.8	9.30	Unknown*
7	0.63	26.2	0.67	65.5	7.10	0.68	64.1	2221.6	5.84	Unknown*
8	0.69	64.5	0.71	85.2	9.23	0.73	78.9	2646.2	6.96	Unknown*
9	0.73	79.5	0.73	86.8	9.41	0.76	70.2	2223.1	5.85	Unknown*
10	0.77	73.1	0.84	272.4	29.54	0.88	0.1	13551.4	35.64	Unknown*

Rf: Retention factor, ALCO: Alcoholic

shows HPTLC chromatogram of three different extracts. Petroleum ether extract (Figure 4a) chloroform extract (Figure 4b), ethanolic extract (Figure 4c).

DISCUSSION

Medicinal plant material is obtained from different heterogeneous sources which may lead to variation in therapeutic values and variation in phytochemistry. The HPTLC-fingerprinting profile is very important parameter of herbal drug standardization for the proper identification for medicinal plants. This parameter can also be very important tool if adulteration is suspected in medicinal plant material.[6]

CONCLUSION

The results indicated that the seed contain an appreciable amount of bioactive compounds. A novel method for HPTLC analysis of *W. tinctoria* has been presented

Figure 2: Chromatographic profiles of chloroform extract under 254 and 366 nm.

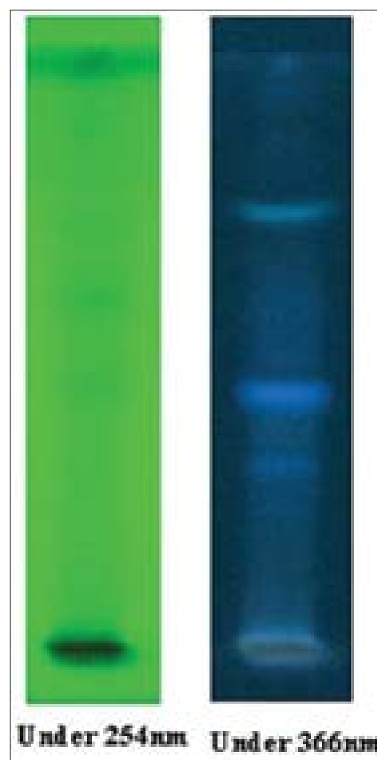

Figure 3: Chromatographic profiles of alcoholic extract under 254 and 366 nm.

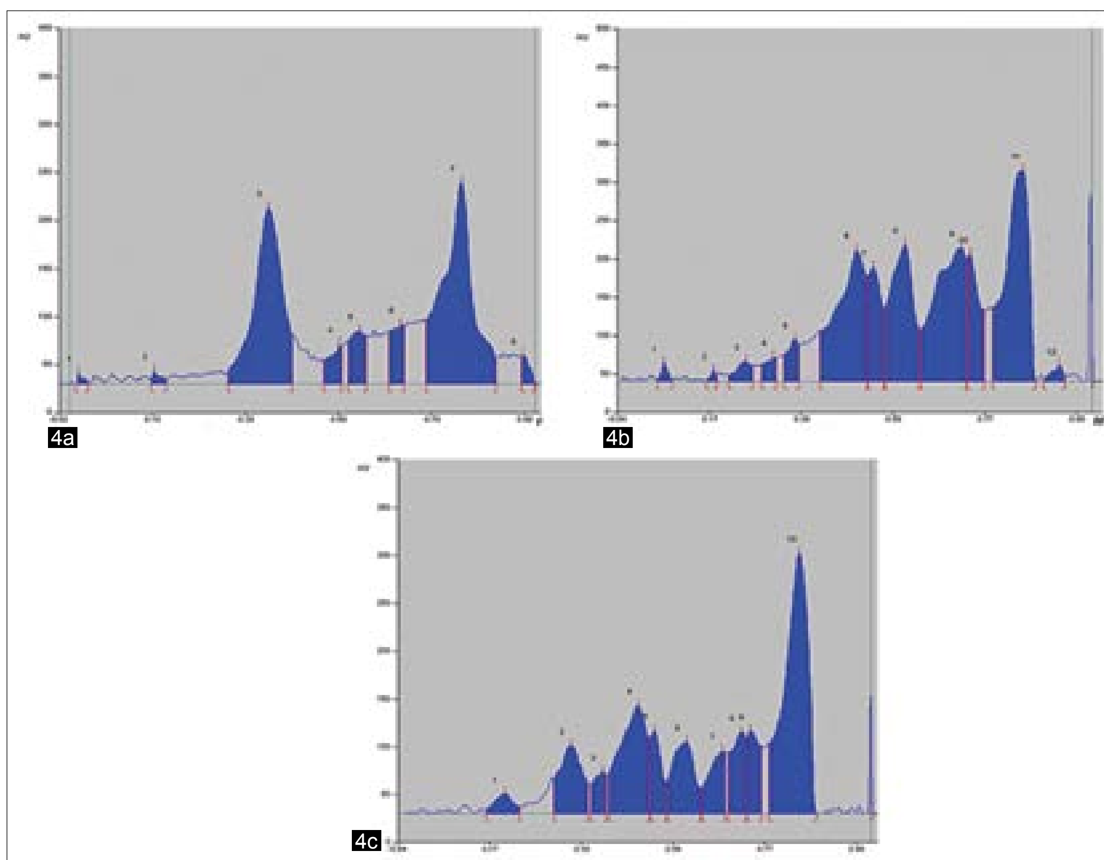

Figure 4: High-performance thin-layer chromatography (HPTLC) chromatogram of petroleum ether extract, (a) HPTLC chromatogram of chloroform extract, (b) HPTLC chromatogram of ethanolic extract (c).

along with results that show the presence of secondary metabolites such as steroids, terpenoids and glycosides in the ethanolic extract of plant material. The essences of these metabolites are beneficial for maintenance of human health and chronic degenerative diseases. The present HPTLC-fingerprinting profile can be used as a diagnostic tool for the identity and to determine the quality and purity of the plant material in future studies. Furthermore, the present study on *W. tinctoria* seed will help in identification and quantification of chemical marker compound.

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Pharmacognostic Study and Physicochemical Analysis of Leaves of *Terminalia arjuna*

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ABSTRACT

Objective: The aim of the present study was to carry out pharmacognostic and physicochemical analysis of *Terminalia arjuna* (TA) (Roxb.) Wight and Arn. (Family; *Combretaceae*) leaf. **Materials and Methods:** The present study deals with pharmacognostic characters as identification parameters of the leaves which were subjected to macro and microscopic studies. Phyto-physicochemical studies were done using WHO recommended parameters, and fluorescent behavior of the leaf sample was also tested. **Results:** The microscopy study revealed the presence of anomocytic stomata, trichome, xylem fibers, calcium oxalate crystals, vascular bundles, etc. Macroscopic study showed alternate thick-coriaceous base obtuse-subcordate while margin was crenate-serrate, obtuse or sub-acute at apex. Physiochemical parameters such as ash values, loss on drying, extractive values, fluorescence analysis were also determined. Preliminary phytochemical screening showed the presence of alkaloids, flavonoids, tannins, triterpenes, cardiac glycosides and saponins. **Conclusion:** The microscopic and physiochemical analysis of the *T. arjuna* leaf is useful in standardization for quality, purity, and sample identification.

Keywords: Leaf, *Terminalia arjuna,* pharmacognostic, physicochemical, phytochemical

INTRODUCTION

The Indian traditional system of medicine namely Ayurveda and Siddha are primarily plant based systems. The use of plants as medicines is dated back to early man. They constitute an effective source of traditional and modern medicines and play an important role in health care programs,[1,2] therefore, it becomes extremely important to make an effort toward standardization of the plant material as medicine. The process of standardization can be achieved by stepwise pharmacognostic studies. Pharmacognosy is an essential measure of authentication and quality. Macroscopical and microscopical studies are simplest and cheapest methods to start with, to establish the correct identification of the source materials.[3]

Terminalia arjuna (TA) Wight and Arn. is distributed throughout the greater part of India. The plant TA has a long history of medicinal uses primarily in the preparation of ayurvedic formulations for over three centuries.[4] Leaf has been reported for anti-cancer activity,[5] antihyperglycemic activity,[6] analgesic and anti-inflammatory,[7] antifungal and antibacterial activity.[8] The bark of the plant also possesses hypocholesterolanic effects,[9] antiherpes simplex virus Type 2,[10] useful drug for cardiovascular disorders[11] like coronary risk factors,[12] myocardial necrosis,[13] polyphenols that includes flavonols, flavones and phenyl propanoids are also useful in curing cancer.[14] Phytochemical investigations of the plant revealed the presence of many bioactive compounds like arjunolic acid,[15,16] arjunic acid,[17] arjunoglycoside,[18] ellagic acid,[19] arjunetoside.[20] However, no systematic study was carried out on this plant leaves. Hence, the objective of the present work was to evaluate various pharmacognostic properties including macro and microscopic and physicochemical characterization of the leaves of the TA.

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MATERIALS AND METHODS

Plant collection

The leaves of TA Wight and Arn. (*Combretaceae*) were collected from Rajkot, Gujarat, India in December, 2012. They were separated, washed thoroughly with tap water and

distilled water. They were shade-dried, uniformly powdered and packed in air tight bottles.

Pharmacognosy and phytocochemical study of TA

Macroscopic study

An organoleptic and external morphological character of freshly collected leaves was observed under magnifying lens.[21]

Microscopic study

Microscopic studies were done by preparing a thin hand section of leaf. Free hand cross transverse sections of leaf were taken and stained with safranine to confirm its lignifications. Coarse powder was used to study microscopical characters of leaf powder.

Fluorescence analysis

Fluorescence study of leaf powder was performed as per reported procedure.[22] A small quantity of the powder was placed on a grease free clean microscopic slide, and 1-2 drops of the freshly prepared reagent solution were added, mixed by gentle tilting the slide and waited for 1-2 min. Then the slide was kept inside the ultraviolet (UV) cabinet and observed in visible light, short (254 nm), and long (365 nm) ultraviolet radiations.

Physicochemical study

In this study, air-dried leaf powder was used for determination of physicochemical parameters like total ash, water soluble ash, acid insoluble ash and extractive value, solubility, etc., were determined as per WHO guidelines.[23]

Statistical analysis

All experiments were repeated at least three times. Results are reported as mean ± standard error of the mean.

RESULTS

Macroscopic characteristics

TA leaves were 15-25 cm in length and 6-7.5 cm in width. The leaves were simple, alternate thick-coriaceous, base obtuse-subcordate. Margin was crenate-serrate, apex obtuse or sub-acute, pale green above, pale brown beneath; shallowly crenate-serrate, petiole was 0.6-0.9 cm long. Oil gland was observed at abaxial side of the leaf near petiole (Figure 1).

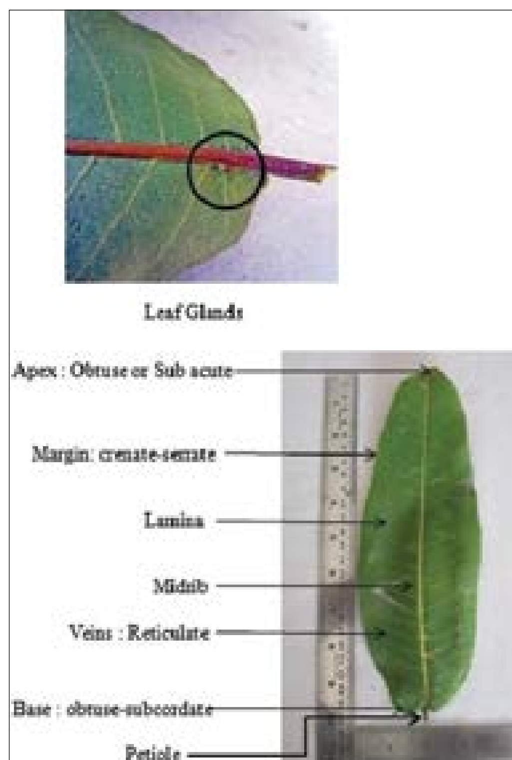

Figure 1: Macroscopic characteristics of *Terminalia arjuna* leaf.

Microscopic characteristics

Leaf microscopic

Transverse section of leaf passing through midrib showed a single layer of the epidermis on both surface and was covered with cuticle. Upper and lower epidermis bears unicellular glandular and non-glandular trichomes. Abundant cluster crystals of calcium oxalate were present in parenchymatous tissue. In the midrib region, several layers of thick walled collenchymatous and thin walled parenchymatous tissues surround the central vascular bundle which is open, bicollateral type. Xylem was lignified while phloem was nonlignified (Figures 2 and 3).

Powder microscopic study

Leaf

The leaf powder was greenish in color, cluster crystals, rosette crystals, and square-shaped calcium oxalate crystals were present; trichomes with unicellular covering were present, anomocytic stomata were present (Figure 4).

Physicochemical analysis

Physicochemical parameters of powder of TA leaf are shown in Table 1. Studies of physicochemical constants

can serve as a valuable source of information and are usually used in judging the purity and quality of drug. The moisture content of dry powder of leaves was 7.05% which seems to be lower than that necessary to support the growth of microbes such as bacteria, fungal, and yeast to bring any change in the composition of the drugs. In physicochemical parameters, ash value was determined in three forms such as total ash, water soluble ash, and acid insoluble ash. The total ash was 10.15% while water soluble ash and acid insoluble ash was 1.5% and 0.5%, respectively (Table 2).

The extractive values give an idea about chemical constitution of drug. The extractive value of TA leaf is shown in Table 2. The maximum extractive value was in methanol solvent and minimum was in petroleum ether. The methanol extract of TA leaf was evaluated for its solubility in 10 solvents with varied polarities (Table 3).

Phytochemical analysis

The results of qualitative phytochemical analysis of the crude powder of TA leaf are shown in Table 4.

