Botanical pharmacognosy of *Andrographis paniculata* (Burm. F.) Wall. Ex. Nees

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ABSTRACT

Introduction: *Andrographis paniculata* Nees is an important medicinal plant belongs to the family Acanthaceae. It is being used in traditional medicine, as a remedy for the cold, fever and detoxification of the body since time immemorial. Kalmegh has successfully halted the spread of 1919 Indian Flue (Influenza) pandemic. Drug has immense therapeutic potentials such as immunomodulatory, antibacterial and anti-inflammatory, laxative, depurative, prophylactic, hepatoprotective and cardiovascular effects. Most of the biological actions of *Andrographis paniculata* have been ascribed to its principal chemical constituent, Andrographolide. **Objectives**: The present work has been designed to delineate the Pharmacognostic profile of the leaves, stem, and root of *Andrographis paniculata* Nees. **Materials and Methods:** Macroscopic, microscopic evaluation, powder analysis, fluorescence standards of the drug and quantitative microscopy were carried out using the stem, root and leaves of *Andrographis paniculata* Nees **Results**: The presence of eucamptodromous pinnate venation with small, polygonal shaped areoles, upper epidermis lacking stomata, abaxial diacytic stomata, small palisade ratio, small stomatal index and fairly large Cystoliths in both upper and lower epidermis were features characteristics of the species. Quadrangular stem with dense collenchyma strands at the angles, uniseriate medullary rays with greater quantities of lignified fibers in the wood, abundant deposition of calcium oxalate in characteristic sites of the epidermal tissues of the lamina, ground tissues of petiole, stem, secondary xylem vessels of the root are also diagnostic characters of the taxon. **Conclusion:** The present study on botanical pharmacognosy of stem, root and leaves of *Andrographis paniculata* Nees thus provides useful information for quality control parameters for the crude drugs. Macro, microscopic, powder, quantitative and fluorescence standards discussed here can be considered as identifying parameters to substantiate and authenticate the drug. It could also fill the lacuna of our understanding about botanical pharmacognosy of *Andrographis paniculata* Nees.

Keywords: *Andrographis paniculata* Nees, palisade ratio, vein-islets, calcium oxalate, powder microscopy, fluorescent property

INTRODUCTION

Andrographis paniculata (Burm. F.) Wall. Ex. Nees. is a herbaceous medicinal plant, native to India, Taiwan and China. It belongs to the family Acanthaceae. All parts of the plant have bitter taste and it is commonly known as 'Kalmegh or King of Bitters. The global flu epidemic of 1919 was one of the most devastating infectious outbreaks in world history, killing millions worldwide. However, it is believed that the remarkable prophylactic benefits of Kalmegh have successfully halted the spread of Indian Flue (Influenza) pandemic. The aerial part of *A. paniculata* is commonly used in Chinese medicine. According to Chinese medicine theory, the plant possess an important cold property to get rid of internal body heat, inflammation, pain and dispose toxins from body and used as remedy for the cold, fever and detoxification, since ancient times.[1–2] Mostly the leaves and root are used in the traditional system of Indian medicine for the treatment of a wide spectrum of ailments, being as febrifuge, bitter tonic, stomachic, flatulence, wounds, ulcers,

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skin diseases, leprosy, diarrhea, dysentery, anthelmentic and cardiotonic. *A. paniculata* is a predominant ingredient in at least 26 Ayurvedic formulations and in several polyherbal preparations of Indian systems of medicine as a hepatostimulant/hepatoprotective agent.[3] Most of the biological actions of *Andrographis paniculata* have been ascribed to the diterpene lactone, Andrographolide.[1,4]

Since it is an important herbal remedy in traditional medicine, *Andrographis paniculata* has extensively been investigated by various workers for its phytochemical,[4–6] pharmacological,[7–9] microbiological[10–11] physiological[12–13] genetics[14] and seed germination studies.[15] However, there seems to be no systematic study on the botanical pharmacognosy of *A. paniculata* available in literature. Authentication of botanicals is a critical step to ensures sustained quality of herbal preparations, maximize the potentials of clinical efficacy and the reproducibility in future testing, thus could minimize or avoid the menace of potential adulterant/substituent.

The present study was undertaken with the objectives to delineate the pharmacognostical profile of stem, root and leaves of *A. panicula,* it may assist in standardization of samples of whole, cut or powdered plant material which could guarantee accurate means of identifying crude drugs and also fill the lacuna of our understanding about botanical pharmacognosy of *A. paniculata.*

MATERIALS AND METHOD

Plant Material

Andrographis paniculata Nees (Figure 1) is a much branched annual herb, 1–1.5 m height. Stem: sharply quadrangular. Leaves: simple, opposite, lanceolate, glabrous, 5–8 cm long, 1–2 cm wide, entire, acute, upper surface is dark green, pale beneath. Inflorescence: terminal or axillary panicle. Flowers: small, white with purplish or violet markings. Calyx: 5-partite, pubescent. Corolla: bilabiate, hairy upper lip oblong, lower lip 3-lobed. Stamens: two, inserted in the throat, ovary 2-celled. Fruit: capsule, linear-oblong, two celled, compressed, longitudinally furrowed on broad faces, acute at both ends, glandular-hairy. Seeds; small, 6–10, round or ovoid, yellowish brown. Root is cylindrical, curved, tapers, 5–20 cm long and 1.5–5 cm in diameter. Externally it is grayish brown, when fractured, the inside is starchy white.

Methods

Andrographis paniculata Nees was collected from the Thiruvananthapuram District of the State of Kerala and

Figure 1. *Andrographis paniculata* Nees

identification was done using Flora of Presidency of Madras. The investigation on the transverse, tangential, radial and paradermal sections of the stem, root and leaves of the plant materials were undertaken in the Drug Standardization Laboratory of the Government Ayurveda College, Thiruvananthapuram and where voucher specimens were deposited. Microphotographs of sections and powder analysis were made by using Olympus Microscope (Model CX 41; Tokyo, Japan) with CCD camera 2 mega pixel and quantitative measurements were taken using Olympus Image-Pro Plus, version 5.1 software. Fine hand sections of lamina, petiole, stem and root, and epidermal peels were taken using standard procedures and were stained with Aqueous Safranin 1% and mounted in glycerin. Fluorescence analysis of the powder was carried out in UV light (256 nm & 366 nm) using Camang UV apparatus. The descriptive terms of the anatomical features used here as per Hickey[16] Metcalfe and Chalk[17] and Carlquist.[18] Microscopic descriptions of tissues of various plant parts are supplemented with micrographs wherever necessary and magnifications are indicated by the scale-bars in the figures. The number of epidermal cells, stomatal number, stomatal index were calculated per square millimeter of leaf area from intercostal areas of fresh leaves and vein islet number and vein termination

number were calculated from cleared leaves as defined by Salisbury.[19] Palisade ratio was determined based on Wallis[20] and size of Guard Cell Area (GCA) was estimated following Franco's formula.[21] For analysis of the Fluorescence properties of crude drug, the solvents of HPLC/ Chromatographic Grade procured from Merck and Qualigens Fine Chemicals, India were used.

RESULTS AND DISCUSSION

Microscopic Evaluation of Leaves

The leaf of *Andrographis paniculata* is microphyll, which consists of an average length of 5.3 cm and width of 1.2 cm. Leaf is dorsiventrally differentiated. The shape of the T.S. of midrib is characteristic, which projects strongly at two corners on the lower side with a prominent ridge having shallow groove in the middle on the adaxial side (Figure 2). Lamina is flat and much reduced in dimension (Figure 2a). Midrib consists of epidemics, collenchyma, mesophyll and vascular tissues. Collenchymatous hypodermis comprises 7–8 layers. A chlorenchyma zone consists of 2–3 layers are located beneath the hypodermis, which is followed by parenchymatous ground tissues. The midrib possesses an arc of xylem lies in the middle of the ground tissue. Xylem vessels (30–35 µm in diameter) arranged in radial rows of 5–6 and phloem lies on abaxal side. Both the epidermis of the lamina is uniseriate, composed of compactly arranged rectangular cells with cuticle in the outer walls. Cells of the upper epidermis are larger in size than the lower surface. The mesophyll is divided into upper palisade and lower spongy tissues (Figure 2a). A single layer of columnar palisade, cells are filled of plenty of chloroplasts. The palisade ratio was found to be about 2. Spongy parenchyma cells are 3 layered, cells of the spongy mesophyll are loosely arranged with wide intercellular spaces (Figure 2b).

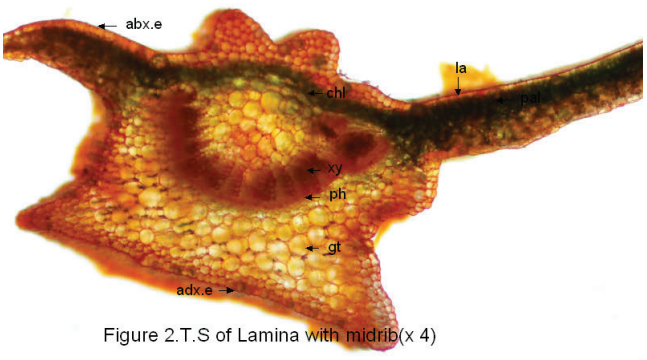

Figure 2. T.S. of Lamina with midrib (× 4).

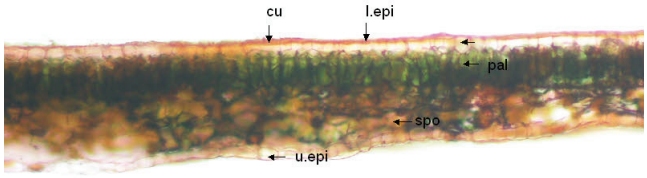

Figure 2a. T.S. of Lamina of *Andrographis paniculata* Nees (× 10).

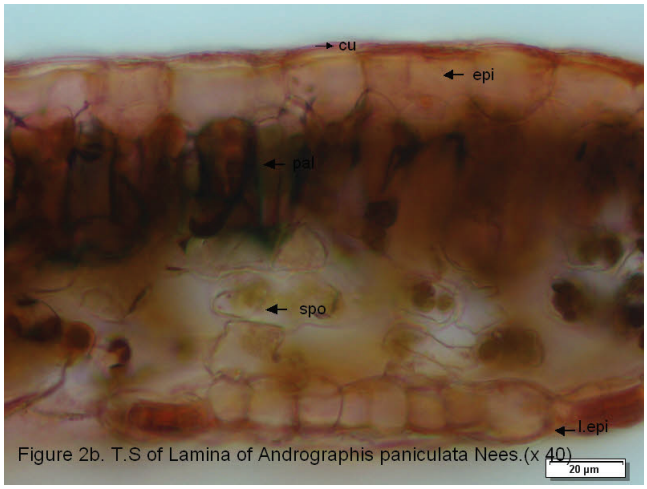

Figure 2b. T.S. of Lamina of *Andrographis paniculata* Nees (× 40).