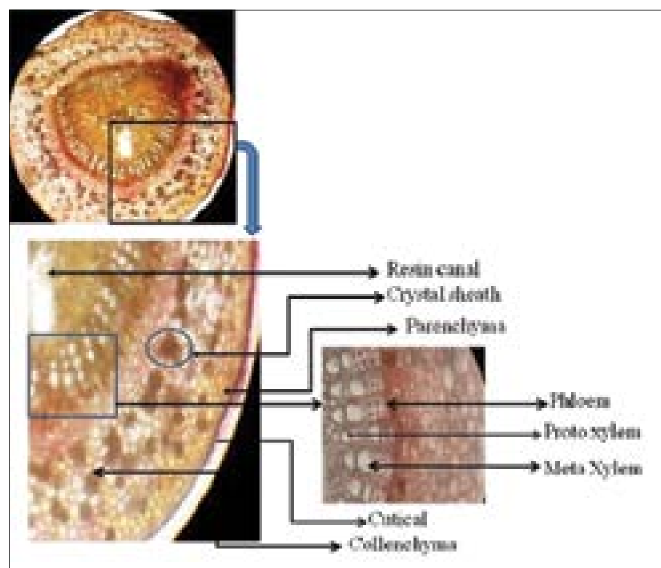

Figure 2: Photomicrograph of microscopic characteristics of *Terminalia arjuna* leaf.

Fluorescence analysis

The fluorescent analysis under day light and UV light by treatment with different chemical reagents and solvents

Table 1: Fluorescence analysis of *T. arjuna* leaf powder

Extractives	Visible light	Short UV	Long UV
Petroleum ether	Dark green	Black	Light green
Ethyl acetate	Yellowish green	Black	Green
Methanol	Green	Black	Light green
Ethyl alcohol	Green	Black	Dark green
Aqueous NaOH	Yellowish brown	Black	Light green
Alcoholic NaOH	Light green	Black	Dark green
H_2SO_4 50%	Dark green	Black	Dark green
HCl 50%	Light green	Black	Dark green
Picric acid	Yellowish green	Black	Yellowish green
Ammonia	Yellowish green	Black	Dark green

UV: Ultraviolet

Table 2: Physicochemical parameters of *T. arjuna* leaf (% W/W)

Parameters	Leaf
Loss on drying	7.05±0.05
Total ash	10.15±0.33
Water soluble ash	1.5±0.16
Acid insoluble ash	0.5±0.16
Petroleum ether extractive value	1.768±0.008
Ethyl acetate extractive value	3.54±0.02
Methanol extractive value	17.58±0.44
Acetone extractive value	3.72±0.07
Water soluble extractive value	14.54±0.20

T. arjuna: Terminalia arjuna

Table 3: Solubility of *T. arjuna* leaf methanol extract in different solvents

Solvent	Solubility (mg/ml)
Non polar solvent	
Petroleum ether	4
Hexane	0
Chloroform	6
Toluene	2
Ethyl acetate	8
Polar solvent	
Acetone	130
Methanol	210
DMSO	290
DMF	245
Water	315

DMSO: Dimethyl sulfoxide, DMF: Dimethyl formamide

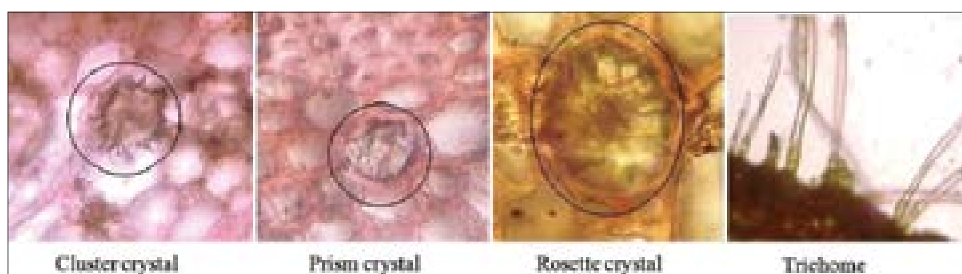

Figure 3: Photomicrograph of microscopic characteristics of *Terminalia arjuna* leaf.

Table 4: Qualitative phytochemical analysis of *Terminalia arjuna* leaf

Phytochemicals	Test	Crude powder
Alkaloids	Dragendroff's test	+
	Mayer's test	+
	Wagner's test	+
Flavonoids	Alkaline reagent	+++
Tannins	$FeCl_3$	++
Phlobatanins	HCl test	-
Triterpenes	H_2SO_4 test	+++
Steroids	Liebermann-Burchard test	-
Saponins	Frothing test	+
Cardiac glycosides	Keller-Kilianni test	++

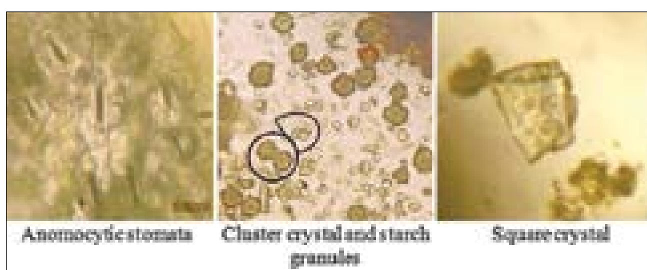

Figure 4: Photomicrograph of microscopic characteristics of *Terminalia arjuna* leaf powder.

showed a different color. Fluorescence is an important phenomenon exhibited by various chemical constituents which show fluorescence in the visible range in day light. The UV light produces fluorescence in many natural products which do not visibly fluorescence in day light. Result of fluorescent analysis of the leaves showed a different color in visible light and UV light when mounted in different solvent and chemical reagent are summarized in Table 1.

DISCUSSION

The standardization of a crude drug is an integral part for establishing its correct identity. Before any crude drug can be included in an herbal pharmacopeia, pharmacognostic parameters, and standards must be established. The morphological characters of leaf can serve as diagnostic parameters.[24] Microscopic evaluation is one of the simplest and cheapest methods for establishing the correct identification of the source of the materials.[25] Total ash values and extractive values are useful in identification and authentication of the plant material.[26,27] Ash values of drug give an idea of earthy matter or the inorganic composition and other impurities present along with drug.[28,29] Extractive values are useful to evaluate the chemical constituents of crude drug.[29]

Some of the diagnostic features of the leaf drug noted from the microscopical study are anomocytic stomata, trichome; maximum extractive value was in methanol. Preliminary phytochemical study showed that the presence of flavonoids, triterpenes, tannins, and cardiac glycosides. The details of pharmacognostic characters, various evaluative parameters, results of preliminary and detailed phytochemical analysis established in the present study will facilitate in identifying the genuine drug and will also be useful in preparation of monographs of leaf of this plant. Fluorescence study of the leaf powder helps in the qualitative evaluation which can be used as a reference data for the identification of adulterations. Pharmacognostic studies on different plants like *Polyalthia longifolia*,[30] *Cissus quadrangularis*,[31] *Woodfordia fruticosa*,[32] *Psidum guajava*,[33] *Mangifera indica*,[34] *Syzygium cumini*[35] are also reported. In conclusion, this study could be used as a diagnostic tool for the standardization of this medicinal plant and will be helpful in the characterization of the crude drug.

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Pharmacognostical Studies of *Bryophyllum pinnatum* (Lam.) Kurz.

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ABSTRACT

Context: *Bryophyllum pinnatum* (Lam.) Kurz. widely used in traditional as well as folk medicinal systems are locally known as Panphuti. Traditionally, it is used for the treatment of kidney stones, urinary tract infection, burns and diarrhoea. **Aims**: In the present study, pharmacognostic studies of root, stem, and leaf of *B. pinnatum* (Lam.) Kurz. is carried out in order to standardize the plant for its phytochemical, phyiso-chemical and pharmacognostical. **Materials and Methods:** For standardization of plant material morphological and anatomical characterization was carried out. Physico-chemical parameters viz. ash content, extractive values, heavy metal content was carried out as per Ayurvedic Pharmacopoeia of India. Phytochemical investigations were made to know the presence of various bioactive molecules, amino acid composition. **Results:** Intra-stelar and extra-stelar secondary growth with wood and periderm formation along with deposition of starch grains were observed in the pith region of the root and cortical region of the stem. Calcium oxalate crystals were also present in the cortical region of the stem. Leaf lamina showed spongy parenchyma in mesophyll region and anisocytic type of stomata. Anthocyanin pigment was present below epidermal cells in petiole. Physico-chemical results can be serves as quality control data. Quantitatively carbohydrate, protein, flavonoids, phenolic compounds, saponins and pro-antocyanidins were found to be present in the root, stem and leaf part of *B. pinnatum* (Lam.) Kurz. **Conclusion:** The results of the study could be useful in setting some diagnostic indices for the identification and preparation of a monograph of the plant.

Keywords: *Bryophyllum pinnatum (Lam.) Kurz.,* HPTLC, pharmacognosy, physico-chemical, phytochemical

INTRODUCTION

Herbal materials are categorized according to sensory, macroscopic and microscopic characteristics. An examination to determine these characteristics is the first step towards establishing the identity and the degree of purity of such materials. Wherever possible, authentic specimens of the material to be identified and samples of pharmacopoeial quality should be available to serve as a reference. Visual inspection provides the simplest and quickest method to establish identity, purity and quality. If a sample is found to be considerably different from the specifications in terms of color, consistency, odor or taste, it is considered as not fulfilling the requirements. Macroscopic identification of herbal materials is based on shape, size, color, surface characteristics, texture, fracture characteristics and appearance of the cut surface.[1]

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Bryophyllum pinnatum (Lam.) Kurz. (*Crassulaceae*) is a perennial herb growing widely and used in folkloric medicine in tropical Africa, tropical America, India, China, and Australia. The divine herb contains a wide range of active compounds, including alkaloids, triterpenes, glycosides, flavonoid, steroids, bufadienolides, lipids and organic acids, have been isolated from this species. The plant is widely used in traditional medicine for the treatment of a variety of ailments and well known for its haemostatic and wound healing properties. The plant have been found to possess pharmacological activities as immunomodulator, central nervous system depressant, analgesic, antimicrobial, anti-inflammatory, antiallergic, antianaphylactic, antileishmanial, antitumorous, antiulcer, antibacterial, antifungal, antihistamine, antiviral, febrifuge, gastroprotective, immunosuppressive, insecticidal, muscle relaxant, sedative.[2]

Literature survey revealed that there is no work on pharmacognostic studies of root, stem and leaf of *B. pinnatum* (Lam.) Kurz. Therefore, in the present work, pharmacognostic studies on root, stem and leaf (fresh material and powder) of *B. pinnatum* (Lam.) Kurz. was

carried out, which may be useful for proper identification and authentication of crude drug.

MATERIALS AND METHODS

Plant material

The plants of *B. pinnatum* (Lam.) Kurz. were obtained from Pathare Nursery, Kalyan and grown in the Botanical Garden, Birla College, Kalyan. The plant was identified and authenticated from Blatter Herbarium, St. Xavier's College, Mumbai. The shade dried material of root, stem, leaf and whole plant were separately cut into small pieces and powdered using mixer grinder. The powdered materials were stored separately in labeled air tight bottles.

Anatomical studies

Morphological features, odor, color and taste of root, stem and leaf were studied. Transverse sections of root, stem, and leaf were taken, stained with safranin and mounted in glycerin. Semi-permanent slides were prepared and observed under compound microscope. Photographs of the sections were taken under ×10 and ×45 magnifications using Nikon camera.[3]

Powder characteristics of root, stem and leaf were studied by staining powder in safranin and observed under compound microscope at ×10 and ×45 magnifications. Histochemical studies, sections of root, stem and leaf were stained with different chemical reagents for the localization of alkaloids, phytosterols, lignins, calcium oxalate crystals and tannins.

Physico-chemical analysis

Root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. were used to study foreign organic matter, loss on drying, total ash content, acid insoluble ash, water soluble ash, alcohol and water soluble extractives and heavy metal content. The above parameters were studied as per standard method of Ayurvedic Pharmacopoeia of India guidelines.[3]

Qualitative phytochemical analysis

Petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of root, stem, leaf and whole plant were tested for the presence of alkaloids by Dragendroffs, Mayers and Wagners reagent; anthraquinones by alcoholic potassium hydroxide and amino acids by ninhydrin reagent,[4] Carbohydrates by Molich, Benedict, iodine and phenol-sulfuric acid reagent,[5] proteins by Lowry method,[6] glycosides by sulfuric acid method, saponins by foam test and flavonoids by sodium hydroxide, lead acetate reagent,[7] phenolic compounds and tannins by ferric chloride reagent,[4] cardiac glycosides by Killer–Kilani reagent, terpenoids by Salwoskii and sterols by Libermann–Burchard reagent.[8]

Quantitative estimation of phytochemical

Root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. were quantitatively estimated for total carbohydrate content by anthrone method,[5] total protein content by Lowry method,[6] total phenolic content by Folin–Ciocalteu method,[9] total flavonoid content by aluminium chloride method,[10] proantocyanidin content by vanillin-hydrochloric acid method[11] and total saponin content by vanillin-sulfuric acid method.[12]

Methanolic extracts of the root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. were separated on silica gel G F_{254} pre-coated plates by using following solvent systems for detection of anthraquinone:ethyl acetate:methanol:distilled water (10:1.5:1); terpenoid:toluene:ethyl acetate:methanol (7:2:1), derivatizing reagent used was Lieberman–Burchard; phenolic compounds:toluene:ethyl acetate:formic acid (5:4:1). Derivatizing reagent used was 5% ethanolic ferric chloride; flavonoids:ethyl acetate:formic acid:glacial acetic acid:distilled water (10:1:1:1) and saponin:chloroform:glacial acetic acid:methanol:distilled water (7:2:0.5:0.5).

RESULTS

Macroscopical studies

The roots were simple, tap root, greenish brown in color when young and light brown when old. Root was 7-10 cm in length (Figure 1). Root powder had a pleasant odor and was sweet in taste. Stem of *B. pinnatum* (Lam.) Kurz. was light green in color when young and light brown in color when old (Figure 1). Old stem was rough and had lenticels on surface. Stem powder had a pleasant odor and slightly bitter in taste. The leaves were opposite, decussate, succulent, 10-20 cm in length. The lower leaf were simple, whereas, the upper leaf is 3-4 foliate with long petiole with dark green in color and fleshy, which are distinctively scalloped and trimmed in red. The leaves are furnished with rooted vegetative buds, and leaf apex is obtuse. Petiole was 2-4 cm in length; leaflet blades were oblong to elliptic, margin crenate with each notch bearing a dormant bud competent to develop into a healthy plantlet (Figure 1).