Epidermal Characters

Surface features of both upper and lower epidermis revealed many fairly large cystoliths (Figure 3a & 3b) measuring an average length of 38.9 µm and width 16.5 µm (ranges 31.6–46.6 µm in length × 13.9–19.2 µm in width). The adaxial epidermis is devoid of stomata (Figure 3b & 3c), the abaxial (lower) epidermis shows diacytic stomata (Figure 3 & 3a). The mean length and

Figure 3. Surface features of abaxial epidermis with stomata (× 40).

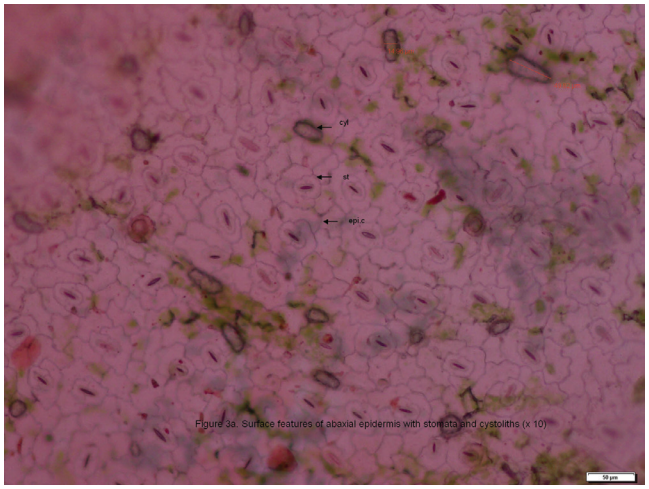

Figure 3a. Surface features of abaxial epidermis with stomata and cystoliths (× 10).

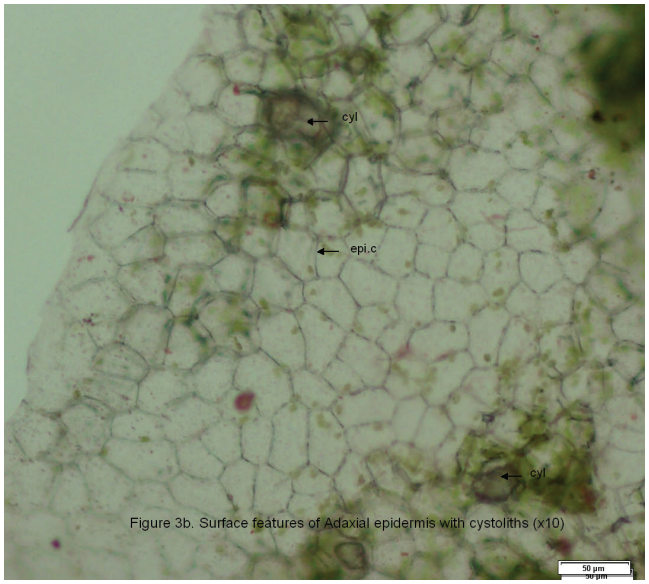

Figure 3b. Surface features of adaxial epidermis with cystoliths (× 10).

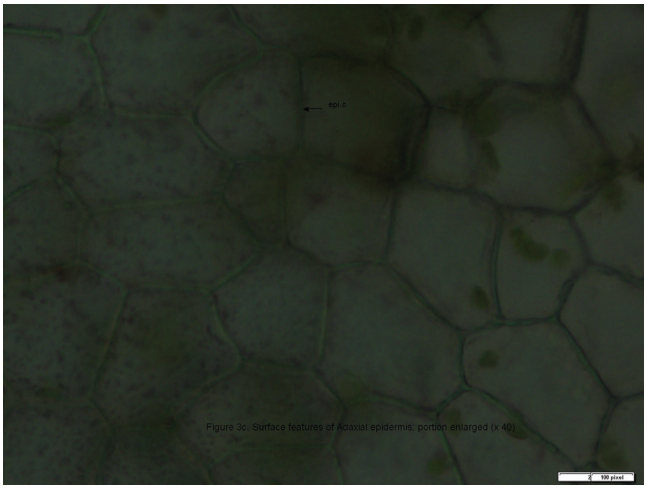

Figure 3c. Surface features of adaxial epidermis: portion enlarged (× 40).

breadth of stoma was observed as16.9 μm × 8.2 μm and Guard cell area (GCA) was found to be 107.3 μm^2. Mean density of abaxial (lower) stomata per square millimeter area of leaf was found to be 172.1 (Table 1). Epidermal cells were wavy in nature. The number of abaxial epidermal cells per square millimeter area of the leaf was observed as 1110.9 and stomatal index for the lower surface was found to be 13.4.

Petiole

In cross sectional view, the petiole has a characteristic structure with shallow adaxial grooves (Figure 4). A chlorenchyma zone consisting of 2–3 layers are located beneath the epidermis, which is followed by parenchymatous ground tissues. The 15–16 vascular bundles are arranged in crescent shape, lies in the middle of the ground tissue. Each vascular strand is separated from one another by wide areas of ground tissues. Xylem vessels are aligned in radial rows of 5–6 and phloem lies on abaxal side. Some of adaxial epidermal cells of the petiole are provided with non-glandular trichomes. Trichomes are uniseriate and 3-celled.

Table 1. Quantitative microscopy of *Andrographis paniculata.*

Parameters	Mean value	Range
Stomata length (μm)	19.3	16.8–20.9
Stomata width (μm)	11.3	8.2–13.4
*Guard cell area GCA (μm^2)	171.3	108.2–220.0
Stomatal density/mm^2 (lower)	172.1	155.1–192.8
Epidermal cell density/mm^2	1110.9	1045.4–1211.6
Stomatal index	13.4	12.9–13.7
Vein-islet number/mm^2	0.76	0.75–0.82
Vein-termination/mm^2	0.86	0.8–0.91
Palisade ratio	2.2	2.0–2.2
Size of areoles (mm)	1.0	0.96–1.1

** Franco's formula.*

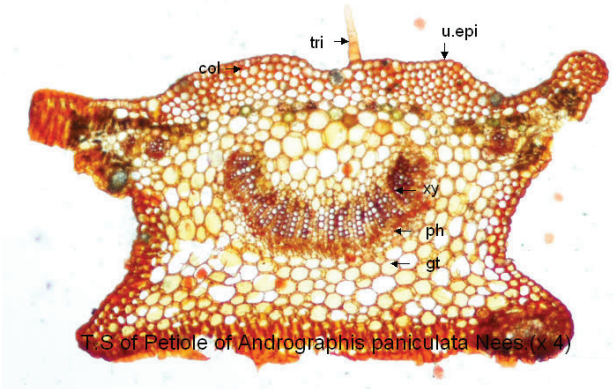

Figure 4. T.S. of Petiole of *Andrographis paniculata* Nees (× 4).

Venation Pattern

Leaf architectural characters could provide useful anatomical information for characterization of the taxon.[22] Venation patterns of cleared leaves were studied and terminology used for description of architecture is as per Hickey.[16] Petiolate simple leaves with entire margins had observed eucamptodromous pinnate venation under low (× 2) magnification. Areolation was poorly developed. Areoles are small, area of areoles ranges from 0.96 to 1.07 mm[20] and areoles are polygonal in shape (Figure 5). Within the areoles terminal vein-endings was absent. Marginal ultimate veins were looped (Figure 5a). The minor venation pattern viz. mean number of vein islet number/mm^2 of leaf was found to be 0.756. Veinlets termination number/mm^2 were found 0.864 and average size of areoles was observed as 1.02 mm. The number of areoles/mm^2 was found to be 0.324 when critically analyzed microscopically.

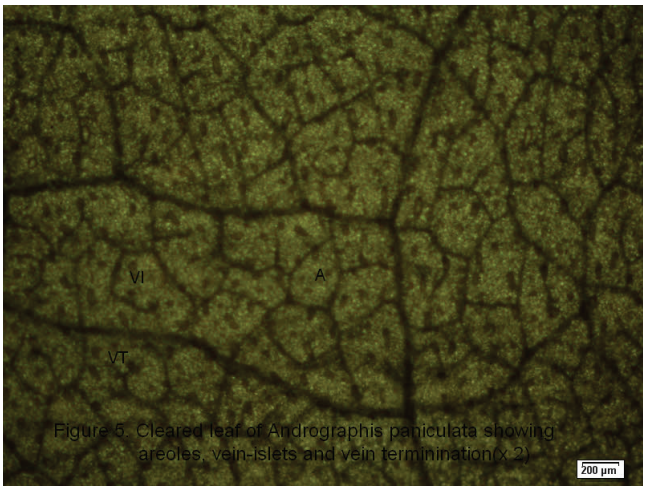

Figure 5. Cleared leaf of *Andrographis paniculata* Nees showing areoles, vein-islets and vein termination (× 2).

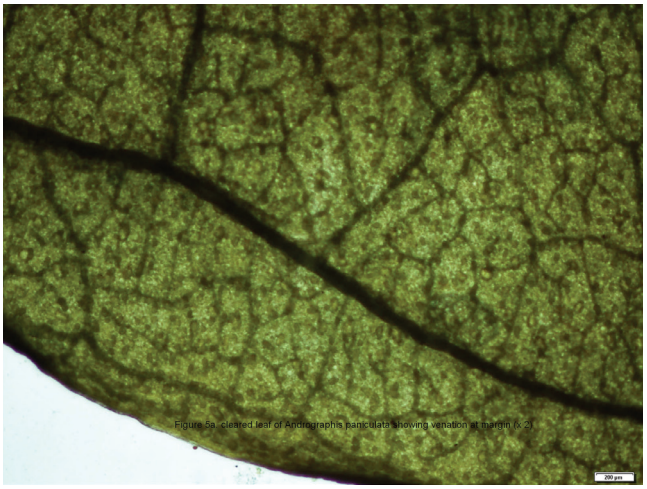

Figure 5a. Cleared leaf of *Andrographis paniculata* Nees showing venation of the leaf margin (× 2).

Microscopic Evaluation of Stem

T.S. of the stem possesses a quadrangular outline with dense collenchyma strands at the four angles (Figure 6) of the stem. Epidermis is composed of single layer of rectangular cells. There is a group of 2–3 layered collenchymas cell zone with secretary cavities having white colored deposition are present under the epidermis. Cortex forms a narrow zone, composed of 5–6 layers of parenchyma cells with chloroplast. Solitary sclereids and a group of sclereids of 4–6 are present in the cortex followed by a layer of thick-walled endodermis and parenchyma contains chloroplastid. Solitary sclereids are present in secondary phloem tissues. Xylem is very prominent and occupies the major portion of the stem **(**Figure 6a**)**. Vessels are mostly solitary and small sized, majority of them are arranged in radial rows. Vessels are circular or polygonal in shape (Figure 6c). The Diameter of vessel

Figure 6. T.S. of stem of *Andrographis paniculata* Nees (× 4).