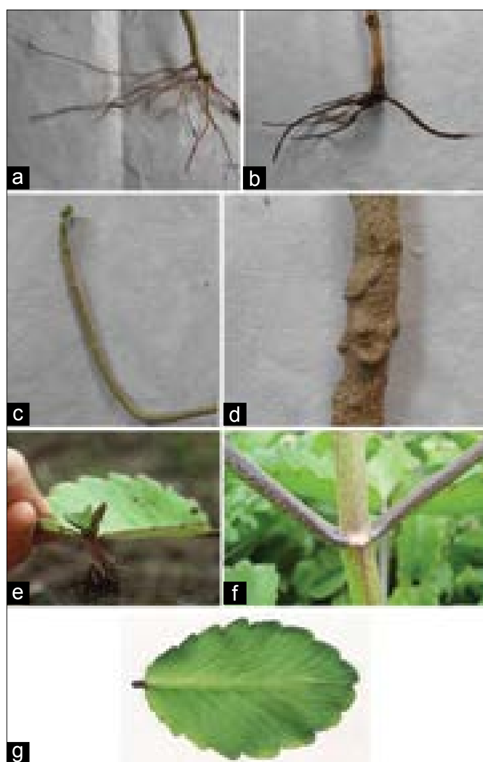

Figure 1: Macroscopical characters of root stem and leaf of *Bryophyllum pinnatum* (Lam.) Kurz. a: Young root; b: Old root; c: Young stem; d: Old stem; e: Leaf with new rooted vegetative bud; f: Purple colored glabrous petiole and g: Crenate shaped leaf.

Microscopical characters of young root

Young root was circular in outline and showed outer epiblema, followed by outer and inner cortex. Outer cortex was 3-4 layered thick walled made up of sclerenchymatous cells known as exodermis. Inner cortex was thin walled made up of parenchymatous cells with deposition of starch grains. Stelar region showed presence of vascular tissue (xylem and phloem). Metaxylem was prominent towards center surrounded by protoxylem toward periphery. Parenchymatous pith occupied the central portion of the root section (siphonostele) (Figure 2).

Microscopical characters of the old root

Old root was circular in outline with prominent secondary growth in extra and intra stelar region. Extrastelar secondary growth showed the presence of periderm, which get differentiated into phellem, phellogen and phelloderm. Parenchymatous cells of secondary cortex or phelloderm showed the deposition of starch grains. In the intra-stelar growth secondary xylem (wood) occupied the major portion in the form of ring. Primary xylem and phloem get pushed towards the center surrounding the pith. Secondary xylem chiefly made up of tracheids along with fibers, xylem

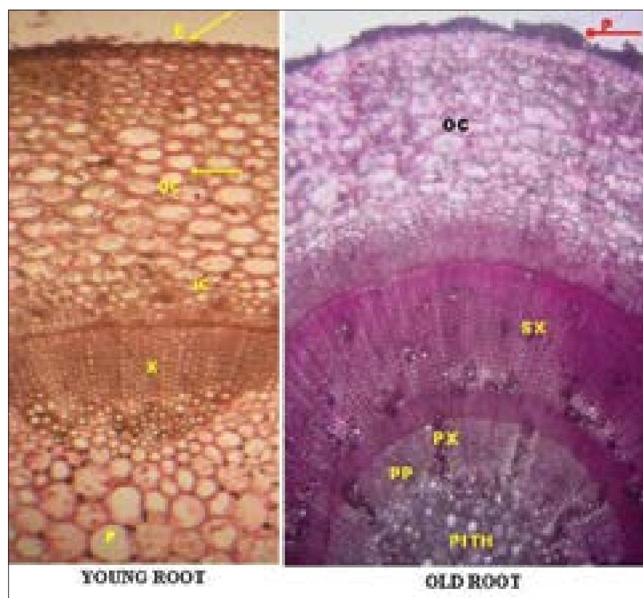

Figure 2: Transverse section of young and old root of *Bryophyllum pinnatum* (Lam.) Kurz. Young root-E: Epiblema; OC: Outer cortical region; IC: Inner cortex; X: Xylem; and P: Pith. Old root-P: Periderm; OC: Outer secondary cortical region; SX: Secondary xylem; PP: Primary phloem; PX: Primary xylem and PITH: Pith.

parenchyma and few vessels. Pith showed deposition of starch grains (Figure 2).

Microscopical characters of young stem

Young stem was circular in outline and showed outer layer of thick walled epidermis with cuticle. Beneath the epidermis 3-4 layered hypodermis made up of sclerenchymatous cells was observed. Inner cortex was thin walled parenchymatous, loosely arranged with deposition of starch grains. Endodermis was not prominent. In stelar region, vascular bundles were arranged in a ring. Each vascular bundle was conjoint, collateral and open. Xylem elements were mainly in the form of tracheids, xylem parenchyma fibers with few vessels. Parenchymatous pith in the center showed the deposition of starch grains (Figure 3).

Microscopical characters of old stem

Old stem was wavy in outline and showed both extra-stelar and intra-stelar secondary growth. Extra-stelar secondary growth gave rise to periderm composed of phellum, phellogen and phelloderm forming a bark. While, intra-stelar secondary growth showed development of a broad region of secondary xylem (wood). Secondary xylem was made up of pith, which was broad made up of thin walled parenchyma cells with depositions of starch grain and calcium oxalate crystals (Figure 4).

Microscopical characters of leaf

Leaf of *B. pinnatum* (Lam.) Kurz. showed upper and lower epidermis with cuticle. Midrib region was broad with distinct upper and lower epidermis. The cells between upper and lower epidermis were homogenous and parenchymatous deposited with starch grains and chlorophyll with two vascular bundles found in the center. Each vascular strand was conjoint, collateral with xylem facing toward the upper side. The mesophyll region of the lamina was homogenous and chlorenchymatous and showed spongy parenchyma. Lamina showed distinct upper and lower epidermis with presence of anisocytic type of stomata specifically on the lower epidermis (Figure 5).

Figure 3: Transverse section of stem (young) of *Bryophyllum pinnatum* (Lam.) Kurz. Epi: Epidermis; Cor: Cortical region; Phl: Phloem; Xyl: Xylem and Pith: Pith.

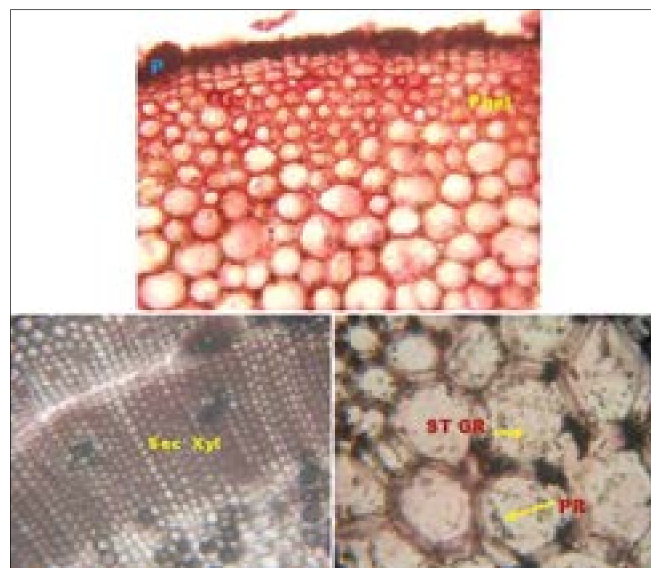

Figure 4: Transverse section of old stem of *Bryophyllum pinnatum* (Lam.) Kurz. P: Phellum (Cork); Phel: Phelloderm (secondary cortex); Sec XYL: Secondary xylem; PR CRS: Prismatic crystal and ST GR: Starch grain.

Microscopical characters of petiole

Petiole of *B. pinnatum* (Lam.) Kurz. was circular in outline with single layered outer cuticularized epidermis. Beneath the epidermis was a broad region of ground tissue made up, loosely arranged thin walled parenchymatous cells with chlorophyll, starch grains and calcium oxalate crystals. Outermost few cells of ground tissue toward epidermis showed deposition of pink colored anthocyanin pigments, which turned to purple color after staining with safranine. Vascular tissue was grouped together to form crescent-shaped structure with phloem surrounding xylem on one side (Figure 6).

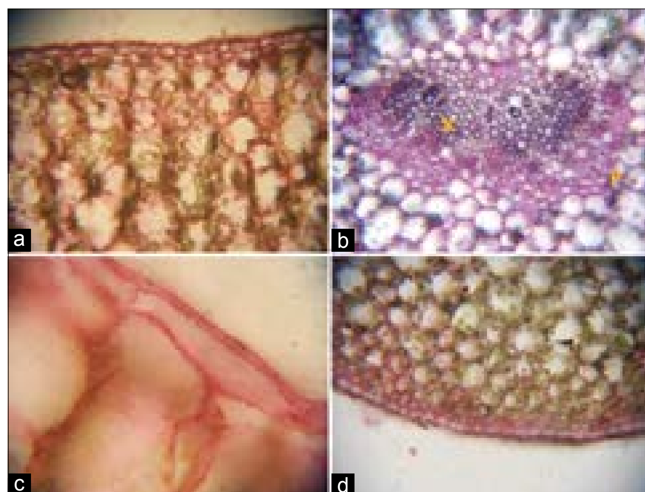

Figure 5: Transverse section (T.S.) of leaf of *Bryophyllum pinnatum* (Lam.) Kurz. a: T.S. of midrib with upper epidermis; b: T.S. of leaf midrib showing vascular bundle – X: Xylem; P: Phloem; c: Cuticularized epidermis; d: Midrib showing lower epddidermis.

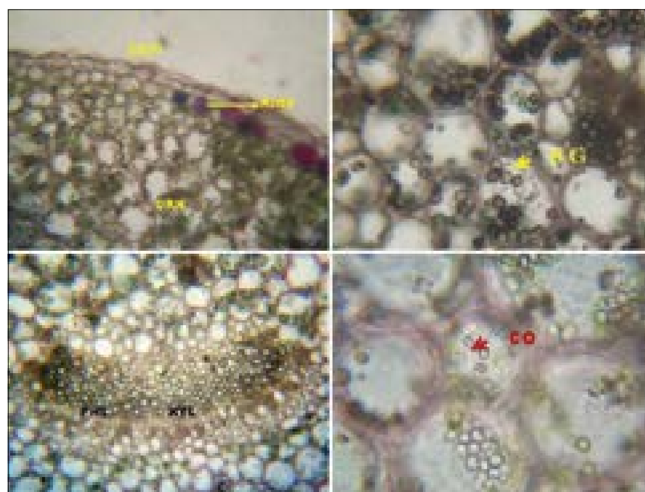

Figure 6: Transverse section of petiole of *Bryophyllum pinnatum* (Lam.) Kurz. CEPI: Cuticularized epidermis; Anto: Anthocyanin pigment; GRN: Ground tissue; PHL: Phleom, XYL: Xylem starch grain and CO: Calcium oxalate crystal.

Powder of root, stem and leaf was stained with safranine and microscopic observation was carried out. Powder microscopy of root showed presence of cork cells, fiber, calcium oxalate crystals and xylem vessels with annular thickening. Stem showed trachieds with simple pits on the lateral wall, vessels with spiral wall thickening, xylem parenchyma with profused deposition of starch grains, and fibers. Leaf showed presence of prismatic oxalate crystals, starch grains, stomata with epidermal cell and pitted vessels annular wall thickening.

Physico-chemical analysis

Plant materials free from visible signs of contamination by molds or insects and other animal contaminants were collected. It was without abnormal odor, discoloration, etc. Thus, the plant material was free from any foreign organic matter.

Root, stem, leaf and whole plant powder of *B. pinnatum* (Lam.) Kurz. were studied to find out total ash, acid insoluble ash and water soluble ash content. Total ash content in the root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. was 10.30 ± 0.30%, 13.50 ± 0.43%, 13.26 ± 1.01% and 11.03 ± 0.32% respectively. Root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. showed 0.86 ± 0.05%, 0.93 ± 0.11%, 1.73 ± 0.05% and 1.03 ± 0.11% of acid insoluble ash content respectively. Water soluble ash content in the root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. was 1.30 ± 0.26%, 2.53 ± 0.25%, 2.13 ± 0.15% and 0.96 ± 0.11% respectively (Table 1).

Extractive value for the root, stem, leaf and whole plant powder of *B. pinnatum* (Lam.) Kurz was estimated by using ethanol and distilled water as solvents. Ethanol soluble extractive value for the root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. was 6.48 ± 0.08%, 5.49 ± 0.44%, 10.18 ± 0.12% and 23.58 ± 0.41% respectively (Table 1), whereas water soluble extractive value for root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. was 18.50 ± 0.44%, 11.70 ± 0.16%, 22.53 ± 0.12% and 29.30 ± 0.20% respectively (Table 1).

Loss on drying was found to be 80.06 ± 0.75%, 77.66 ± 0.83%, 91.46 ± 0.80% and 79.00 ± 0.60% in root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. respectively (Table 1).

Arsenic content in root, stem and leaf of *B. pinnatum* (Lam.) Kurz. was 0.712, 1.876 and 0.516 ppm, respectively (Table 2). However metals like cadmium and mercury were absent in root, stem and leaf of *B. pinnatum* (Lam.) Kurz. Lead content in root, stem and leaf of *B. pinnatum* (Lam.) Kurz. was 0.045, 0.089 and 0.093 ppm, respectively (Table 1).

Phytochemical analysis

Preliminary phytochemical analysis showed the presence of terpenoids, glycosides, anthraquinones, flavonoids, sterols, proteins, amino acids and carbohydrates in different extracts of the root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. Preliminary phytochemical evaluation may help further in the estimation of the phytoconstituents quantitatively from different parts of *B. pinnatum* (Lam.) Kurz.

Quantitative phytochemical estimation by standard methods showed a higher content of carbohydrates and phenolic compounds in root. Proteins, flavonoids, saponins, pro-anthocyanidins were found to be higher in leaf of *B. pinnatum* (Lam.) Kurz. (Table 2).

Phytochemicals present in the root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. were extracted and separated using thin layer chromatography (TLC). The different phytoconstituents were identified by phytochemical analysis. The R_f values obtained can be

Table 1: Physico-chemical analysis of *B. pinnatum* (Lam.) Kurz.

Parameters	Root	Stem	Leaf
Foreign organic matter	-	-	-
Percentage loss on drying	80.06±0.75	77.66±0.83	91.46±0.80
Percentage total ash	10.03±0.30	13.50±0.43	13.76±1.01
Percentage acid insoluble ash	0.86±0.05	0.93±0.11	1.73±0.05
Percentage water soluble ash	1.30±0.26	2.53±0.25	2.13±0.15
Percentage alcohol soluble extractive	6.48±0.08	5.49±0.44	10.18±0.12
Percentage water soluble extractive	18.50±0.44	11.70±0.16	22.53±0.12
Lead	0.045	0.089	0.093
Arsenic	0.712	1.876	0.516
Cadmium	ND	ND	ND
Mercury	ND	ND	ND

-: Not present, ND: Not detected, *B. pinnatum: Bryophyllum pinnatum*

Table 2: Quantitative estimation of phytochemicals from *B. pinnatum* (Lam.) Kurz.