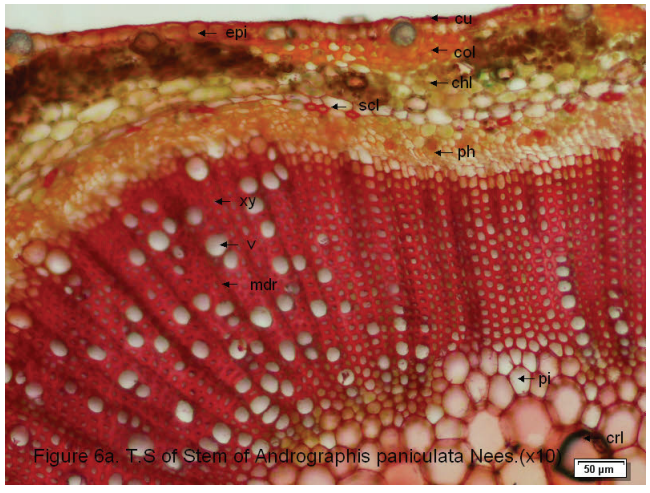

Figure 6a. T.S. of stem of *Andrographis paniculata* Nees (× 10).

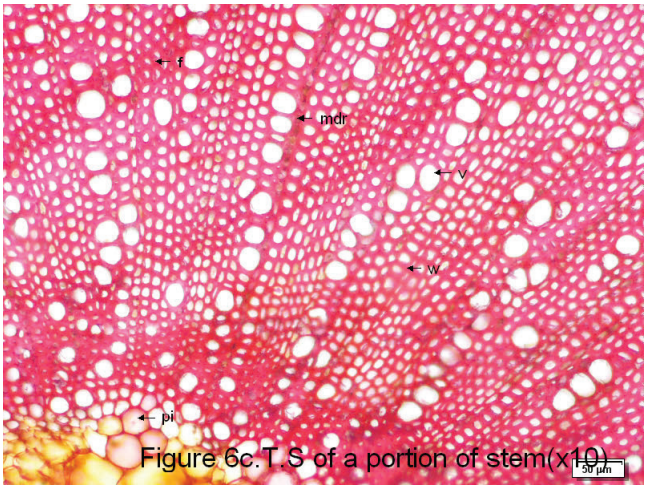

Figure 6c. T.S. of stem of *Andrographis paniculata* Nees: a portion enlarged (× 10).

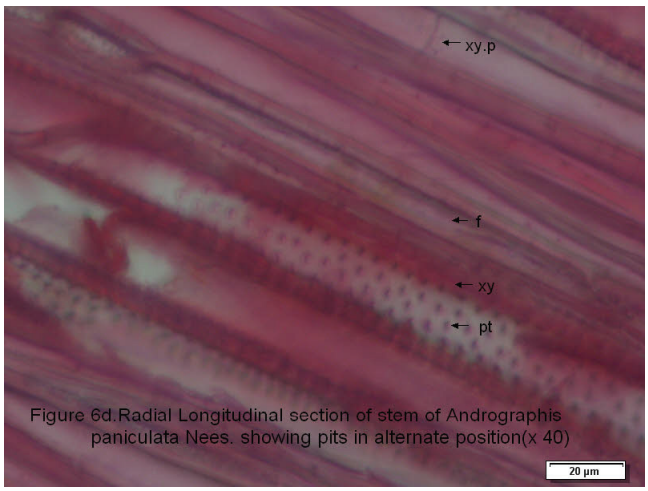

Figure 6d. R.L.S of stem of *Andrographis paniculata* Nees showing pits in alternate position (× 40).

lumen ranges from 18.3 µm to 35.8 µm. Mean number of vessels per square millimeter of the xylem was found to be 393.8 (ranges 419.8 to 345.4). Wood with spiral, reticulate and pitted xylem vessels were revealed. The vessels with bordered pits and intervessel pitting were of alternate position (Figure 6d) were observed. The bulk of the xylem is constituted by fibers. Medullary rays are very conspicuous and many in number. Rays are mostly uniseriate, biseriate rays are also found occasionally. Centrally placed pith with large parenchymatous cells and cells are polygonal in shape. Some of the pith cells contain prismatic crystals of calcium oxalate (Figure 6a).

Microscopic Evaluation of Root

Conspicuous cork is composed of 8–10 layers (Figure 7). Outer 2–3 layers are thick walled cells filled with reddish brown content, followed by 6–7 layers of thin walled square or rectangular cells, a narrow band of parenchyma interior to the cork has numerous sclereids aligned in more or less a ring (Figure 7a) and pit apertures about 2.17 µm in diameter. Wood is very prominent and occupies the major portion of the root. Wood consists of a large number of vessels. Vessels are mostly solitary and small sized, majority of them are arranged in radial rows. The vessel groupings of radial multiples of four or more are quite common. Vessels groupings in radial multiples of two and three were also occasionally found. Vessels are circular, elliptical or polygonal in shape. The Diameter of vessel lumen ranges from 12.61 µm to 39.6 µm. Mean number of vessels per square millimeter of the wood was found to be 417.4 (ranges 389.8 to 425.4). Tylosis are occasionally found. The bulk of the wood is constituted by fibers (Figure 7f).Medullary rays are very conspicuous and many in number. Medullary rays are mostly uniseriate and biseriate (Figure 7c). Cells of the xylem vessels contain prismatic crystals of calcium oxalate (Figure 7d);

Figure 7. T.S. of root of *Andrographis paniculata* Nees (× 4).

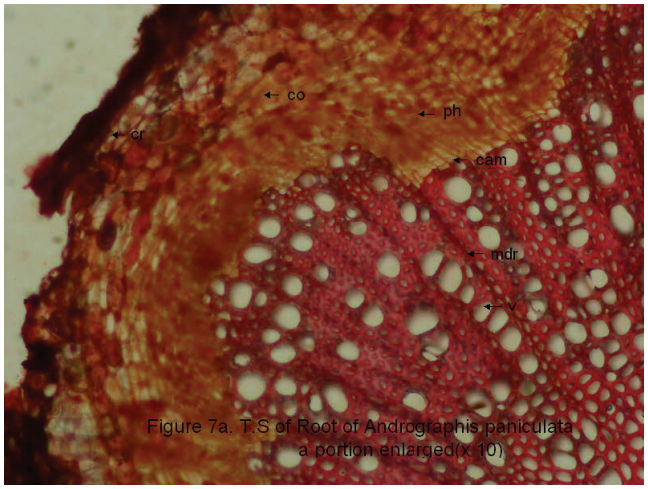

Figure 7a. T.S. of root of *Andrographis paniculata* Nees: a portion enlarged (× 10).

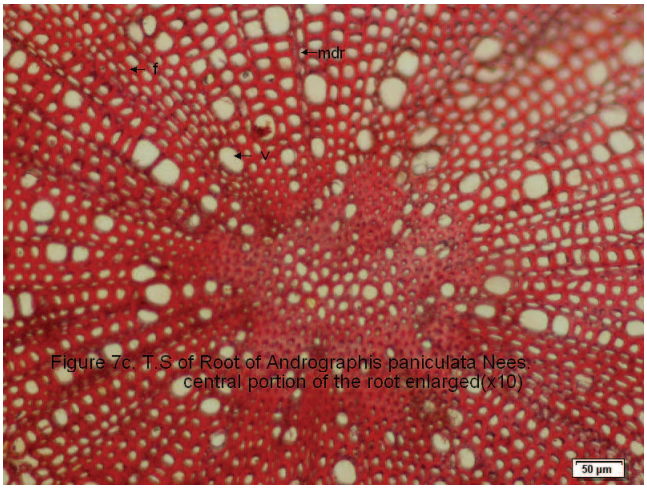

Figure 7c. T.S. of root of *Andrographis paniculata* Nees: central portion enlarged (× 10).

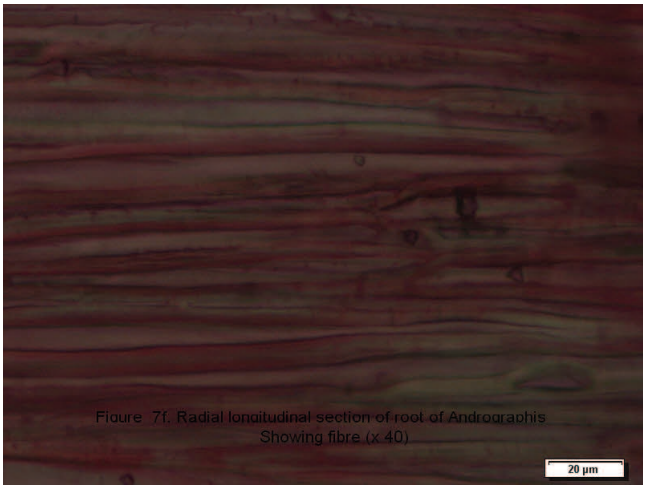

Figure 7f. R.L.S. of root of *Andrographis paniculata* Nees showing fibres (× 40).

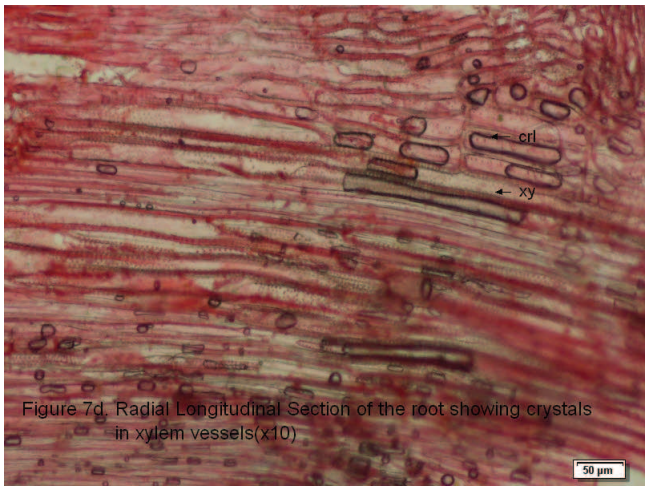

Figure 7d. R.L.S. of root showing crystals in xylem vessels (× 10).

ranges from 7.4–11.6 µm and 24.7–42.2 µm in length. Radial longitudinal section showed the vessel to vessel pitting was in alternate position (Figure 7e). The pits moderately sparse and pit aperture about 2.69 µm in diameter.