	Content in mg/100 g*		
Phytoconstituents	Root	Stem	Leaf
Carbohydrate	87.44±1.90	78.55±1.92	54.10±1.90
Proteins	17.16±0.28	47.16±0.57	73.88±0.28
Total phenolics	106.22±0.38	-	12.88±0.38
Total flavonoids	59.13±1.15	33.13±0.23	89.13±1.15
Total saponins	22.26±1.15	18.26±1.15	40.93±1.15
Total proanthocyanidin	41.83±1.44	21.83±1.44	46.83±1.44

*Values are mean of three determinants, *B. pinnatum: Bryophyllum pinnatum*

used as a reference to check the quality of the plant during bulk collection. TLC method developed in the present work is reliable, fast and economic. The fingerprint pattern obtained by TLC was distinctive for different phytoconstituents present in *B. pinnatum* (Lam.) Kurz. (Figure 7).

DISCUSSION

If the monograph of plants is given or mentioned in pharmacopoeia, it provides the simplest and quickest means of establishing the identity and purity of herbal raw material. Microscopic analysis is based on observation of specific microscopic characteristics. The anatomical characters can also be used to identify the spurious drugs from the original one.

In the present work, macroscopic characters of root, stem and leaf were studied, which may help to identify the plant by its external morphology. Microscopic section of root showed broad region of secondary xylem, stem showed development of periderm after secondary growth. Leaf showed anisocytic type of stomata, presence of starch grains. Presence of anthocyanin pigment and prismatic crystals was seen in petiole. These anatomical characters can be used for proper identification of *B. pinnatum* (Lam.) Kurz.

The major problem faced in the herbal industry is the identification of authenticated raw material and in the absence of data one can use adulterant in the drug formulation.[13] The detailed systematic pharmacognostical evaluation of plant and plant material provides means of standardization of a herb that can be used as a drug or as raw material.[14]

The anatomical characters studied can be used for proper identification and will avoid the use of the adulterant of plant raw material to be used in herbal pharmaceutical industries. Data obtained from macroscopic and microscopic studies may be considered as a distinguishing parameter to identify and decide the authenticity of the plant material, and this can be included as pharmacognostic standards in pharmacopoeia.

As per the WHO guidelines, the quality control of medicinal or herbal plant is mandatory before using for consumption.[15] The physico-chemical parameters will help in judging the purity and quality of the herbal plants.

From the present study it was observed that foreign organic matters were absent in the root, stem and leaf of *B. pinnatum* (Lam.) Kurz. this may be due to collection of plant material from non-polluted area. Ash content was less in root, stem, leaf and whole plant powder of *B. pinnatum* (Lam.) Kurz. due to low content of carbonates, phosphates, silicates and silica. The results showed that the root, stem, leaf and whole plant of *B. pinnatum* (Lam.) Kurz. showed higher water soluble extractive value in comparison with the alcohol extractive values. It indicates the presence of a considerable amount of polar compounds and large quantity of water soluble constituents such as sugar, glycosides, phenolic and tannins.

Amongst the four heavy metals analyzed, cadmium and mercury were absent in the root, stem and leaf of *B. pinnatum* (Lam.) Kurz. Arsenic and lead were in permissible limits in root, stem and leaf of *B. pinnatum* (Lam.) Kurz.[16] Data obtained from physico-chemical parameters *viz.* ash value, extractive value, loss on drying and heavy metal content will be useful to check the quality and to confirm the authenticity of *B. pinnatum* (Lam.) Kurz.

Phytochemicals, generally have a wide range of pharmacological activities or actions.[8] Most of these

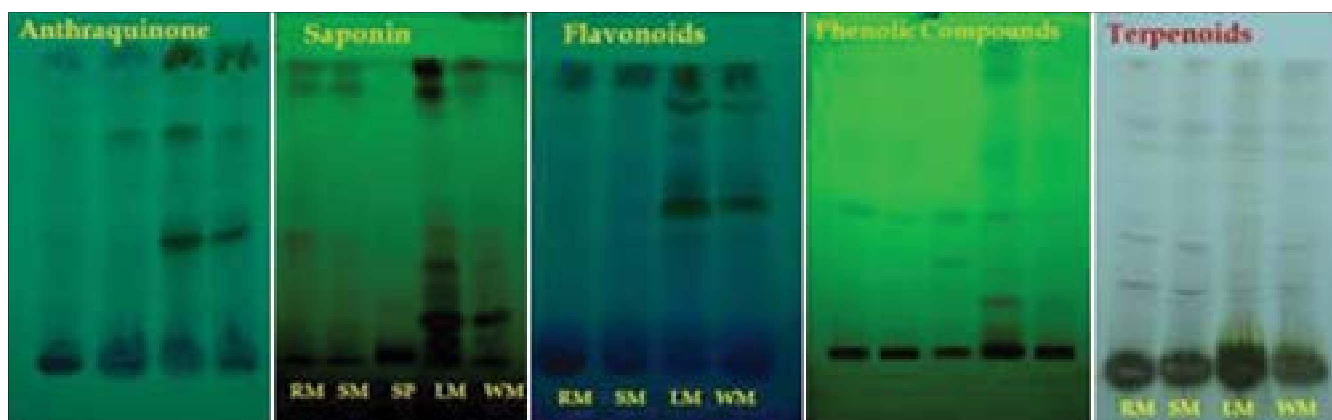

Figure 7: Thin layer chromatography fingerprints of phytochemicals from *Bryophyllum pinnatum* (Lam.) Kurz. RM: Methanolic extract of root, SM: Methanolic extract of stem, LM: Methanolic extract of leaf and WM: Methanolic extract of whole plant.

phytochemical constituents are potent bioactive compounds found in medicinal plant parts which are precursors for the synthesis of useful drugs.[7] Natural products are of great significance to man, fulfilling the roles of medicines, stimulants, perfumes, spices, antimicrobial agents, hallucinogens and as components of industrial products. Phytoconstituents have been known to possess various health benefits *viz*. antimicrobial, anti-inflammatory, cancer preventive, anti-diabetic and antihypertensive effects. Phytochemical analysis can be an important path of information for selection of active constituents in pharmacological studies as secondary metabolites are responsible for various biological activities.

CONCLUSION

The present study confirms the presence of bioactive constituents such as flavonoid, terpenoid and mucilage in root, stem leaf and whole plant of *B. pinnatum* (Lam.) Kurz. Further studies are required to be carried out for; *in vivo* pharmacokinetic evaluation and assess the bioavailability of *B. pinnatum* (Lam.) Kurz.; efficacy study, which may provide the necessary evidence for rational use of the plant as potent herbal medicine.

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Chemical Constituents with Free-Radical-Scavenging Activity from the Leaves of *Lantana montevidensis* (Spreng.) Briq.

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ABSTRACT

Background: Seventy percent aqueous methanolic extract of the leaves of *Lantana montevidensis* exhibited antibacterial, anti-inflammatory, anti-pyretic, antioxidant, and analgesic activities. Previous phytochemical study of the leaves led to the isolation of various flavones, pentacyclic triterpenoids, β-sitosterol and benzoic acid. **Materials and Methods:** The 70% aqueous methanolic extract of *L. montevidensis* (Spreng.) Briq. leaves was partitioned between water and *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, successively. By using a series of different chromatographic techniques, the $CHCl_3$ and EtOAc extracts afforded nine compounds. The isolated compounds were identified by spectroscopic techniques and by comparison with literature values and/or authentic samples. The free radical scavenging activity of some of the isolated compounds was evaluated. **Results:** Hispidulin-7-*O*-glucurnoide methyl ester (1), hispidulin-7-*O*-glucoside (2), cinaroside (3), nepetin-7-*O*-glucoside (4) pectolinarigenin (5), apigenin (6), luteolin (7) in addition to salicylic acid (8) and β-sitosterol-3-*O*-glucoside (9) have been isolated. Compounds 1-4 are first report from the genus *Lantana,* whereas compounds 5, 8 and 9 are first report from this plant. The structures of these compounds were elucidated on the basis of spectral methods, including 2D nuclear magnetic resonance experiments, and confirmed by comparing with the literature data and/or authentic samples. Additionally, some of the isolated flavonoids exhibited free radical scavenging activity. **Conclusion:** *L. montevidensis* (Spreng.) Briq. cultivated in Egypt is rich in biologically active ingredients.

Keywords: Apigenin, cinaroside, free radical scavenging activity, hispidulin-7-O-glucurnoide methyl ester, hispidulin-7-O-glucoside, *Lantana montevidensis,* luteolin, nepetin-7-O-glucoside, pectolinarigenin

INTRODUCTION

Lantana montevidensis (Spreng.) Briq. (weeping or trailing *Lantana*, F. *Verbenaceae*) is introduced in many countries as an ornamental plant and considered as an invasive species in many parts of the world.[1] The tea and infusions of the dried leaves have been used in folk medicine as carminative and antiseptic as well as for the treatment of itching, stomach ache, rheumatism, wounds, biliary fever, toothache, influenza, bronchitis, headaches, and sunstroke.[1-3] The leaves methanolic extract and the flavonoid fraction from the leaves presented anti-proliferative activity against tumor cells *in vitro*.[4] The leaves essential oil in addition to the ethanolic extracts of the leaves and roots exhibited promising antimicrobial activity.[2,3,5-7] Furthermore, the 70% aqueous methanolic extract of the leaves presented antibacterial, anti-inflammatory, anti-pyretic, antioxidant and analgesic activities.[8] Various flavones had been reported from the leaves of *L. montevidensis*.[4,9] Our previous phytochemical study on 70% methanolic extract of *L. montevidensis* leaves led to the isolation of pentacyclic triterpenoids.[10] In continuation of our investigation, we report herein the isolation and identification of seven flavonoids (1-7) in addition to salicylic acid (8) and β-sitosterol-3-*O*-glucoside (9). Furthermore, some of the isolated flavonoids were evaluated for their free radical scavenging activity.

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MATERIALS AND METHODS

General

1D and 2D Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ and DMSO-d_6 using JEOL Oxford NMR YH-400 and Varian AS 400 spectrometer (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR). Electron impact (EI) mass spectra were obtained by JEOL JMS 600 H and time of flight (TOF) mass spectra were recorded using Agilent spectrometer. Ultraviolet (UV) spectra were recorded in methanol on Ultrospec 1000, UV-Visible spectrophotometer with matched 1 cm quartz cells (Pharmacia Biotech, Cambridge, England). Column chromatography was carried out on silica gel 60 (70-230 mesh, E. Merck) and Sephadex LH-20 (25-100 mm, E. Merck). Moderate pressure liquid chromatography (MPLC); CIG column system (22 mm i.d. × 30 cm) packed with RP-18 (Kusano Scientific Co., Tokyo, Japan). Analytical thin-layer chromatography (TLC) was conducted on precoated aluminium sheets of RP-18 F_{254} (0.25 mm, E. Merck) and silica gel 60 GF_{254} (0.25 mm, E. Merck) and the spots were detected by UV irradiation (254 and 366 nm), followed by spraying with 10% H_2SO_4 reagent and heating at 110°C for 5 min.

Plant material

The leaves of *L. montevidensis* (Spreng.) Briq. were collected during the flowering stage in the period of February to November 2009 from the gardens of Assiut University, Assiut, Egypt. The plant was kindly identified and authenticated by Prof. Dr. Naeem E. Keltawy, Professor of Ornamental Horticulture and Floriculture, Faculty of Agriculture, Assiut University. A voucher sample (no. 2009 LM) has been deposited in the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

Chemicals and drugs

1,1-Diphenyl-2-Picryl-Hydrazyl radical (DPPH) was obtained from Sigma and quercetin (reference antioxidant) was obtained from El-Nasr Pharmaceutical and Chemical Co., Egypt (Adwic).

Extraction and isolation

The air-dried powdered leaves of *L. montevidensis* (Spreng.) Briq. (5 kg) were extracted with 70% aqueous methanol and fractionated with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH as described previously.[10]

About 30 g of the EtOAc extract was fractionated on silica gel column (900 g, 150 cm × 5 cm). Elution was performed using $CHCl_3$-MeOH gradient, where four groups were obtained: E-I (5.5 g, eluted with $CHCl_3$-MeOH 95:5), E-II (7 g, $CHCl_3$-MeOH 90:10), E-III (12 g, $CHCl_3$-MeOH 80:20) and E-IV (3 g, $CHCl_3$-MeOH 70:30). Group E-II (7 g) was re-chromatographed on silica gel column (210 g, 100 × 5 cm) using $CHCl_3$-MeOH gradient, the fractions eluted with $CHCl_3$-MeOH (90:10) were collected together and subjected to further purification on Sephadex LH-20 column using MeOH to obtain compound 1 (500 mg). A part of group E-II (7 g) was re-chromatographed on a silica column (210 g, 100 × 5 cm) using $CHCl_3$-MeOH gradient, two sub-fractions were obtained: E-III-1 (2.1 g, eluted with $CHCl_3$-MeOH, 85:15) and E-III-2 (4.6 g, eluted with $CHCl_3$-MeOH, 80:20). Sub-fraction E-III-1 was purified on Sephadex LH-20 column using MeOH to yield compound 2 (15 mg). A part of sub-fraction E-III-2 (1.5 g) was subjected to MPLC using RP-18 packed column (30 × 22 cm i.d.) and MeOH-H_2O (10:90 and 20:80), then repurified on Sephadex LH-20 column using MeOH to obtain pure compounds 3 (20 mg) and 4 (18 mg), respectively.