Powder Microscopy

The dried leaves, root and stem of *Andrographis paniculata* were analyzed for powder characteristics. Microscopic examination showed fragments of leaf epidermis with diacytic, stomata (Figure 8g) and fairly large cystoliths in upper and lower epidermis (Figure 8 and 8f). Stem and root powder showed abundant pyramidal calcium oxalate crystals (Figure 8c).Vessel elements with bordered pits and intervessel pitting in alternate position (Figure 8b) and lignified fibers with pointed ends (Figure 8d) were observed. Fragment of parenchymatous tissues of the cortex (Figure 8h) were also observed.

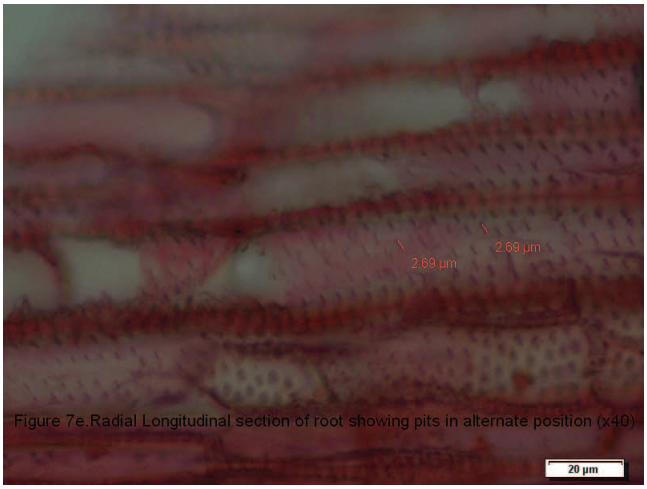

Figure 7e. R.L.S. of root of *Andrographis paniculata* showing pits in alternate positions (× 40).

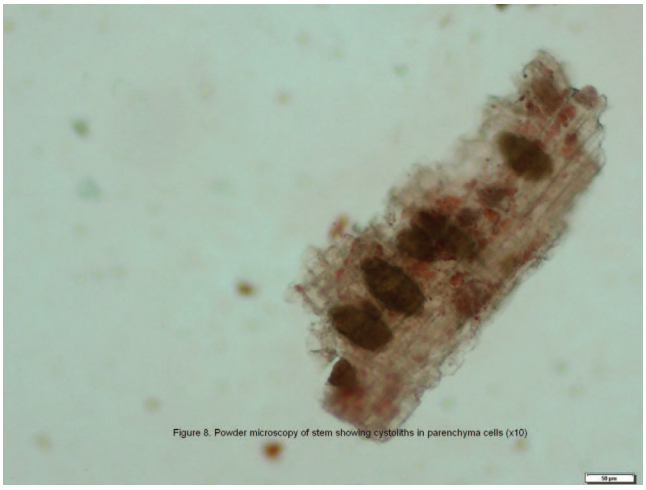

Figure 8. Powder microscopy of stem showing cystoliths in parenchymatous cells (× 10).

Figure 8b. Powder microscopy of root- vessel elements (× 40).

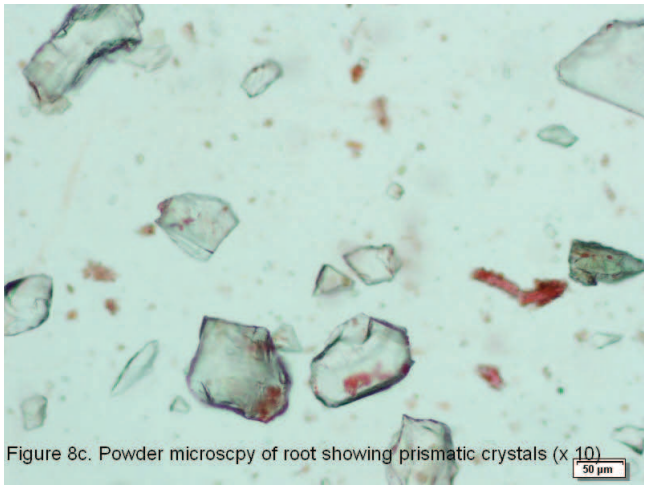

Figure 8c. Powder microscopy of root showing prismatic crystals (× 10).

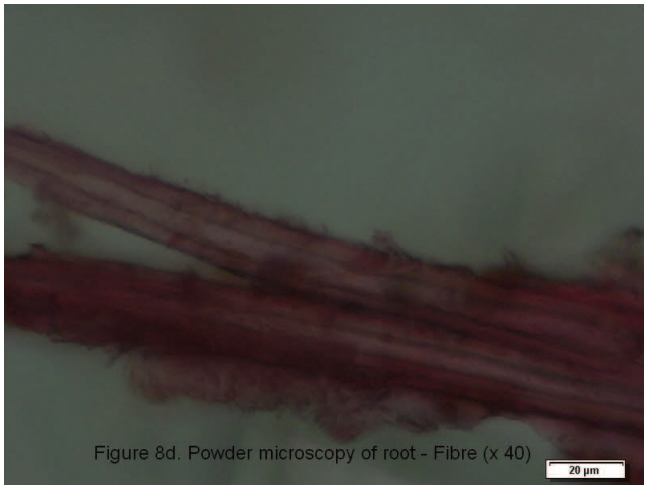

Figure 8d. Powder microscopy of root-Fibre (× 40).

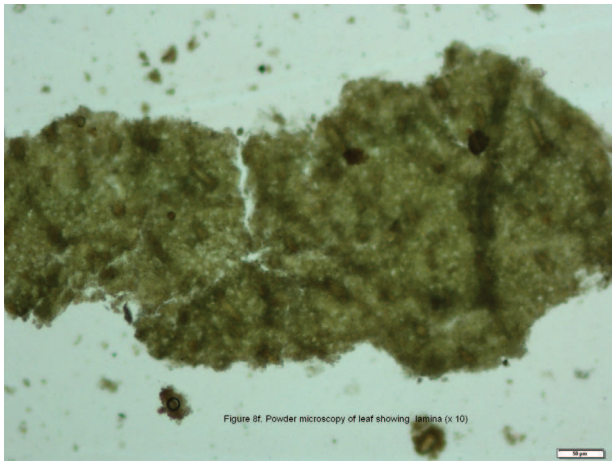

Figure 8f. Powder microscopy of leaf showing lamina (× 10).

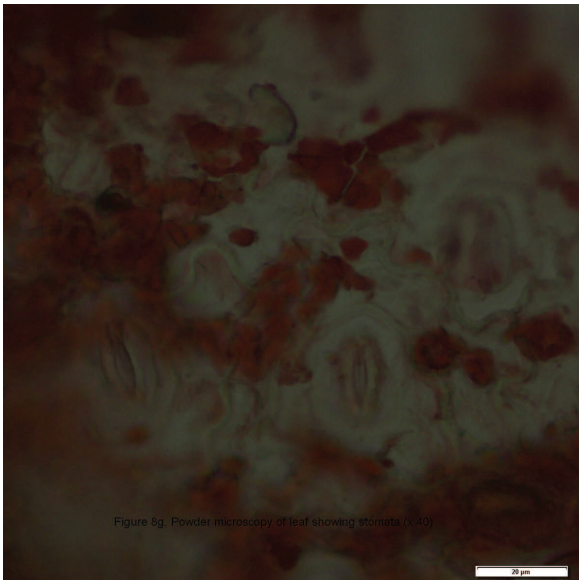

Figure 8g. Powder microscopy of leaf showing stomata (× 40).

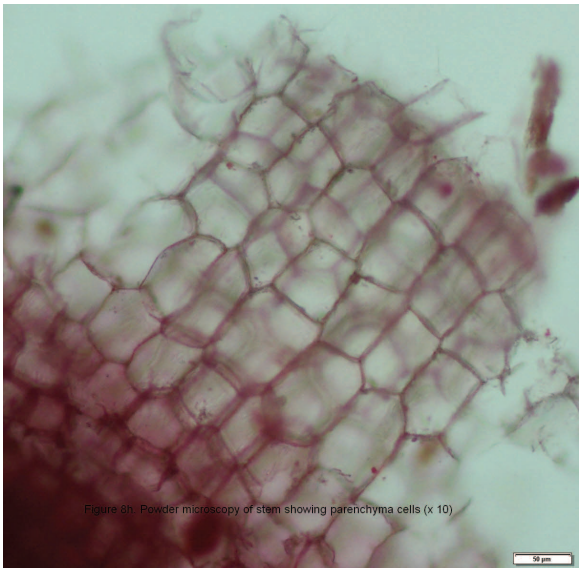

Figure 8h. Powder microscopy of stem showing parenchyma cells (× 40).

Fluorescence Analysis

The use of fluorescence can be very useful adjunct to botanical pharmacognosy, since it can be applied as rapid and easy test to verify certain identifications of the botanicals. When exposed to the day light, root powder of *Andrographis paniculata* was found to be white color, stem powder was green and leaf powder was found to be of dark green color. Powder of whole plant mixture was found to be light green color. The Fluorescence property of the powdered drug extracts taken in different solvent systems was analyzed under UV light (long and short). Specimens were recorded as either fluorescent (with color and intensity) or not fluorescent and their responses under UV light are presented in Table 2.

CONCLUSIONS

The present study on botanical pharmacognosy of *Andrographis paniculata* provides useful information for quality control parameters for the crude drugs. Macro, microscopic, powder, quantitative and fluorescence standards discussed here can be considered as identifying parameters to substantiate and authenticate the drug.

Abbreviation used in the Figures are

A = areola; abx = abaxial; abx.e = abaxial epidermis; adx = adaxial, adax.e = adaxial epidermis; b.c = basal cell; cam = cambium; ck, cr = cork, co = cortex; col = collenchyma; crl = crystal; cut = cuticle; cyl = cystoliths; ep, epi.c = epidermal cell; epi = epidermis; f = foot cell; gu.c = guard cell; gt = ground tissue; la = lamina; l. up = lower epidermis; mdr = medullary rays; pal = palisade cell; ph = phloem; pi = pith; scb = sclerenchymatous bundle sheath; spa, spo = spongy parenchyma; s.co = secondary cortex; s.ph = secondary phloem; st = stomata; stcl = stone cell; str = starch grain; s.xy = secondary xylem; tri = trichome; t.c = stalk cell; xy = xylem; u.epi = upper epidermis; tyl = tylosis; vb = vascular bundle; VI = vein islet; VT veinlet termination; wd = wood.

Table 2. Fluorescence properties of the extract of root, stem and leaves of *Andrographis paniculata* in various solvents.