About 8 g of the $CHCl_3$ extract was subjected to silica gel column chromatographic fractionation (240 g, 100 × 5 cm) using gradient elution system of $CHCl_3$-MeOH to produce three groups: C-I (1.3 g, eluted with $CHCl_3$), C-II (2.2 g, eluted with $CHCl_3$-MeOH 95:5) and C-III (2.5 g, eluted with $CHCl_3$-MeOH (90:10). Group C-II (2.2 g) was re-chromatographed on silica gel column (66 g, 50 × 3 cm) using $CHCl_3$-MeOH gradient to give two sub-fractions: C-II-1 (0.5 g, eluted with $CHCl_3$-MeOH 95:5) and C-II-2 (1.5 g, eluted with $CHCl_3$-MeOH 90:10). Sub-fraction C-II-1 was purified on Sephadex column using MeOH as eluent to obtain compound 5 (10 mg). Repeated crystallization of sub-fraction C-II-2 from MeOH afforded compound 9 (200 mg). Group C-III (2.5 g) was subjected to further purification on silica gel (75 g, 70 × 3 cm) using mixtures of *n*-hexane-acetone as mobile phase, three sub-fractions were obtained: C-III-1 (0.2 g, eluted with *n*-hexane-acetone 80:20), C-III-2 (1g, eluted with *n*-hexane-acetone 75:25) and C-III-3 (1g eluted with *n*-hexane-acetone 70:30). Repeated crystallization of sub-fraction C-III-1 from MeOH gave compound 8 (8 mg). Each of sub-fraction C-III-2 and C-III-3 was subjected to further purification on silica gel column (30 g, 50 × 3 cm) using $CHCl_3$-MeOH (95:5) to yield compounds 6 (8 mg) and 7 (7 mg), respectively.

Compound 1: Yellow amorphous powder; UV (MeOH) λ_{max}: 274, 335; +NaOMe: 275, 379; +$AlCl_3$: 287, 395;

+$AlCl_3$/HCl: 285, 393; +NaOAc: 278, 380; +NaOAc/H_3BO_3: 276, 336 nm. ^{1}H NMR (DMSO-*d6*, 400 MHz): δ 3.19~3.42 (m, sugar protons), 3.65 (3H, s, 6''-OC$\underline{H}_3$), 3.75 (3H, s, 6-OC$\underline{H}_3$), 4.19 (1H, d, J=9.6 Hz, H-5''), 5.34 (1H, d, J=6.8 Hz, H-1''), 6.84 (1H, s, H-3), 6.93 (2H, d, J=8.8 Hz, H-3'/H-5'), 7.05 (1H, s, H-8), 7.93 (2H, d, J=8.8 Hz, H-2'/H-6'), 12.90 (1H, br s, 5-O$\underline{H}$); ^{13}C NMR (DMSO-*d6*, 100 MHz): δ 52.44 (6''-OC$\underline{H}_3$, q), 60.75 (6-OC$\underline{H}_3$, q), 71.76 (C-4'', d), 73.25 (C-2'', d), 75.74 (C-5'', d), 76.11 (C-3'', d), 94.35 (C-8, d), 99.85 (C-1'', d), 103.14 (C-3, d), 106.35 (C-10, s), 116.45 (C-3'/5', d), 121.52 (C-1', s), 129.00 (C-2'/6', d), 132.97 (C-6, s), 152.54 (C-9, s), 153.10 (C-5, s), 156.36 (C-7, s), 161.82 (C-4', s), 164.79 (C-2, s), 169.79 (C-6'', s), 182.74 (C-4, s).

Compound 2: Oily residue; UV (MeOH) λ_{max}: 275, 338; + NaOMe: 279, 381; +$AlCl_3$: 287, 383; +$AlCl_3$/HCl: 285, 381; +NaOAc: 278, 363; +NaOAc/H_3BO_3: 276, 340 nm. ^{1}H NMR (DMSO-*d6*, 400 MHz): δ 3.19~4.69 (m, sugar protons), 3.75 (3H, s, 6-OC$\underline{H}_3$), 5.11 (1H, d, J=7.2 Hz, H-1''), 6.86 (1H, s, H-3), 6.93 (2H, d, J=8.4 Hz, H-3'/H-5'), 7.01 (1H, s, H-8), 7.94 (2H, d, J=8.4 Hz, H-2'/H-6'), 12.90 (1H, br s, 5-O$\underline{H}$); ^{13}C NMR (DMSO-*d6*, 100 MHz): δ 60.27 (6-O$\underline{C}H_3$), 60.54 (C-6''), 69.74 (C-4''), 73.15 (C-2''), 76.70 (C-5''), 77.23 (C-3''), 94.18 (C-8), 100.13 (C-1''), 102.45 (C-3), 105.56 (C-10), 116.03 (C-3'/5'), 120.70 (C-1'), 128.53 (C-2'/6'), 132.35 (C-6), 152.06 (C-9), 152.31 (C-5), 156.41 (C-7), 161.66 (C-4'), 164.19 (C-2), 182.31 (C-4).

Compound 3: Yellow powder; UV (MeOH) λ_{max}: 255, 349; +NaOMe: 263, 393; +$AlCl_3$: 274, 430; +$AlCl_3$/HCl: 273, 388; +NaOAc: 259, 404; + NaOAc/H_3BO_3: 259, 373 nm. ^{1}H NMR (DMSO-*d6*, 400 MHz): δ 3.14~3.31 (m, sugar protons), 5.07 (1H, d, J=7.2 Hz, H-1''), 6.43 (1H, d, J=2.0 Hz, H-6), 6.75 (1H, s, H-3), 6.78 (1H, d, J=2.0 Hz, H-8), 6.89 (1H, d, J=8.0 Hz, H-5'), 7.41 (1H, d, J=2.0 Hz, H-2'), 7.44 (1H, dd, J=8.0, 2.0 Hz, H-6'), 12.96 (1H, br s, 5-O$\underline{H}$); ^{13}C NMR (DMSO-*d6*, 100 MHz): δ 61.03 (C-6'', t), 69.95 (C-4'', d), 73.53 (C-2'', d), 76.79 (C-3'', d), 77.56 (C-5'', d), 95.15 (C-8, d), 99.95 (C-6, d), 100.28 (C-1'', d), 103.58 (C-3, d), 105.76 (C-10, s), 113.96 (C-2', d), 116.42 (C-5', d), 119.62 (C-6', d), 121.80 (C-1', s), 146.19 (C-3', s), 150.34 (C-4'), 157.36 (C-9, s), 161.15 (C-5, s), 163.34 (C-7, s), 164.88 (C-2, s), 182.32 (C-4, s).

Compound 4: Yellow powder; UV (MeOH) λ_{max}: 255, 346; +NaOMe: 266, 402; +$AlCl_3$: 274, 420; +$AlCl_3$/HCl: 260, 380; +NaOAc: 258, 377; +NaOAc/H_3BO_3: 259, 373 nm. ^{1}H NMR (DMSO-*d6*, 400 MHz): δ 3.19~3.70 (m, sugar protons), 3.74 (3H, s, 6-OC$\underline{H}_3$), 5.12 (1H, d, J=6.4 Hz, H-1''), 6.73 (1H, s, H-3), 6.89 (1H, d, J=8.4 Hz, H-5'), 6.96 (1H, s, H-8), 7.40 (1H, br s, H-2'), 7.43 (1H, br d, J=8.4 Hz, H-6'), 12.99 (1H, br s, 5-O$\underline{H}$); ^{13}C NMR (DMSO-*d6*, 100 MHz): δ 60.33 (6-O$\underline{C}H_3$), 60.63 (C-6''), 69.53 (C-4''), 73.20 (C-2''), 76.69 (C-3''), 77.26 (C-5''), 94.21 (C-8), 100.12 (C-1''), 102.77 (C-3), 105.71 (C-10), 113.58 (C-2'), 115.96 (C-5'), 119.15 (C-6'), 121.46 (C-1'), 132.48 (C-6), 145.81 (C-3'), 149.92 (C-4'), 152.16 (C-5), 152.49 (C-9), 156.49 (C-7), 164.52 (C-2), 182.22 (C-4).

Compound 5: Yellow powder; UV (MeOH) λ_{max}: 277, 333; +NaOMe: 280, 360; +$AlCl_3$: 276, 386; + $AlCl_3$/HCl: 280, 386; +NaOAc: 279, 366; +NaOAc/H_3BO_3: 277, 335 nm. ^{1}H NMR (DMSO-*d6*, 400 MHz): δ 3.73 (3H, s, 6-OC$\underline{H}_3$), 3.84 (3H, s, 4'-OC$\underline{H}_3$), 6.61 (1H, s, H-8), 6.85 (1H, s, H-3), 7.09 (2H, d, J=8.4 Hz, H-3'/H-5'), 8.02 (2H, d, J=8.4 Hz, H-2'/H-6'), 13.01 (1H, br s, 5-O$\underline{H}$); ^{13}C NMR (DMSO-*d6*, 100 MHz): δ 56.01 (4'-OC$\underline{H}_3$, q), 60.39 (6-O$\underline{C}H_3$, q), 94.76 (C-8, d), 103.50 (C-3, d), 104.55 (C-10, s), 115.03 (C-3'/5', d), 123.30 (C-1', s), 128.76 (C-2'/6', d), 131.83 (C-6, s), 152.87 (C-9, s), 153.20 (C-5, s), 157.84 (C-7, s), 162.74 (C-4', s), 163.79 (C-2, s), 182.60 (C-4, s). Positive-ion TOF MS *m/z* (rel. int. %): 315 $[M+1]^+$ $C_{17}H_{14}O_6$ (100).

Compound 6: Yellow powder; UV (MeOH) λ_{max}: 268, 337; +NaOMe: 275, 391; +$AlCl_3$: 276, 386; +$AlCl_3$/HCl: 276, 380; +NaOAc: 275, 375; +NaOAc/H_3BO_3: 268, 338 nm. ^{1}H NMR (DMSO-*d6*, 400 MHz): δ 6.17 (1H, brs, H-6), 6.47 (1H, brs, H-8), 6.78 (1H, s, H-3), 6.91 (2H, d, J=8.0 Hz, H-3'/H-5'), 7.92 (2H, d, J=8.0 Hz, H-2'/H-6'), 12.96 (1H, br s, 5-O$\underline{H}$).

Compound 7: Yellow powder; UV (MeOH) λ_{max}: 254, 349; +NaOMe: 280, 402; +$AlCl_3$: 268, 416; +$AlCl_3$/HCl: 268, 380; +NaOAc: 270, 374; +NaOAc/H_3BO_3: 262, 375 nm. ^{1}H NMR (DMSO-*d6*, 400 MHz): δ 6.17 (1H, brs, H-6), 6.43 (1H, brs, H-8), 6.66 (1H, s, H-3), 6.87 (1H, d, J=8.4 Hz, H-5'), 7.37 (1H, dd, J=8.4, 2.4 Hz, H-6'), 7.42 (1H, d, J=2.4 Hz, H-2'), 12.96 (1H, br s, 5-O$\underline{H}$).

Compound 8: White powder; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.94 (1H, t, J=7.6 Hz, H-5), 7.02 (1H, d, J=8.0, H-3), 7.54 (1H, ddd, J=8.0, 7.6, 1.6 Hz, H-4), 7.94 (1H, dd, J=7.6, 1.6 Hz, H-6), 10.38 (1H, br s, 2-O$\underline{H}$). EI MS *m/z* (%): 138 (55), 120 (100), 92 (65).

Free radical scavenging activity

The free radical scavenging activity of compounds 1, 3, 4, 6 and 7 were measured by spectrophotometric method. One milliliter of ethanolic solutions of the test compound (50 and 100 μg/ml) were mixed with 1 ml of the ethanolic solution of DPPH (200 μM). Similarly, 1 ml of ethanolic

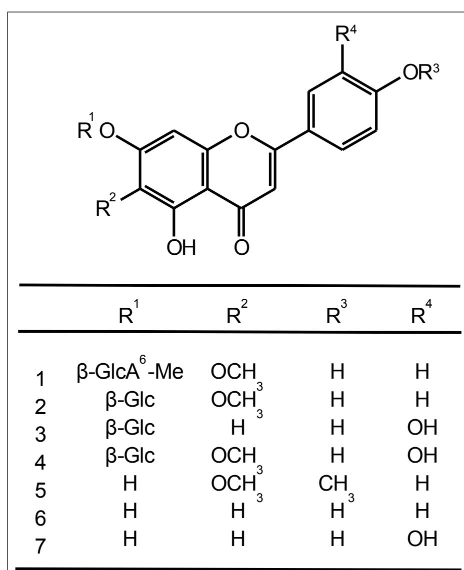
solutions of quercetin (50 and 100 μg/ml) were mixed with 1 ml DPPH solution and used as a positive control. 1 ml of ethanol was used instead of the sample solution as a blank. After mixing, all the solutions were incubated in dark for 20 min and absorbance was measured at 517 nm.[11] The experiments were performed in triplicate and percent scavenging activity was calculated as follows:

$$\text{Scavenging } \% = \frac{\text{Absorbance of control} - \text{Absorbance of test compound}}{\text{Absorbance of control}} \times 100$$

RESULTS AND DISCUSSION

Seventy percent aqueous methanolic extract of *L. montevidensis* (Spreng.) Briq. leaves was partitioned between water and *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, successively. By using a series of different chromatographic techniques, the EtOAc and $CHCl_3$ extracts afforded nine compounds including seven flavonoids (1-7) (Figure 1) in addition to salicylic acid (8) and β-sitosterol-3-*O*-glucoside (9).

The UV spectral data in methanol for compounds 1 and 2 suggested their structures to be flavones having free hydroxyl groups at the 5- and 4'- and lacking free hydroxyl group at the 7-position.[12] ^{1}H-NMR spectrum of 1 displayed signals for apigenin derivative by the appearance of a signal at δ_H 6.84 (1H, s) characteristic for H-3, two signals attributable to *p*-substituted benzene ring at δ_H 7.93 (2H, d, J= 8.8 Hz, H-2', 6') and 6.93 (2H, d, J= 8.8 Hz, H-3', 5'), a singlet signal of ring A at δ_H 7.05 (IH, s). Also, it revealed



	R1	R2	R3	R4
1	β-GlcA6-Me	OCH3	H	H
2	β-Glc	OCH3	H	H
3	β-Glc	H	H	OH
4	β-Glc	OCH3	H	OH
5	H	OCH3	CH3	H
6	H	H	H	H
7	H	H	H	OH

Figure 1: Structures of flavonoids (1-7) isolated from the leaves of *Lantana montevidensis* (Spreng.) Briq.

the presence of two methoxyl groups appeared at δ_H 3.65 and 3.75 (each 3H, s) and an anomeric proton resonated at δ_H 5.34 (1H, d, J=6.8 Hz, H-1'').[12,13] The ^{13}C-NMR spectrum revealed the presence of 23 carbons owing to apigenin derivative and methyl ester of β-glucuronide moiety (52.44, 71.76, 73.25, 75.74, 76.11, 99.85, 169.79).[14-16] The hetero-nuclear multiple bond connectivity (HMBC) spectrum appeared long-range correlations between the anomeric proton at δ_H 5.34 and C-7 (δ_C 156.36) confirmed the *O*-glycosidic linkage at C-7. Furthermore, the proton signals of the methoxyl groups at δ_H 3.65 and 3.75 showed long-range correlation with C-6'' (δ_C 169.79) and C-6 (δ_C 132.97) indicated the location of the methoxyl groups at position-6'' of the glucuronic acid and C-6 of aglycone, respectively. Based on these data and comparison with literature data,[17] 1 was determined to be hispidulin-7-*O*-glucuronide methyl ester.