Solvent	Under UV (256 nm)	Under UV (366 nm) (color & intensity)
Aqueous	$NF^{S, L, R}$	milky white^{S,} $NF^{L, R}$
Methanol #	$NF^{S, L, R}$	white ^{S,} milky white$^{L, R}$
Ethyl Alcohol #	$NF^{S, L, R}$	pink- orange $^{S, L, R}$
Acetone *	$NF^{S, L, R}$	(medium) pink ^{S,} (high) pink^{L}, milky white^{R}
Chloroform *	$NF^{S, L, R}$	white$^{S, R}$ NF^{L}
Ethyl acetate *	$NF^{S, L, R}$	(medium) pink^{S,} (high) pink^{L,} milky white^{R}
Benzene #	$NF^{S, L, R}$	(light) pink^{S,} (high) pink^{L,} NF^{R}
Toluene #	$NF^{S, L, R}$	(medium) pink^{S,} (high) pink^{L,} NF^{R}
Cyclohexane #	$NF^{S, L, R}$	(light) pink$^{S, L,}$ NF^{R}

*from *MERCK*; # from *Qualigens*; *NF = not fluorescent* ^{R} *= extract of root,* ^{S} *= extract of stem,* ^{L} *= extract of leaf*

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Pharmacognostic studies on *Tephrosia jamnagarensis* Sant. – An endemic species of India

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ABSTRACT

Introduction: *Tephrosia jamnagarensis* Sant. is a rare endemic species, restricted to the semiarid region of India. **Methods:** The present study gives insight into the anatomical, pharmacognosy, histochemical and preliminary phytochemical analysis of the root, stem and leaf of *Tephrosia jamnagarensis*. The analysis's were done according to WHO norms. **Result:** The anatomical study of all of root and stem showed the characteristic rhomboidal calcium oxalate crystals in medullary rays. The paedomorphic ray and medullary phloem were observed in the mature stem. Micro–morphological parameters such as the stomatal index, palisade ratio and vein-islet number were distinctly quantified. The powder study showed the presence of warty unicellular trichomes and anisocyctic stomata in leaf and stem whereas calcium oxalate crystals and pitted xylem vessels were observed in roots, stem and leaves. The histochemical tests performed indicate the presence of mucilage and alkaloids. Phytochemical screening shows the presence of alkaloids, phenolic acids, flavonoids and mucilage. **Conclusion:** The standardization parameters provide referential information for correct identification of the plant material and will also be useful in preparation of monographs on these plants.

Keywords: *Tephrosia jamnagarensis,* endemism, rhomboidal crystals, phytochemical analysis, histochemical test

INTRODUCTION

Tephrosia genus comprise of 393 species distributed all over the world,[1] of which 53 species are rare and endangered.[2] In India, 35 species of *Tephrosia* are recorded.[3] Among them 12 species are recorded from semi-arid region[4] of which two species are endemic (*T. jamnagarensis* Sant. and *T. collina* Sharma).[5] *T. jamnagarensis* is an endemic plant of Saurashtra peninsula, Gujarat, India. It was first discovered by Santapau and was identified as new species based on the fruit characters.[6,7] The first detailed morphological description of this plant with flower was given by Smith *et al.*; stating it as a new species *Tephrosia axillaris*[8]. Later, this species was reconfirmed by Raghava *et al.* reported that *T. axillaris* was assigned as the synonym of *T. Jamnagarensis*.[9] *T. jamnagarensis* is an erect or sub erect, annual herbs; stem simple or sparsely branched, covered with whitish appressed hairs. Leaves, 3–5.2 × 5–9 cm, linear, glabrous adaxially, densely hairy abaxially, petioles 2–3 mm, hairy; stipulates subulate, 3–4 mm hairy. Flower single or in pairs axillary; pedicels 2–3 mm densely hairy. Calyx 1–3 mm, hairy, teeth subulate, filiform subequal. Corolla mauve, 2.5–3 mm abaxially hairy and with oil gland. Legumes compressed 20 × 5 mm, densely and patently hairy with greyish tinge oblique at both end, apiculate; seed 5–6 renifrom, dull or matt, brownish. Flowers and fruits: Sep–Oct. (Plate: 1- A, B, C)

As on today only morphological description is available, thus this paper deals with the anatomical, pharmacognosy ,histochemical and preliminary phytochemical analysis of roots, stem and leaf of *T. jamnagarensis*.

MATERIALS AND METHODS

Collection

T. jamnagarensis was collected in October 2011 from Khadkambaliyavidi, Jamnagar and was grown at arboretum of The M. S. University of Baroda, Vadodara,

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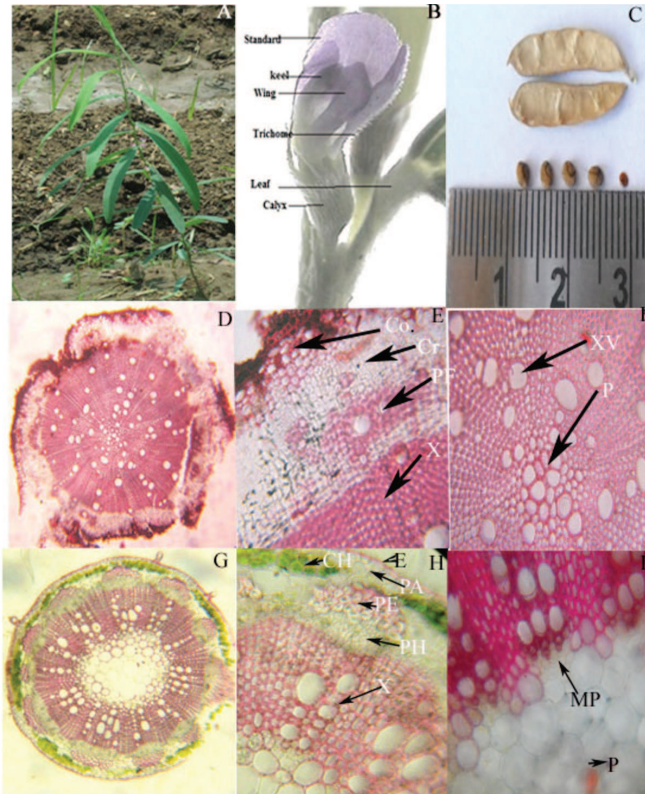

Plate 1. A) - Habit of *T. Jamnagarensis*, B) - Flower, C) - Seed, D, E, F) - T.S. of root, G, H, I) - T S of stem Co-cork, Cr-Cortex, PF-Phloem Fiber, X-Xlyem, XV-Xylem vessel, P-Pith, E-Epidermis, CH-Chlorenchyma, PA-Parenchyma, PE-Pericycle, PH-Phloem, MP-Medullary Phloem.

Gujarat, India. It was identified, authenticated and deposited at Botanical Survey of India (BSI) Arid zone Jodhpur, Rajasthan. (The voucher specimen No.-BSI/AZC/I12012/Tech/2011-12(Pl.Id)/548).

Anatomy

The free hand sections of all the three plant parts were done. Sections of 10–15 μ thickness were selected. These selected sections were stained with Safranin (0.5%) in water and the mounted in 50% glycerine. The slides were then observed under the microscope and the sizes of various cells observed in the tissues were measured using an ocular micrometer. The least count of the micrometer was calculated for this purpose. The sections were photographed under Lecia DM 2000 microscope connected to Digital Canon Camera.

Micromorphology

Fresh plant materials were washed and small fragments of leaves were taken from the middle region of the lamina of mature leaves. The epidermal layer was peeled off with the help of pointed needle and blade and was washed in water, stained with safranin (0.5%) and then mounted of slide and viewed under the microscope. Stomatal index, palisade ratio, vein termination number and vein islet number were then calculated using standard procedures.[10]

Powder Studies

Completely dried plant material was finely powdered and sieved through BSS mesh No. 44. The fine powder obtained was stained using safranin in water. The stained powder was mounted on a slide and observed under a microscope to locate and identify the distinguishing characters. The characters observed were photographed under Lecia DM 200 microscope connected to a Digital Canon camera.

Histochemical Tests

Specific reagents for identification of important classes of compounds were prepared according to procedures prescribed in the WHO guidelines. Sections and powder of root, stem and leaves were treated with reagents and mounted on slides for observations under microscope.[11]

Preliminary Phytochemical Screening

The coarsely powdered leaves, stem and roots were extracted with different solvents by using Soxhlet's apparatus and analysed using simple chemical tests for preliminary screening of various groups of phyto-constituents such as alkaloids, flavonoids, phenolic acids, iridiodes and so on, as per WHO guidelines.[11,12]

RESULTS AND DISCUSSION

Anatomy

The roots of *T. jamnagarensis* Sant. are cylindrical, slender, brown of 0.45–0.54 mm in diameter. The roots showed the presence of rootlets and possess no characteristic taste. The transverse section of root is circular in outline (Plate: 1 D, E, F). The outer most layers shows 7–10 layered dark brown cork cell. The cortex is narrow composed of 7–8 layers parenchymatous cells. The stelar region shows absence of the endodermis and pericycle. The cortex is interrupted by the phloem (125–85 μm) which shows prominent phloem fibre patches (25–62.5 μm). The xylem tissue composed of xylem vessels, xylem fibres and xylem parenchyma which stores rhomboidal calcium oxalate crystals. Pith is absent.The stem is erect, cylinder, pubescent, slender and green in colour of diameter 1–0.81 mm. In T.S. of stem is

circular (Plate: 1-G, H, I). The epidermis is single layered shows barrel shaped cells lined by thin cuticle and warty unicellular trichomes (375 × 18.75 μm). Hypodermis consists of one layer of collenchyma (27.5 μm) followed by cortex of 2–3 layers of chlorenchyma (26.67 μm) patches alternating with parenchyma patches. Endodermis is distinct above pericycle. Pericycle is composed of interrupted ring of sclerenchyma patch containing of calcium oxalate crystal. Phloem is found in patches separated by large medullar cells. Xylem consists of angular vessels (23.8 μm), paedomorphic ray with calcium oxalated crystals and xylem fibres. Medullary phloem patches are seen towards the pith. Pith is large, round and parenchymatous.The transverse section of leaf shows lamina segregated into upper epidermis, palisade, spongy and lower epidermis (Plate: 2 -J). The upper and lower epidermis composed of large barrel shape cells covered by thick cuticle with anisocytic stomata. The trichomes present on the lowerside are warty unicellular trichome (343.5 × 65 μm). The mid rib shows the presence of large vascular bundle (28.8 μm) having pericycle on the either side of vascular bundle. The pericycle composed of the sclerenchyma cells containing rhomboidal calcium oxalate crystal. The vascular bundle composed of xylem and phloem. Mesophyll consists of three to four layered palisade (89.6 μm) and closely packed spongy tissues (22 μm).

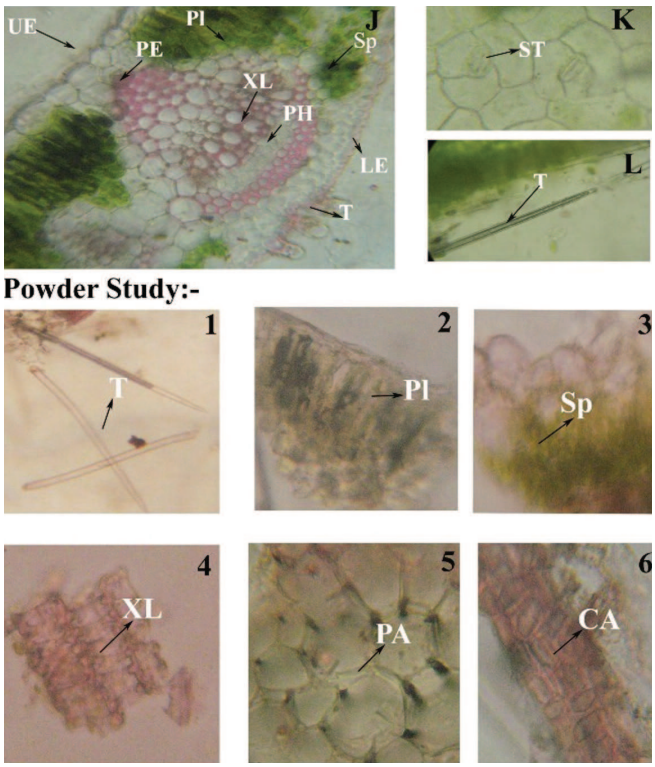

Plate 2. J) T S of Leaf, K, L) peel position showing stomata and tri-chome, 1, 2, 3, 4, 5, 6) powder study. UE-Upper epidermis, LE-Lower epidermis, PI-Palisade, SP-Spongy, XL-Xylem, PE-Pericycle, PH-Phloem, ST-Stomata, T-Trichome, PA-Parenchyma, CA-Calcium oxalate csystal in xylem fiber.

Micromorphology

The leaves and stem of the *T. jamnagarensis* show the presence of the anisocytic stomata (Plate: 2) and warty unicellular trichomes (Plate: 2). The values of the stomatal index, vein termination no, vein islet no and palisade ratio are given in Table 1.

Powder Study

The powder of leaves and stem are green in colour whereas root is brownish in colour. The powder all the three parts are coarse in texture, bitter in taste and have a characteristic odour. The microscopic observations of all three plants showed following characteristic. The warty unicellular elongated trichome, anisocytic stomata, palisade, spongy, parenchyma, collenchyma in powder of leaves and stem, chlorenchyma in stem, cork cell in root powder, xylem vessel showing large rhomboidal calcium oxalate crystals, xylem vessel with annular and pitted thickening. (Plate: 2)

Histochemical Test

The results of histochemical detection are furnished in Table 2.

Preliminary Phytochemical Screening

Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phenolic acids and gums and mucilage's. (Table 3)

Table 1. Results of Leaves Micrometry of *T. jamnagarensis* Sant.

Stomatal index	18.4 ± 3.13
Palisade ratio	4.4 ± 0.5
Vein islet no.	3.6
Vein termination no.	1 or 2

Table 2. Histochemical Test on the Sections of *T. jamnagarensis* Sant.

Cell content	Reagent used	Root T.S.	Stem T.S.	Leaves T.S.
Tannin	Ferric chloride	–	–	–
Calcium oxalate	Concentrated Hydrochloride	+	+	+
Starch grain	Iodine solution	–	–	–
Alkaloid	Dragendroff	+	+	ND
Mucilage	Ruthenium red	+	+	+
Cellulose	Iodine and sulphuric acid	+	+	+
Alueron grain	Iodine	–	–	–

+: mean test is positive, −: mean test is negative, ND: mean not determine.

Table 3. Result of Preliminary Phytochemical Screening on Powder of *T. jamnagarensis.*

Group of phytoconsituents	Roots	Stem	Leaves
Alkaloid	+	+	+
Saponins	–	–	–
Flavonoids	+	+	+
Phenolic acids	+	+	+
Gums and mucilage's	+	+	+
Iridiodes	–	–	–
Anthocyanin	–	–	–

(–) = Absent (+) = Present.

DISCUSSION

The above observations and analysis are the first report providing information regarding the identification and pharmacognostic studies of the endemic species *T. jamnagarensis* Sant. In general the microscopy characters like presence of warty unicellular trichomes, rhomboidal calcium oxalate crystal and anisocytic stomata are important characteristic feature while paedomorphic ray and medullary phloem in stem are integral part for identifying and quality of a crude drug. The preliminary phytochemical screening of the all the plant parts showed the presence of the flavoniods, phenolic acids, gums and mucilage's as well as alkaloids. Thus this researches helps us developed some basic profile of the endemic *T. jamnagarensis* which would be further helpful in developing the detail monograph for identification.

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Pharmacognostic evaluation of *Leucas cephalotes* spreng leaves

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ABSTRACT

Background: The reliability and integrity of traditional systems of medicine depend upon properly identified sample of drugs ready to use as therapeutic agents for safety reason of the consumers. **Objective:** Keeping this background in mind this study was carried out on the leaf of *Leucas cephalotes*, basically a folklore drug also used in Unani Medicine and Ayurveda, on pharmacognostical parameters for producing enough data for correct identification of the plant. **Materials and Methods:** Pharmacognostic parameters such as morphology, anatomy, macrometry, micrometry, and quantitative microscopy, observation of isolated elements, physicochemical evaluation, preliminary phytochemical evaluation and fluorescence analysis of leaf were carried out using standard methods described by Johnson, Trease and Evans, British pharmacopoeia, Jenkins et al. and Kokoshi et al. HPLC and Spectrophotometery of aqueous and methanol extracts of leaf were also made. **Results:** Detailed results have been shown in figures and tables. **Conclusion:** The study provided useful information which can effectively be used to ascertain the authenticity of the available sample of the drug.

Keywords: anatomy, HPLC, micrometry, physicochemical studies, spectrophotometery.

INTRODUCTION

Medicinal plants and traditional systems of medicine have been indissolubly linked as medicinal plants are back bone of these systems. Since the credibility and reliability of these systems of medicine depend on appropriately identified crude drug samples, it becomes crucial to have authentic samples ready to use as therapeutic agents which are largely concerned with the safety of consumers because herbal drugs can be used safely only when their safety, efficacy and quality standards are up to the mark. Reproducibility of the effectiveness of herbal formulations is another concern for which availability of homogenous starting material is inevitable. Unfortunately, these values have not been given due consideration in traditional systems of medicine. In view of the continuously rising demand and interest of people in herbal drugs that have called for greater exactitude in appraisal of these drugs, it becomes critical to ascertain standard samples of crude single drugs for referential information.[1] The rapid expansion of various aspects of crude drugs has necessitated a systemic approach to study these drugs with methodical and appropriate methods of standardization.[2]

Sophisticated analytical instruments play significant role in the evaluation of crude drugs and formulations and are used for standardization. Though, it is obligatory to use instrumental techniques to obtain information required for solving analytical problems, significance of classical methods of analysis can't be underestimated. Therefore, combination of some physical and chemical operations on the samples of crude drugs substantiated with modern analytical tools will be better for checking the genuineness of crude drug samples.

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Leucas cephalotes (LC) commonly known as Gumma in India, belonging to the family Lamiaceae,[3] primarily a folklore medicine also used in Unani medicine and Ayurveda, is an annual herb and an upland rainy season weed,[4] usually found in roadsides, meadows, waste lands and cultivated grounds throughout the greater part of India.[5] The entire plant as well as its different parts, in isolation, are used medicinally. Whole plant possesses stimulant, laxative,[6] diaphoretic,[7] antiseptic,[8] anthelminthic,[9] insecticidal,[10] germicidal,[11] fungicidal,[12] emmenagogue,[13] expectorant and antipyretic [14] properties. Though, fruit and seed of this plant have been evaluated for some pharmacological actions and chemical constituents,[15–19] but, very few reports on its pharmacognostic aspects are available. Therefore, pharmacognostic study of the leaf of this plant was taken up for generating data for referential information.

MATERIALS AND METHODS

Material

Collection and authentication of the plant

Fresh plant was collected from the forest of *Satpura* range of Burhanpur (MP), India, in the month of July. The plant was identified and authenticated by botanists of National Ayurveda Dietetic Research Institute, Bangalore vide authentication no. Drug Authentication/SMPU/NADRI/BNG/2009-10/896. Fresh material was used for morphological and anatomical studies, whereas the material was dried well in shade and powdered in electric grinder for other studies. The study was carried out in the pharmacognosy laboratory and Central Instrumentation Facility Laboratory, National Institute of Unani Medicine (NIUM), Bangalore.

Preparation of extract

Coarse powder of air dried drug (100 g) was subjected to Soxhlet apparatus for 8 h for hot extraction with distilled water, methanol, acetone, di-ethyl ether, petroleum ether and chloroform, separately. The extracts were filtered and the filtrate was evaporated to dryness. The percentage yield of each solvent was calculated with reference to the air dried drug and expressed in gm%±SEM.

Macroscopic studies

Fresh leaf was examined by naked eye for morphology and organoleptic characters.

Microscopic studies

Transverse sections of leaf were cut according to the method described by Johnson.[20] The sections were stained, mounted and observed under microscope. Photographs were taken by digital camera (Sony10.1MP). Micrometry of various cells was done with the help of a micrometer (stage micrometer and ocular micrometer) by the method described by Trease and Evans and expressed in micron (μm). Study of isolated elements was also carried out.[21]

Physico-chemical studies

For estimation of ash values, extractive values, and pH, standard methods described in British pharmacopoeia[22] were applied. Moisture content was determined by the method of Jenkin et al.[23] Florescence analysis of powdered drug was carried out according to the method of Kokoshi et al.[24]

Preliminary phytochemical studies

Preliminary phytochemical screening for detection of various phytochemical was done by the method of Bhattacharji and Das.[25]

High performance liquid chromatography (HPLC)

HPLC of aqueous extract of leaf was run on an ultra fast liquid chromatography (UFLC) system (Shimadzu, Japan) with a LC-20AD pump and 20A auto-sampler, Phenomenex Luna C_{18} (2) column (250×4.6 mm id) 5 micron was maintained at 40°C. Mobile phase solvents were filtered through 0.45 μ membrane Millipore, PVDF under vacuum. The sample for analysis was filtered through the 0.22 μ membrane. The mobile phase A, solvent was double distilled water. The mobile phase B, solvent was HPLC analytical grade methanol. The flow rate was 0.5 ml/minutes using methanol: water (70:30) as mobile phase solvent, under a pressure of 100 f/sq.cm, run time of 10 minute and an injection volume of 20 μL. at 240, 205, 254, and 238 nm. Analyst 1.4 software was used to control all the parameters.

Spectrophotometry

Spectrum scan curves of aqueous and methanol extracts of leaf were obtained by using UV-Vis Spectrophotometer 3000 (Labindia). After preheat time, spectrophotometer was assessed to spectrum scanning mode. The parameters were set, the photometric mode was assessed to Abs, scanning speed was set as middle, and the wave

length range was set to 190–660 nm. Base line correction was performed with the blank cell, and then samples of extracts of drug were scanned.