The ^{1}H-NMR and ^{13}C-NMR spectra of compound 2 exhibited signals for hispidulin derivative resembling those of 1 except for the appearance of β-D-glucopyranosyl moiety (60.54, 69.74, 73.15, 76.70, 77.23 and 100.13) instead of the methyl ester of β-glucuronide moiety.[12-16] By comparison of the spectral data with the reported data,[17] the structure of compound 2 was concluded to be hispidulin-7-*O*-glucoside.

Compounds 3 and 4 had UV spectral data of flavones having free hydroxyl groups at C-5, 3' and 4' and lacking free hydroxyl group at C-7.[12] The ^{1}H-NMR spectrum of compound 3 exhibited signals for luteolin nucleus by the appearance of a characteristic singlet for H-3 of a flavone at δ_H 6.75 (1H, s), two signals of ring A at δ_H 6.43 and 6.78 (each 1H, d, J=2.0 Hz) were assigned for H-6 and H-8, respectively and three signals for protons of ring-B appeared at δ_H 6.89 (1H, d, J=8.0 H-5'), 7.41 (1H, d, J=2.0 Hz, H-2') and 7.44 (IH, dd, J=8.0, 2.0 Hz, H-6').[12,13] Furthermore, it revealed an anomeric proton signal appeared at δ_H 5.07 (1H, d, J=7.2 Hz) indicated the presence of β-D-glucopyranosyl moiety.[13] The ^{13}C-NMR spectrum revealed the presence of twenty-one signals owing to luteolin and one β-D-glucopyranosyl.[14-16] The HMBC spectrum showed long-range correlation between the anomeric proton signal H-1'' (δ_H 5.07) and C-7 (δ_C 163.34) supported the *O*-glycosidation at C-7. Based on these spectral data and by comparison with the literature data,[18] compound 3 was concluded to be luteolin-7-*O*-glucoside (cinaroside).

The ^{1}H- and ^{13}C-NMR spectra of compound 4 displayed signals owing to luteolin derivative and one β-glucopyranosyl moiety resembling those of three.[12-16] In addition, the

spectra revealed the presence of one methoxyl group [δ_H 3.74 (3H, s), δ_C 60.33] which is characteristic for the methoxyl group at C-6 (δ_C 132.48).[15,16] Thus, compound 4 was identified as 6-methoxyluteolin-7-*O*-glucoside (nepetin-7-*O*-glucoside; nipitrin).

Compound 5 displayed characteristic UV absorption maxima for flavone skeleton having free hydroxyl groups at positions 5 and 7 and lacking free hydroxyl groups at position 4'.[12] Its ^{1}H- and ^{13}C-NMR spectra displayed signals for a flavone derivative in addition to two methoxyl groups (δ_H 3.73 and 3.84 [each 3H, s], δ_C 60.39 and 56.01).[12,13,15,16] The placement of the methoxyl groups at positions C-6 and C-4' are supported by the HMBC spectrum, the protons of the methoxyl groups at δ_H 3.73 and 3.84 showed correlations with the carbons at position 6 (δ_C 131.83) and 4' (δ_C 162.74), respectively. By comparison with the reported literature,[17] compound 5 was deduced as pectolinarigenin (5,7-dihydroxy-6,4'-dimethoxyflavone).

Compounds 6-8 were identified as apigenin, luteolin[12,13,16] and salicylic acid[19,20] by comparison of their spectral data with literature data, while compound 9 was identified as β-sitosterol-3-*O*-glucoside by direct comparison with an authentic sample (co-TLC).

Compounds with *ortho*-dihydroxy system in the ring B exhibited strong antioxidant activity as luteolin (7), luteolin-7-*O*-glucoside (3) and nepetin-7-*O*-glucoside (4), while the compounds having only free hydroxyl group in position 4', as apigenin (5) and hispidulin-7-*O*-glucournoide methyl ester (1) showed weak antioxidant activity (Table 1).

CONCLUSION

Seven flavonoids, salicylic acid and *β*-sitosterol-3-*O*-glucoside have been isolated from the leaves of *L. montevidensis* (Spreng.) Briq. Moreover, some of the isolated flavonoids isolated from this plant exhibited strong to moderate free radical scavenging activity.

Table 1: Free radical scavenging activity of some flavonoids isolated from *L. montevidensis* (Spreng.) Briq. leaves

Compounds	Antioxidant %	
	50 µg	100 µg
Quercetin (Reference)	96.5	96.5
Hispidulin-7-*O*-glucurnoide methyl ester (1)	38.9	53.1
Luteolin-7-*O*-glucoside (3)	62.1	68.9
Nepetin-7-*O*-glucoside (4)	60.3	66.3
Apigenin (6)	49.4	55.7
Luteolin (7)	72.1	88.3

L. montevidensis: Lantana montevidensis

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Anti-proliferative Activity of Crude Extract and Fractions Obtained from *Digera muricata* on HeLa Cell Lines of Human Cervix and A_{549} Cell Lines of Human Lung

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ABSTRACT

Aim: *Digera muricata* (Linn.) of family Amaranthaceae is an ethanobotanically important plant species traditionally used against various disorders. **Materials and Methods:** Cytotoxic potential of methanolic extract and its fractions were investigated against HeLa and A_{549} cell lines. Crude extract of *D. muricata* was prepared in methanol by continuous hot soxhlation technique. Crude extract was fractionated into two organic and one aqueous fraction by the help of column chromatography. 3-(4,5-dimethylthiazol-2yl)-2,4 diphenyltetrazolium bromide assay was used to evaluate the reduction of viability of the cancer cell lines. Cell viability was inhibited by crude extract of *D. muricata* in a dose dependent manner ranging from 25 μg/ml to 250 μg/ml. **Results:** Apoptosis assays using nucleic acid stains namely propidium iodide (PI) exclusion assay and hoestch/PI assay were performed by the help of fluorescence microscopy. Morphological analysis was done by calculation of apoptotic ratio and percentage apoptosis. **Conclusion:** Our results suggest that methanolic and aqueous fraction of the extract of *D. muricata* can be a good source of cytotoxic compounds.

Keywords: 3-(4,5-dimethylthiazol-2yl)-2,4 diphenyltetrazolium bromide assay, A_{549} cell line, cytotoxic, *Digera muricata,* HeLa cell line

INTRODUCTION

Digera muricata (L.), Family Amaranthaceae, wild edible plant commonly known as "Latmahuria" is commonly distributed throughout India. In Ayurveda, the herb is considered as a cooling, astringent to the bowels and also used as a laxative. The flowers and seeds be used to treat urinary discharges.[1] Boiled root infusion is given to mothers after child birth for lactation purpose.[2] *D. muricata* is also used to treat renal disorders in folk medicine practices. The generation of reactive radical species has been implicated in carbon tetrachloride-induced nephrotoxicity, which are involved in lipid peroxidation and accumulation of dysfunctional proteins, leading to injuries in kidneys.[3] The treatment with the plant augments the antioxidant defense mechanism against carbon tetrachloride induced toxicity and provides evidence that it may have a therapeutic role in free radical mediated diseases.[4]

The phytochemical investigation on the plant *D. muricata* has revealed the presence of flavonoids in it that have a diverse effect on improvement and protection of deficits. Hence, the present work was undertaken to investigate the effect of crude extract and flavonoid rich fractions of *D. muricata* as an anticancer drug.

Cancer belongs to a group of disorders that are difficult to be treated and are sometimes incurable. Nowadays, considerable scientific and commercial interests have increased for discovering new anticancer agents with natural sources.[5-8] Plants have been sources of the well known anticancer drugs such as campthothecin, podophyllotoxin and paclitaxel.[9] The potential of natural products as anticancer agents was recognized for the first

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time in the 1950 by the U.S. National Cancer Institute, and after that many investigations have been performed to find out new natural anticancer agents.[10] Different methods are used for screening of anticancer agents. One of the techniques is 3-(4,5-dimethylthiazol-2yl)-2,4 diphenyltetrazolium bromide (MTT) assay which is a simple and reliable method for evaluation of anticancer agents.[11]

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT is reduced by metabolically active cells, by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis; it measures the reduction in cell viability.

The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type, the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation and a higher absorbance rate indicates an increase in cell proliferation.

Cervical cancer is an important area of action for any cancer control program because of the burden of disease and the potential for effective prevention via screening. Cervical cancer comprises approximately 12% of all cancers in women. It is the second most common cancer in women worldwide but the commonest in developing countries such as Indonesia. Annual global estimates around the year 2000 are for 470,600 new cases and 233,400 deaths from cervical cancer annually. 80% of these cases occur in developing countries.[12] Incidence of cancer in Indonesia was estimated to be 100 per 100,000 people per year or about 200,000 population per year, and cervical cancer is the first most common cancer in women of Indonesia. Cervical cancer is malignant neoplasm of the cervix uteri or cervical area. It may present with vaginal bleeding, but symptoms may be absent until the cancer is in its advanced stages. The most important risk factor in the development of cervical cancer is infection with a high-risk strain of human papilloma virus. Symptoms of advanced cervical cancer may include: Loss of appetite, weight loss, fatigue, pelvic pain, back pain, leg pain, single swollen leg, heavy bleeding from the vagina, leaking of urine or feces from the vagina, and bone fractures. Natural compounds from plants have provided many effective anticancer agents in current use. Currently, over 50% of drugs in clinical trials for anticancer activity have been isolated from natural sources.[13]

Worldwide, lung cancer is another most common cancer in terms of both occurrence and mortality, with the highest rates in Europe and North America. Despite rapid advances in diagnostic and operative techniques, lung cancer remains one of the most difficult human malignancies to treat. The American Cancer Society estimates that 214,440 persons in the United States developed lung cancer in 2009, with 159,390 deaths. Lung cancer-dependent deaths constituted 30% (men) and 26% (women) of the estimated total cancer-related deaths in 2009. Data indicate that while the overall incidence of lung cancer is declining, it continues to rise in women.[14] The relative 5-year survival ratio of the patients that had lung or bronchus cancer from 1995 to 2001 was still quite low (15%) and was not improved very much compared to the 1970's (12%). From the 1950s, the incidence of lung adenocarcinoma started to rise relative to other types of lung cancers.[15]

Recently, multiple new chemotherapeutic agents have been developed, and some are in clinical trials.[16,17] Although some of them have produced promising results, their therapeutic spectrum is narrow along with toxicity. This toxicity problem at therapeutic concentration induced scientists to search for anticancer compounds derived from natural sources such as phytochemicals. Keeping this in mind, the aim of the present work was to explore the anticancer property of *D. musica* on A_{549} human lung cancer cell lines and help cell lines of cervical cancer.

MATERIALS AND METHODS

Plant material

The whole plant of *D. muricata* was collected in the month of August, 2011 from fields behind the Faculty of Pharmacy, Integral University, Lucknow, U.P, India. For identification and taxonomic authentication, sample of plant material was given to National Botanical Research Institute, Lucknow, India, which confirmed the authenticity of the plant specimen (voucher specimen no. NBRI/263/2011). The

fresh plant was used for the examination of macroscopic and microscopic characters whereas the dried powder was used for the determination of physicochemical parameters. Preliminary phytochemical investigation was done as per standard methods.

Preparation of extract

The whole plant was dried in an oven at 50 ± 0.5°C up to approximately 5-7% moisture content. This powder was extracted with methanol in a soxhlet apparatus for 24 h. The extract was concentrated under reduced pressure by a rotary vacuum evaporator. This extract was then fractionated by column chromatography into three flavonoid rich fractions namely chloroform, ethylacetate and residual water. The methanolic extract and all the fractions were dissolved in Di-methyl-sulphoxide to prepare a series of concentration in a range of 25-250 µg/ml. This extract and its fractions were screened for their anticancer activity.

Cytotoxicity assays

Cell lines

Two cancerous cell lines HeLa (human cervix) and A_{549} (human lung carcinoma) were obtained from cell repository at NCCS, Pune.

Reagent and chemicals

HeLa cell line was cultured in Eagle's minimal essential medium (MEM, Himedia) with 2.0 mM L-glutamine, 1.5 g/L $NaHCO_3$, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate and supplemented to contain 10% fetal calf serum (Himedia). Similarly, A_{549} cell was cultured in Ham's F12K medium (Himedia) with 2.0 mM L-glutamine adjusted to contain 1.5 g/L $NaHCO_3$ and 10% fetal calf serum (Himedia).

MTT reagent, phosphate buffer saline, dimethyl-sulphoxide, propidium iodide (PI) and Hoechst stain kit, paraformaldehyde, triton.

Microculture tetrazolium toxicity assay

The cytotoxic potential was evaluated using the MTT assay.[18] Cells were grown at 37°C in 5% CO_2 incubator. The medium was replaced with fresh supplemented medium containing different concentrations (25-250 µg/ml) of methanolic extract and fractions of the plant obtained during fractionation by column chromatography. Cells were then incubated at 37°C for 48 h. After incubation, 10 µl of a 5 mg/ml stock solution of MTT in phosphate buffer solution (PBS) was added to each well containing the cells and incubated again for 4 h. Then, the supernatant without the cells was aspirated from each well, and 100 µl of dimethyl sulfoxide was added to dissolve the dark blue formazan crystals resulting from MTT reduction by homogenization in plate shaker. The extent of MTT reduction to formazan within cells was measured by taking absorbance at 550 nm using a scanning microplate reader (Biorad, Model 680). The percentage cell proliferation was calculated by using the following formula:

$$\text{Percentage cell proliferation} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of treated}}{\text{Absorbance of control}}\right] \times 100$$

Statistical analysis

Data of cell proliferation were expressed as the mean ± standard deviation from three independent experiments. One-way ANOVA and Dunnett's multiple comparison tests were performed using Graph Pad prism (version 5.01 Software Inc. 2013) for the significance test, using a $P \leq 0.05$.

Morphological examination of cancer cells[19-21]

Calculation of the apoptotic ratio

The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei under inverted fluorescent microscope using G-2A filter at ×400 magnification.

Calculation of the percentage apoptosis

By scoring the number of apoptotic cells with that of the total number of cells involved in the experiment, percentage apoptosis was measured which indicated the level of apoptosis induced by the test drug. Freshly stained cell suspension was dropped into a glass slide and covered by a cover slip. Slides were observed under phase contrast microscope. The percentages of viable, apoptotic, and necrotic cells were determined in more than 200 cells by help of hemocytometer.

PI exclusion assay for necrosis

First, the cell suspension was aspirated and washed by 100 µl PBS, 2-3 times followed by 50 µl of 4% paraformaldehyde (50 µl). It was left as such for 15 min. It was then treated with 50 µl triton for 20 min. After being washed with 100 µl of PBS, 1 µl of PI stain was added. Then, it was kept in the dark for 10 min and visualized under fluorescence microscope.

Hoechst/PI nuclear staining and fluorescence microscopy

First, the cell suspension was aspirated and washed by 100 μl of PBS, 2-3 times, followed by 75 μl of 4% paraformaldehyde and kept at room temperature for 15 min. It was then treated with Methanol and kept for 20 min at room temperature. After being washed with 100 μl of PBS, 1 μl of PI stain and 1 μl of Hoechst stain was added and kept for 15 min. It was then visualized under fluorescent microscope after rinsing with PBS. The morphology of viable, apoptotic and necrotic cells was visualized under fluorescence microscope.

RESULTS

Effect of crude methanolic extract of *D. musica* against A_{549} cell lines

Crude extract of *D. muricata* showed inhibition at all concentrations in a dose dependent manner with a nominal amount of decrease in percentage proliferation (90.83%) at a concentration of 25 μg/ml and a marked decrease in percentage proliferation (29.68%) at a concentration of 250 μg/ml. A marked decrease in percentage proliferation was found at higher concentrations (between 100 and 250 μg/ml), however, this decrease in percentage proliferation gradually slowed down and finally became more or less constant beyond 250 μg/ml.

Effect of crude methanolic extract of *D. musica* against HeLa cell lines

Crude extract of *D. muricata* showed inhibition at all concentrations in a dose dependent manner with a minimum decrease of percentage proliferation (72.88%) at a concentration of 25 μg/ml and a maximum decrease in percentage proliferation (27.61%) at a concentration of 250 μg/ml. A marked decrease in percentage proliferation was found at higher concentrations (250 μg/ml) amounting to a constant beyond this value.

Effect of the chloroform fraction of *D. muricata* against A_{549} and HeLa cell lines

The chloroform fraction of *D. muricata* showed effect against A_{549} cell lines in a dose dependent manner with a nominal decrease of percentage proliferation (95.83%) at a concentration of 25 μg/ml and a decrease of up-to 27.62% proliferation at a concentration of 250 μg/ml. A significant decrease of percentage proliferation was found at higher concentrations (250 μg/ml). The Chloroform fraction of *D. muricata* showed effect against HeLa cell lines in a dose dependent manner with a nominal decrease of 88.6% proliferation at a concentration of 25 μg/ml and a decrease of up-to 35.48% proliferation at a concentration of 250 μg/ml.

Effect of ethylacetate fraction of *D. muricata* against A_{549} and HeLa cell lines

The ethylacetate fraction of *D. muricata* showed effect against A_{549} cell lines in a dose dependent manner with a decrease of 76.03% proliferation at a concentration of 25 μg/ml and an increase of up-to 27% proliferation at a concentration of 250 μg/ml. A significant decrease was found at higher concentrations (250 μg/ml).

The ethylacetate fraction of *D. muricata* showed effect against HeLa cell lines in a dose dependent manner with a marked decrease of 59.5% proliferation at a concentration of 25 μg/ml and an increase of up-to 24.95% proliferation at a concentration of 250 μg/ml. A significant decrease was found at higher concentrations (250 μg/ml).

Effect of the aqueous fraction of *D. muricata* against A_{549} and HeLa cell lines

The aqueous fraction of *D. muricata* showed effect against A_{549} cell lines in a dose dependent manner with a marked decrease of 78.08% proliferation at a concentration of 25 μg/ml and an increase of up-to 35.66% proliferation at a concentration of 250 μg/ml. A significant decrease was found at higher concentrations (250 μg/ml).

The aqueous fraction of *D. muricata* showed effect against HeLa cell lines in a dose dependent manner with a marked decrease of 59.5% proliferation at a concentration of 25 μg/ml and an increase of up-to 24.95% proliferation at a concentration of 250 μg/ml. A significant decrease was found at higher concentrations (250 μg/ml) (Figures 1-3).

PI - Exclusion assay for necrosis

Due to their extensive membrane damage, necrotic cells were quickly stained by short incubations with PI. A number of necrotic cells could be seen in the photomicrograph that resulted in increased permeability of the stain. Necrotic debris was seen which increased with concentration (Figures 4-6).

Hoechst/PI double staining and florescence microscopy

Apoptotic cells showed a high Hoechst staining and a low PI staining, since they initially tend to exclude PI (Figure 7).

Necrotic cells were brightly stained with PI while healthy cells were dimly stained by Hoechst and not stained by PI. Live cells showed only a low level of fluorescence; apoptotic cells showed a higher level of blue fluorescence, and dead cells showed low-blue and high-red fluorescence. Late apoptotic cells exhibited an orange nucleus showing condensation of chromatin while necrotic cells displayed an orange nucleus with intact structure. This assay provided a useful quantitative evaluation and was done 3 times. The staining pattern resulted from the simultaneous use of these dyes made it possible to distinguish normal, apoptotic, and dead cell populations by fluorescence microscopy.

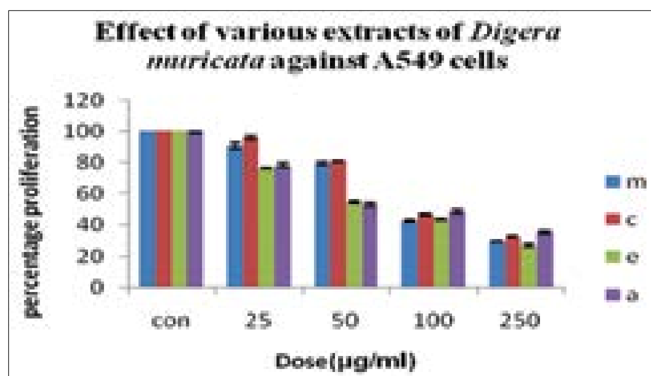

Figure 1: Percentage proliferation of cancerous cells A_{549} after 24 h after treatment with methanolic extract and its fractions obtained from *Digera muricata* evaluated by 3-(4,5-dimethylthiazol-2yl)-2,4 diphenyltetrazolium bromide assay at indicated concentrations. Values are mean ± standard deviation from three independent experiments. Triplicates of each treatment group were used in each independent experiment. Results were found to be statistically significant difference as compared to control ($P < 0.001$ for each).

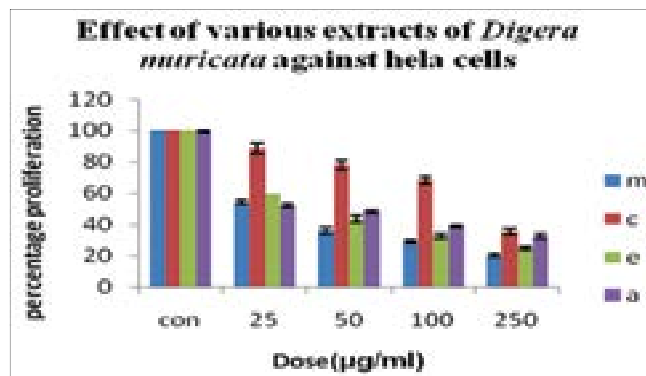

Figure 2: Percentage proliferation of cancerous cells HeLa after 24 h after treatment with methanolic extract and its fractions obtained from *Digera muricata* evaluated by 3-(4,5-dimethylthiazol-2yl)-2,4 diphenyltetrazolium bromide assay at indicated concentrations. Values are mean ± standard deviation from three independent experiments. Triplicates of each treatment group were used in each independent experiment. Statistically significant difference as compared to control ($P < 0.001$ for each).

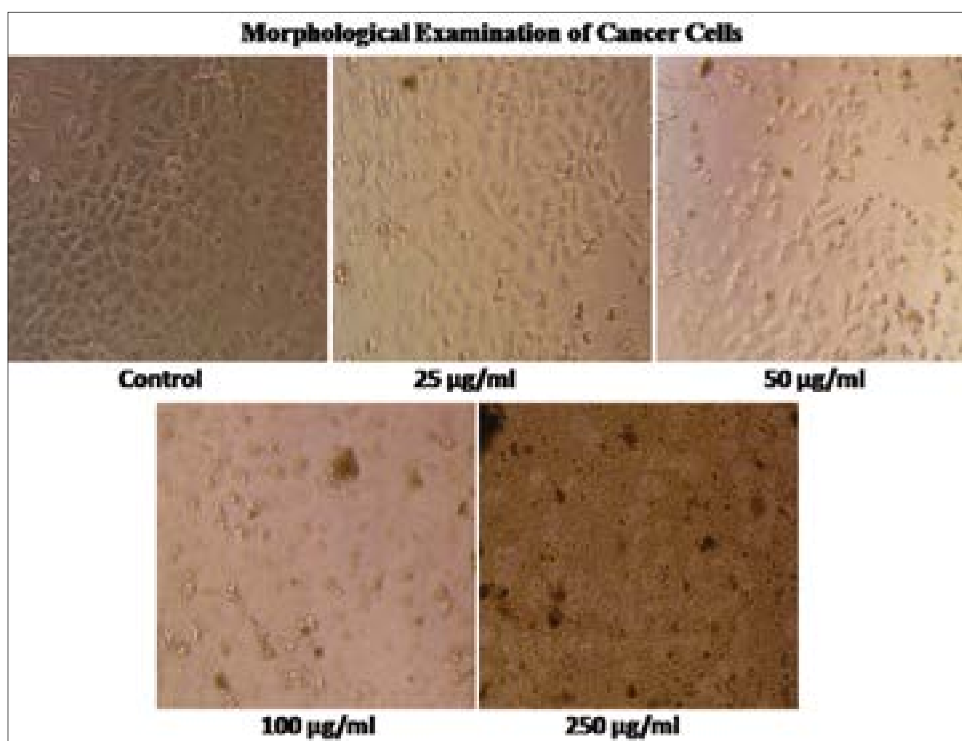

Figure 3: The morphological changes as seen through phase contrast microscope in various doses of methanolic extract of *Digera muricata*.

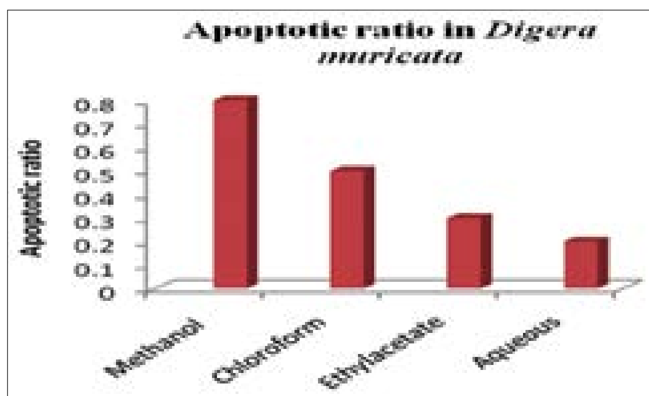

Figure 4: Apoptotic ratio in *Digera muricata* using HeLa cell lines at a dose of 250 µg/ml.

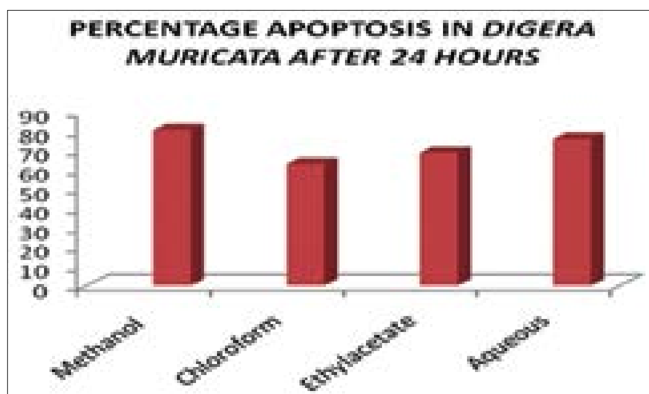

Figure 5: Percentage apoptosis in *Digera muricata* using HeLa cell lines after 24 h.

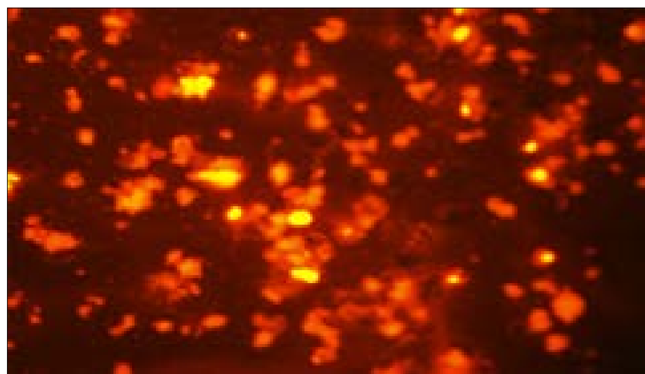

Figure 6: Photomicrograph of methanolic extract of *Digera muricata* showing extensive necrosis using propidium iodide exclusion assay.

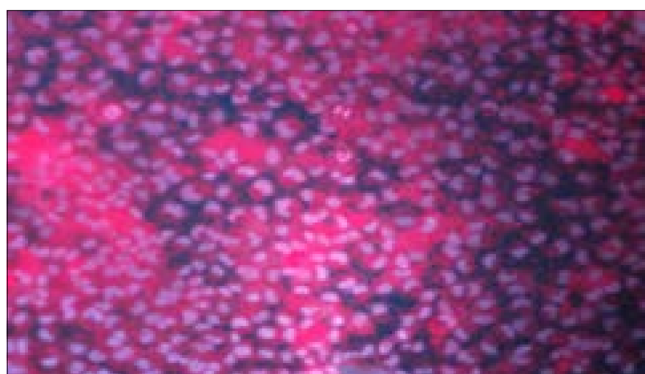

Figure 7: Photomicrograph of methanolic extract of *Digera muricata* showing extensive necrosis using Hoestch/propidium iodide double staining technique.