RESULTS

Leaf shortly petioled, oblong-elliptic, opposite, decussate, simple, petioles pubescent; lamina ovate, widest in the middle and tapering to pointed apex, bases obtuse, margin serrulate, crenate, tips acute, unicostate, reticulate, upper surface pubescent, lower surface puberulent, the veins more conspicuous and membranaceous, petioles 6–11 mm long, lamina 3.5–7.0 cm.×1.5–3 cm. and green in colour (Figures 1a & b). Transverse section of leaf was through midrib and lamina showed the single layered epidermis of elongated epithelial cells with wavy cell wall measured 46.12–62.26–80.71 μ in length and 34.59–36.89–46.12 μ in breadth. Numerous covering trichome uniseriate, multicellular, acute tip, 1–4 celled present on upper and lower surface of the section measured 57.65–85.32–126.83 μ in length and 11.53 μ in breadth. The Diayctic (Caryophyllous or cross celled) stomata measured 23.06 μ in length and 11.53 μ in breadth. Mesophyll comprises of palisade and spongy parenchyma cells. Palisade cells arranged in single layer, compact with radially elongated cells below upper epidermis measured 34.59–43.81–46.1 μ in length and 8.12–10.16–11.53 μ in breadth. Spongy parenchyma multilayered, isodiametric, measured 34.59–50.73–69.18 μ in length and 34.59–46.12–80.71 μ in breadth. In the midrib portion the palisade cells absent and epithelial cells followed by the double layer of the collenchyma cells present at below upper epidermis measured 11.53 μ in length and 11.53 μ in breadth and thickness of layers of collenchymas above lower epidermis measured 57.65–76.09–103.75 μ. ransverse section of midrib vascular bundles slightly arc shaped with lignified Xylem and non lignified Phloem measured 172.95–244.53–299.78 μ in length and 115.3–138.36–172.95 μ in breadth. The size of xylem measured 8.12–13.15–23.06 μ in length and 8.12–10.84–11.53 μ in breadth. Phloem measured 8.12 μ (Figures 2a–2d). Results of micrometry and quantitative microscopy of leaf and micrometry of trichome are given in Tables 1, 2, 3, respectively. Isolated elements are given in Figures 3l–3h. Results of fluorescence analysis of powder are shown in Table 4.

The mean percentage values of total ash, acid insoluble ash, water soluble ash and water insoluble ash was found to be 6.24 ± 0.31; 15.19 ± 0.10, 6.35 ± 0.10, 5.78 ± 0.14. The percentage of moisture content with reference to air dried drug determined by Azeotropic volumetric method using Dean stark apparatus was found to be 6.8 ± 0.37. The extractive values determined in Petroleum Ether, Diethyl Ether, Chloroform, Acetone, Methanol, and Distilled Water where found to be 4.37 ± 0.15, 5.07 ± 0.30, 6.83 ± 0.12, 6.41 ± 0.09, 14.14 ± 0.17 and 38.79 ± 2.31, respectively. The preliminary phytochemical screening of leaf showed presence of phytosterols, fixed oil, carbohydrate, phenolic compound & tannin, protein amino acid, glycoside, cardiac glycosides and flavonoids.

HPLC analysis of aqueous extract (Aq Ext LC-L) at 254 nm showed three peaks (Figure 4). Spectrum scanning of aqueous extract (Aq Ext LC-L) gave nine peaks and three valleys whereas methanolic extract (Met Ext LC-L) gave 3 peaks and four valleys (Figures 5 a&b).

DISCUSSION

Quality of raw materials which plays central role in guaranteeing purity, safety, efficacy and stability of herbal preparations is often challenging, but it can be triumphed

Table 1. Macrometry of leaf of *Leucas cephalotes* Spreng.

	Leaf (l x b) (cm)	Petiole (mm)	Whorls (diameter (cm))	Whole plant	Seed
Mean	5.38 x 2.2	8.4	9.6/3.05	72	0.28 x 0.1
SD	1.31 x 0.5	72.07	1.19/0.38	5.74	0.04 x 0.0
SEM	0.58 x 0.25	0.92	0.53/0.17	2.56	0.02 x 0.0

Table 2. Micrometry of trichomes of leaf of *Leucas cephalotes* Spreng.

	Large		Small	
	Length (μ)	Width (μ)	Length (μ)	Width (μ)
Range	611.09–1291.36	34.59–57.65	34.59–219.07	–
Mean	882.42	43.04	112.23	11.53
SD	201.09	8.11	57.56	0.00
SEM	52.07	2.09	14.86	0.00

Table 3. Quantitative microscopy of leaf of *Leucas cephalotes* Spreng.

	Pallisade ratio	Stomatal index	Stomatal number	Vein islet. number	Vein termination number	Stomata size (μ)
Range	–	–	92–115	4–6.5	12–15	–
Mean	1:10.95	30.82%	101	5.45	13.35	23.06 x 11.53
SD	0.41	0.97	7.39	1.00	1.24	0.00
SEM	0.18	0.43	2.33	0.45	0.07	0.00

Table 4. Fluorescence analysis of powder of leaf of *Leucas cephalotes* Spreng.

S. No.	Tests	Observations	
		Day light	U/V light
1.	Powder as such	Dark olive	Light green
2.	Powder+1NHCL	Dark yellowish brown	Light yellow
3.	Powder+1NNaOH	Dark saddle brown	Lawn green
4.	Powder+1NNaOH+Methanol	Dark olive	Green
5.	Powder+50%KOH	Saddle brown	Lime
6.	Powder+50%H_2SO_4	Saddle brown	Lawn green
7.	Powder+Conc.H_2SO_4	Dark red	Dark slate grey
8.	Powder+50%HNO_3	Saddle brown	Lime green
9.	Powder+ Conc.HNO_3	Dark red	Yellowish green
10.	Powder+ Acetic Acid	Olive	Spring green
11.	Powder+ Iodine solution	Dark olive green	Spring green
12.	Powder+ Distilled water	Olive	Lawn green
13.	Powder+ Chloroform	Olive	Medium spring green
14.	Powder+ Acetone	Olive	Lime green
15.	Powder+ Picric Acid	Olive	Lime

over by making appropriate strategies for standardization of herbs and their preparations. The approach includes a range of classical and analytical methods such as macroscopic, microscopic, physico-chemical, phytochemical and analytical studies.

Microscopic characters of a plant material such as types and arrangements of different cells, typical shape of trichomes, stomata, vascular bundle and other cells, micrometry and quantitative microscopy are not only helpful for identification but are also indispensible, specially for those parts of the plants which are avilable in pieces. Therefore, these methods are often used in association with other analytical methods.[25]

Physico-chemical standards such as ash values, extractive values, moisture content, pH, fluorescence analysis of powdered drug, and qualitative and quantitative analysis of chemical constituents are widely accepted parameters. Ash value is an important parameter for detection of adulteration in herbal drugs.[26] Another valuable parameter is the extractive value in different solvents, to check the quality of drug. A specific solvent extracts specific phytochemical in specific amount. The amount of extract in a particular substance plays an important role in establishing the index of the purity. Any adulteration or substitution may cause change in extractive values.[26] An excess of water in medicinal plant material encourages microbial growth and deterioration. Estimation of moisture content is important for the material which deteriorates quickly in the presence of water. Thus, estimation of moisture content may be a good parameter for checking the purity of the drug.[27] Herbal drugs are generally used in powder form which is more susceptible for adulteration. This problem can be solved by observing the powder of the drug under day light and U/V light after treating the powder with different chemicals because the fluorescence characters are diagnostic.

Phytochemical present in plants are mainly alkaloids, glycosides, glucosides, essential oil, tannins, resins, and flavonoids etc. Analysis of these constituents is a receptive parameter for standardization. These phytochemicals not only vary from species to species but also differ in different samples of the same drug; therefore it can be used as an approachable parameter in the quality control of drugs.[28]

Recently, it has been possible to use sophisticated analytical methods such as HPLC, HPTLC, UV/VIS Spectrophotometery for isolation and identification of chemical constituents present in the with high end results. HPLC is a fast, sensitive and most preferred chromatographic technique for routine assay of new drug as well as determination of adulterant of established drugs. UV/VIS absorption technique may be used for analysis of a variety of natural products. In the present study HPLC of aqueous extract and UV/VIS Spectrophotometery of aqueous and methanol extracts were carried out. These two studies done by us were of preliminary type, hence no major inference can be drawn and could not be interpreted with the reported phytochmicals, however, may be used as standard. Further study is needed in this regard. It was also not possible to compare our findings with any other data as no such study on has been done, hence our findings may also be considered as an addition to the existing reserve of knowledge.

CONCLUSION

In the light of the present study it can be concluded that the findings can serve as the source of information to ascertain the authenticity and standardization of the available sample of the drug.

CONFLICTS OF INTEREST

None

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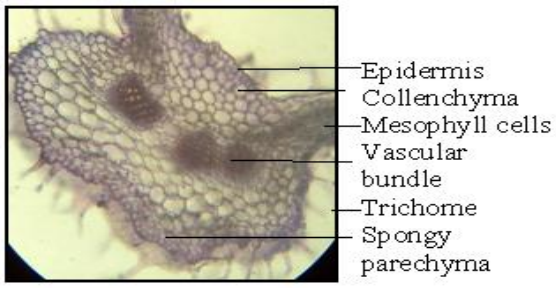
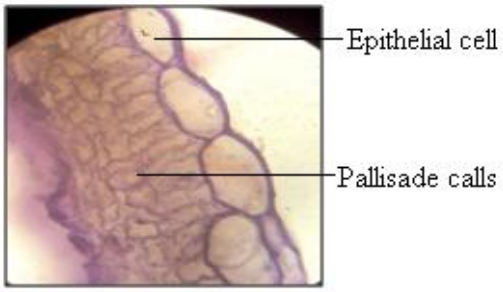
Fig. 1. *Leucas cephalotes* Spreng

(1a) A twig with stem, fruit and flower **(1b) Leaf**

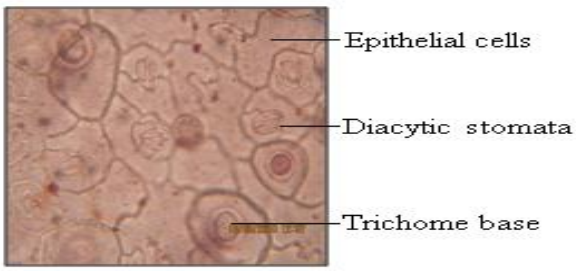
Figure 2: Transverse sections of leaf *of Leucas cephalotes* Spreng