DISCUSSION

The results of cytotoxicity study of methanolic extract of *D. muricata* showed significant cytotoxicity against both the cell lines, but more for HeLa cell lines. The cytotoxicity of methanolic extract of the plants may be due to the presence of flavonoids having mono to poly phenolic groups in the structure. The flavonoids have been reported for their cytotoxic activity due to the presence of phenolic groups.[22] Anticancer activity was at its peak in methanolic extract indicating that most of the active components were extracted with methanol. Cytotoxic changes observed was cell aggregation, cell rounding and cell death. The overall results indicated the promising baseline information for the potential uses of the methanol extracts of the plants as an anticancer agent.

The association between flavonoids and reduced cancer risk has been reported in previous studies that show a decrease in cancer risk with consumption of vegetables and fruits rich with flavonoids.[23] Results of this study are in accordance with this finding since the phytochemical screening showed the presence of flavonoids in the extract and its fractions.[24] The anti-proliferative activity of total flavonoids and alkaloids isolated from different plants has been reported.[25] The significant activity of methanolic extract of the plant can be due to the induction of cell death by apoptosis that was shown in our results.

Our apoptotic test results would support the previous studies especially nuclear condensation and cell shrinkage which were clearly observed. This is not an exception since many commercially available chemotherapeutic agents and folk medicinal plants exert their anticancer effect by inducing cell apoptosis.[26] However, this needs further comprehensive studies to be fully substantiated.

The results suggest a possible selectivity and better activity of the extract against cervical cancer cell lines in comparison to lung cancer cell lines, as observed for the cisplatin compounds, which are preferentially used for testicular and ovarian cancer. The selectivity of action could be related to the differences in morphology and physiology between tested cell lines, although this is not yet proven.

The results are very encouraging and can promote a specific treatment for both the cancers. Although, it has not been reported in the scientific literature as a cytotoxic agent, the activity presented here may be related to the major presence of flavonoids in the extract, or even its performance in synergy with other constituents such as phenolic and flavonic constituents identified in this extract.

CONCLUSION

In the present study, we have observed that methanolic extract of *D. muricata* showed significant cytotoxicity against both the cell lines, but more for HeLa cell lines. Crude methanolic extract of *D. muricata* inhibited the cell viability in a dose dependent manner ranging from 25 μg/ml to 250 μg/ml. Our results from this *in vitro* study suggests that the methanolic and aqueous fraction of the extract of *D. muricata* have significant anti-proliferative activity against HeLa and A_{549} cell lines. On the basis of the present findings, it can be concluded that the crude extract of *D. muricata* can be a good source of cytotoxic compounds.

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Antistaphylococcal Activity of *Xanthium cavanillesii* Lactones

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ABSTRACT

Objective: The genus *Xanthium* L., of the *Asteraceae* Dum. family, (tribe Heliantheae) comprises 30 species of cosmopolitan distribution, many of which, as *X. spinosum* and *X. strumarium* are used as medicinal plants. This genus has been the object of numerous phytochemical investigations being sesquiterpene lactones with guaiane or secoguaiane frameworks the main secondary metabolites. Several sesquiterpene lactones have been demonstrated to have antimicrobial activity, in particular against Gram+ bacteria and in Uruguay the infusion of *Xanthium cavanillesii* Show (common name "Abrojo" or "Abrojo grande") which grows wild, is used as antiseptic in popular medicine. In this work we present the results of the antibacterial analysis of several extracts, fractions and pure compounds from *X. cavanillesii* against both sensitive and resistant strains of *Staphylococcus aureus*. **Materials and Methods:** Compounds were isolated from *X. cavanillesii* aerial parts by several chromatographic and spectroscopic methods antimicrobial analysis were performed according to Clinical and Laboratory Standards Institute guidelines. **Results:** The minimum inhibitory concentration (MIC) found were high for the sensitive 6538p strain when compared with common antibiotics. For the resistant strains, the pure compounds activity clearly outperformed the antibiotics, especially in the case of the multiresistant 700,699 strain with MICs of 31, 236 and 356 μg/mL for the *Xanthium* compounds, gentamicin and oxacillin respectively.

Keywords: Antimicrobial, abrojo, methicillin-resistant *Staphylococcus aureus*, sesquiterpene lactones

INTRODUCTION

In spite of the great advance in chemotherapeutics, infectious diseases are still one of the leading causes of death in the world. The World Health Organisation[1] states that infectious and parasitic diseases account for nearly 11 million among the 57 million total deaths in 2006.

Although it appears to be a great array of antibacterial and antifungal drugs in clinical use, the appearance of resistant organisms makes them sometimes ineffective or lead to recurrence.

Higher plants have shown to be an important source of new bioactive compounds, including antihypertensive, analgesics, cytotoxic compounds, amongst others.[2-5]

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Though no plant derived compound has been found to compete with clinically used antibiotics, to date, the great structural variety found in plants makes them attractive as a source of novel lead compounds. In fact, higher plants frequently exhibit significant potency against human bacterial and fungal pathogens.[6,7]

The genus *Xanthium* L of the Asteraceae family, (tribe Heliantheae) comprises 30 species of cosmopolitan distribution, many of which, as *X. spines* and *X. strumarium* are used as medicinal plants.[8,9] This genus has been the object of numerous phytochemical investigations being sesquiterpene lactones with guaiane or secoguaiane frameworks the main secondary metabolites.[10-13] In particular, in *X. cavanillesii*, the main sesquiterpene lactone constituents are xanthumin and its dihydro derivative.[14]

Several sesquiterpene lactones have been demonstrated to have antimicrobial activity, in particular against Gram+ bacteria,[15-17] and inhibitory activity on NF-κB activation.

The infusion of *Xanthium cavanillesii* Schouw (common name "Abrojo" or "Abrojogrande") which grows wild

in Uruguay is used as antiseptic in ethnomedicine.[18] In previous works we study its antimicrobial activities and toxicity and isolated a new sesquiterpene lactone, named xanchristin.[19]

In this work we present the results of the antibacterial analysis of several extracts, fractions and pure compounds from *X. cavanillesii* against both sensitive and resistant strains of *Staphylococcus aureus*.

MATERIALS AND METHODS

General experimental procedures

Gas chromatography (GC) analysis was performed in a Shimadzu GC 14 apparatus with an SE-52 column using a temperature program from 100 to 280°C with a 5°C/min gradient.

A Brucker micrOTOF-Q-TOF with electrospray ionization source in positive mode was used for mass spectrometry (MS) spectra and a Shimadzu QP 5050 with a SE 52 column was used for the GC-MS analysis.

Thin-layer chromatography (TLC) was performed on silica gel plates (Macherey Nagel, Dürin, Germany) using CH_2Cl_2/acetone (6:1) as solvent and H_2SO_4/heating or *p*-hydroxylbenzaldehyde as detection reagents.[13]

Infrared (IR) analysis was performed in a Nicolet 8700 Fourier transform (FT) - IR. Nuclear magnetic resonance (^{1}H NMR) spectra were obtained at 400 MHz and ^{13}C NMR spectra at 100 MHz, on a Brucker Advance DPX 400 spectrometer, using CD3OD or $CDCl_3$ as solvents and tetramethylsilane (δ_H 0.00) and acetone (δ_C 31.00) as references. 2D (different H,H-COSY, H,C-COSY, HMBC, HMQC and NOESY) and 3D (HSQC-TOCSY) experiments were carried out with programs available in the Brucker software.

Plant material

X. cavanillesii leaves were collected in Solymar (Canelones) near Montevideo and identified by Lic. F. Haretche, Museo y Jardin Botánico "Atilio Lombardo," Montevideo. Voucher specimens are kept in the MVFQ Herbarium, Jardin Botánico, Montevideo.

Extraction and isolation

X. cavanillesii leaves (240 g) were extracted exhaustively with CH_2Cl_2 (3 L) for 72 h in the dark at room temperature. After vacuum evaporation of the solvent the dichloromethane extract (14 g) was submitted to vacuum liquid chromatography on SilicaGel 40 (Merck, Darmstad). The extract was separated into eight fractions (hexane, hexane-CH_2Cl_2 4:1, hexane-CH_2Cl_2 1:1, CH_2Cl_2, CH_2Cl_2-ethyl acetate 1:1, ethyl acetate, acetone, MeOH). Fractions were analyzed by TLC and pooled.

A sample of the ethylacetate fraction was fractionated trough flash chromatography (Si 50 SF 15-24 g Varian) with dichloromethane/acetone 10:1 as eluent and fractions 1-6 pooled and further fractionated using normal phase (Si 50 SF 15-24 g Varian) and reverse phase (C18 SF 15-16 g Varian) flash chromatography. Finally, preparative TLC (Machery-Nagel) gave two compounds (2, 3).

Xanchristin (1) was already isolated as previously reported.[19]

Microbiological analysis

Minimum inhibitory concentration (MIC) was determined by the microdilution technique according to Clinical and Laboratory Standards Institute[20] using sensitive (ATCC 6538p) and resistant (ATCC 43300, ATCC 700699) strains. Gentamicin and oxacillin were used as control.

Bioautographies were made on developed and dried TLC plates according to the agaroverlay method of Rahalison[21] using *S. aureus* (ATCC 6538p).

4-*epi*-Xanthanol. Colourless oil, IR ν_{max} (cm^{-1}, thin film) 3350, 1762, 1735.

MS m/z (rel. int.): 248, 230, 204, 189, 176.

^{1}H and ^{13}C NMR spectroscopy (Table 1).

4-*epi*-Isoxanthanol. Light yellow oil, IR ν_{max} (cm^{-1}, thin film) 3400, 1765, 1740.

MS.): 248, 230, 204, 189, 176.

^{1}H and ^{13}C NMR spectroscopy (Table 1).

RESULTS AND DISCUSSION

The bioactivity guided fractionation of a chloroform extract of *X. cavanillesii* leaves yielded after repeated chromatography, among several others, three sesquiterpene lactones. From chromatographic and spectroscopic data (GC-MS, HR-ESIMS, 1D, 2D and 3D NMR) and

Table 1: NMR spectroscopic data of compounds 2-3

Carbon nº	2		3	
	δC	δH (m, J)	δC	δH (m, J)
1	149.7	-	146.0	-
2	74.8	4.03 (dd, 7.9, 5.3)	78.0	5.31 (dd, 9.8,4.3)
3	41.5	1.73 (m)	42.7	1.83 (m)
4	68.5	4.98 (m)	63.6	1.64 (m)
5	123.3	5.85 (dd, 6.3,3.4)	126.1	3.76 (m)
6	24.6	2.60 (ddd, 15.7, 9.4, 2.3)	24.6	5.96 (dd, 9.4, 3.6)
7	48.1	2.16 (ddd, 15.8, 11.3, 3.4)	47.3	2.63 (ddd, 16.2, 9.7, 2.3)
8	82.9	2.51 (m)	82.6	2.15 (ddd, 16.1, 11.5, 3.7)
9	36.4	4.41 (ddd, 11.0, 2.9, 2.3)	36.5	2.49 (m)
10	29.1	1.80 (m)	29.7	4.39 (ddd, 11.2, 2.8, 2.3)
11	140.0	2.30 (ddd, 12.0, 2.9, 1.5)	139.9	2.28 (ddd, 12.6, 3.1, 1.6)
12	170.7	2.81 (m)	170.8	2.84 (m)
13	117.6	-	117.6	-
14	19.1	-	18.3	-
15	19.8	5.59 (d, 3.2)	22.6	6.11 (d, 3.3)
CH_3CO	20.5	6.11 (d, 3.4)	19.8	5.59 (d, 3.2)
		1.22 (d, 7.4)		1.16 (d, 7.4)
		1.25 (d, 6.2)		1.21 (d, 6.2)
		2.04(s)		2.06 (s)
CH_3CO	171.1	-	170.8	-

NMR: Nuclear magnetic resonance

comparison with bibliographic data these compounds could be identified as xanchristin (1),[19] 4-*epi*-isoxanthanol (2)[22] and 4-*epi*-xanthanol (3) (Figure 1).[23,24]

Compound 1, with a new xanthanolide skeleton, was firstly isolated by us and compounded 2 and 3, although common *Xanthium* metabolites were never isolated before in *X. cavanillesii*.

The antimicrobial activity of the extract, fractions and pure compounds against sensitive and resistant *S. aureus* are presented in Table 2.

The MICs found were high for the sensitive 6538p strain when compared with common antibiotics. For the resistant strains, the pure compounds activity clearly outperformed the antibiotics, especially in the case of the multi resistant 700,699 strain with MICs of 31, 236 and 356 µg/mL for the *Xanthium* compounds, gentamicine and oxacillin respectively.

All the isolated compounds showed very similar activity against all strains that are consistent with previous studies as the principal pharmacophore of these molecules is the a-methylen-γ-lactone moiety, with the rest of the molecule acting as modulator of the activity.[25-28]

Table 2: MICs of samples against S. *aureus strains*

Sample/strain	MIC (µg/mL)		
	ATCC6538p	ATCC 700699	ATCC 43300
Xanthium extract	67	135	269
VLC 5	116	233	233
VLC 6	67	116	233
1	15	31	62.5
2	15	31	62.5
3	15	31	62.5
Gentamicin sulfate	4	236	15
Oxacillin	0.15	356	45

MIC: Minimum inhibitory concentration, VLC: Vacuum liquid chromatography

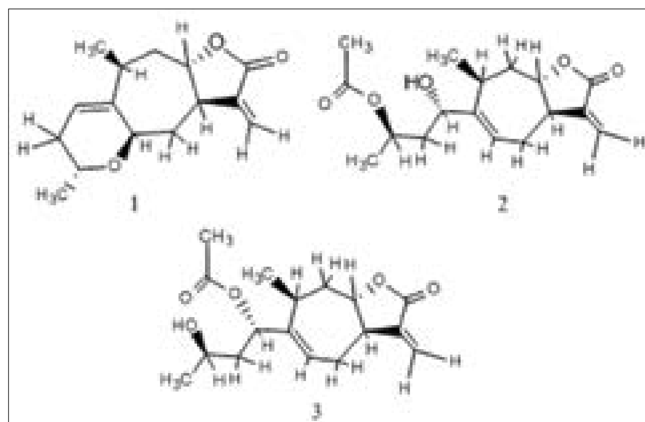

Figure 1: Structure of studied compounds, (1) Xanchristin, (2) 4-epi-Isoxanthanol: R1OH/R2AcO, (3) 4-epi-Xanthanol: R1AcO/R2 OH.

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