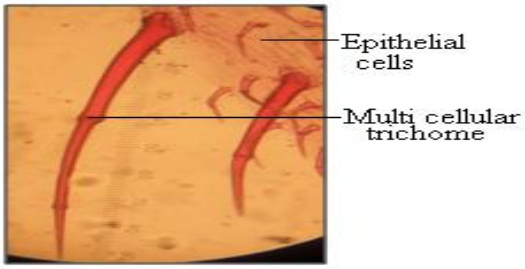
(2a)T.S. (10 X 10)



(2b)T.S. (Pallisade cells)(10 x 45)

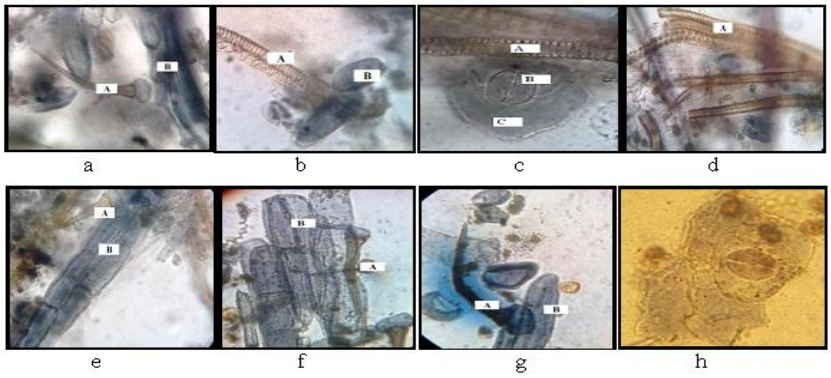


(2c) Stomata of leaf (10 x 45)



(2d) Trichome of leaf (10 x 10)

Figure 3: Isolated elements of leaf of *Leucas cephalotes* Spreng



(3a) A,Trichome,B, epithilial cells(10 x 10) ,(3b) A, Scalariform vessel, B, Parenchymatous cells (10 x 45),(3c) A, Vessel, B, Stomata (Diacytic), C,Epithilial cells (10 x45),(3d) A, Compound vessels (10 x 45),(3e,f) A-Trichome (Base), B, Elongated epithilial cells (10 x 10),(3g) A,Trichome (Base), B, Elongated epithilial cells (10 x10),(3h) Stomatal cell (10 x 45)

Page 19-A

Figure 4: HPLC of aqueous extract of leaf of *Leucas cephalotes* Spreng

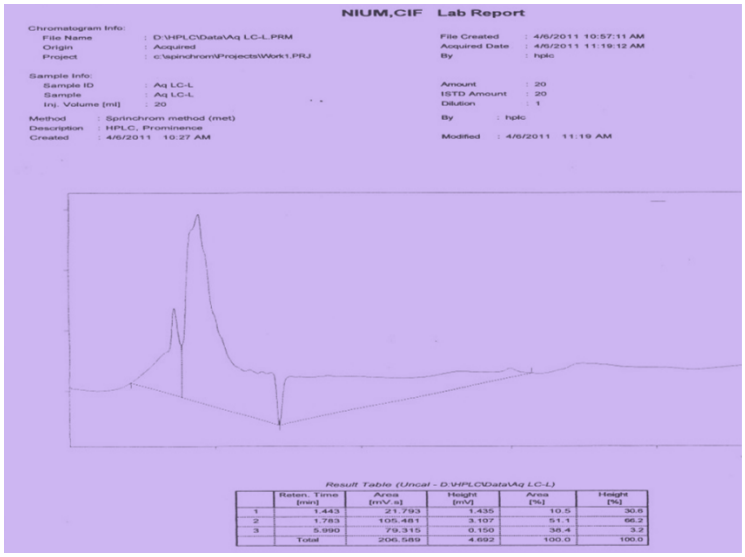
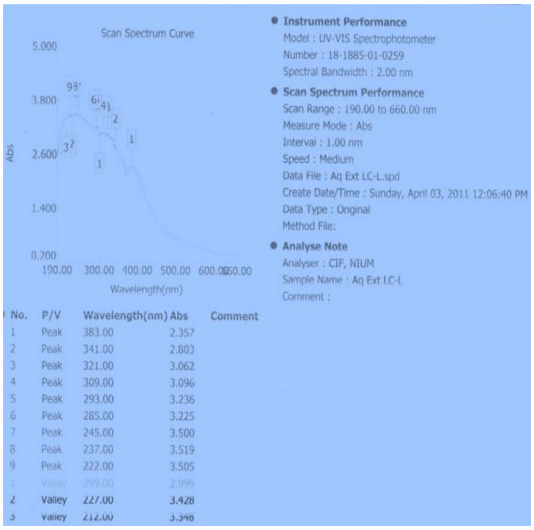
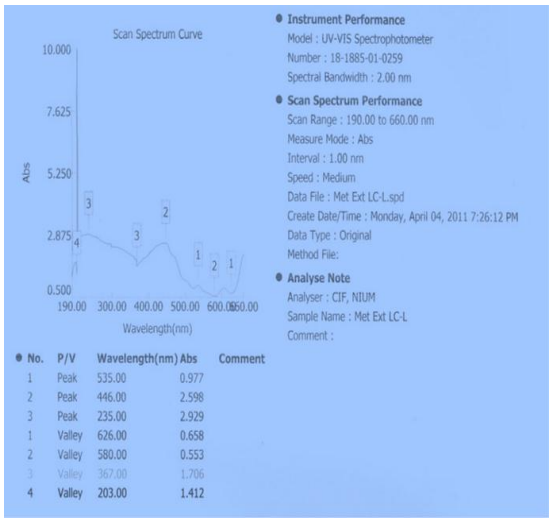


Figure 5: Spectrophotometry of extracts of leaf of *Leucas cephalotes* Spreng





(5a)Aqueous extract (5b) Methanol extract

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Types of endophytic bacteria associated with traditional medicinal plant *Lantana camara* Linn.

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ABSTRACT

Background: Traditionally, *Lantana camara* plant (Family: Verbenaceae) is used in herbal medicine as an antiseptic for wounds, in the treatment of skin itches, and externally for leprosy and scabies. The leaves of this plant possess adulticidal activity against different mosquito species. Endophytic bacteria (EB) can produce bioactive compounds found in their host; hence, investigation to find out what types of EB are associated with *L. camara* is necessary. **Objectives:** The main objective of this study was to isolate and identify EB associated with *L. camara*. **Materials and methods:** *Lantana camara* twigs along with the leaves and fruits samples were collected; and EB were isolated from surface-sterilized tissue samples. The 16S rRNA gene fragments were amplified using PCR method; and endophytic bacterial isolates (EBIs) were identified based on 16S rRNA gene sequence similarity method. **Results:** Cultivable, 50 EBIs were analyzed; and analysis of their 16S rRNA gene sequences suggests that varied 40 types of EB are associated with *L. camara*. Majority (24%) of EBIs were from *Bacillus* genus. **Conclusion:** Thus, we conclude that *Lantana camara* plants harbour a wide array of cultivable endophytic bacteria.

Keywords: 16S rRNA, diversity, endophytes, herbal medicine, Malaysia, natural products

INTRODUCTION

Use of medicinal plants in the treatment of various health ailments is as old as mankind. Plant genus, *Lantana* contains about 150 species and some of the species are used as antibacterial, antirheumatic, biological control, stimulant and as ornamental plant.[1–2] *Lantana camara* is one of the species and used in traditional medicinal system of various countries. *Lantana camara* is a shrub type of plant which belongs to family Verbenaceae. This plant is a native of Africa and America; and it is extensively used as an ornamental plant in some other countries. However, in Malaysia and India, this plant is found in the forests as well as along the roadsides and on the waste land. In some countries like Brazil, this plant is found in most of the regions.[2–3]

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The leaves of *L. camara* are used in the treatment of skin injuries, as an antiseptic for wounds, and externally for leprosy and scabies.[4] Leaves are also used in the treatment of various other health ailments such as biliary fever, bronquitis, rheumatism, scratching, stomachache, and toothache.[2,5] In addition to this, the leaves of this plant are also used in the treatment of pulmonary diseases and rheumatism;[6] and plant is also explored for some other potential pharmaceutical application.[7]

The essential oil of *L. camara* leaves contains a high amount of sesquiterpenes, and oil is known to inhibit the growth of *Pseudomonas aeruginosa*, *Aspergillus niger*, *Fusarium solani* and *Candida albicans*.[5] The oil obtained from *L. camara* also possesses insecticidal and repellent activities; and as stated by Sousa et al. (2010), the essential oil also shows repellent effect against *Aedes* mosquitoes.[8] The research findings reported by Sousa et al. (2010) suggest that the essential oil of *L. camara* could be used as a source of plant-derived natural products with resistance-modifying activity.[8]

In year 2010, Mehanni and Safwat has reported that endophytic microorganisms isolated from medicinal plants do produce the same metabolites as their hosts.[9] Hence, endophytes can be considered as one of the potential sources of the therapeutic compounds. *Lantana camara* is an important medicinal plant; but, despite its various medicinal applications we do not know what types of endophytes are associated with it.[4,10]

The main aim of this study was to understand what types of EB are associated with *L. camara*; and the specific-objective of this study was to isolate and identify the EB from *L. camara*.

MATERIALS AND METHODS

The leaves, twigs and fruits from 15 individual plants of *L. camara* were collected from Taman Tasek Semeling, Kedah, Malaysia. The stem pieces, leaves, and fruits were carefully washed under plenty of running tap water. Surface-sterilization of leaves (along with petioles), stem pieces and fruits samples were carried out as described in earlier publication.[11]

The leaves, petiole and stem tissue pieces, and fruits (by making injuries) were inoculated aseptically in the Petri plates containing nutrient agar. Petri plates containing inoculated tissue samples were incubated at 37°C (± 3°C) for 18 to 20 h in the dark in an incubator.

Isolation and cultivation of endophytes, amplification of 16S rRNA gene fragments, sequencing of 16S rRNA gene fragments and identification of EB was carried out as described in previous publication.[11]

RESULTS

Incubation of Petri plates containing the inoculated tissues sample on nutrient agar enabled cultivable EB to grow; and the grown EB colonies were visible on the margins of the inoculated tissues. In total, fifty (50) EBIs were examined (12, 10, 23, and 5 from stem, petiole, leaf, and fruit tissues, respectively).

All EBIs were identified based on their sequenced 16S rRNA gene sequence BLAST (megablast) hits analysis. The annotated 16S rRNA gene fragment's nucleotide sequences of all 50 EBIs have been submitted to the international DNA database (GenBank/DDBJ/EMBL) under accession numbers: JN835522 – JN835571.

The analysis of the identified EBIs revealed that there were 40 different types of species (Table 1) of the bacteria in isolates; and the majority (24%) of EBIs were from *Bacillus* genus. Results clearly indicate that *L. camara* harbours diverse types of endophytic bacteria.

DISCUSSION

It is strongly believed that almost every plant on the earth harbours EB.[12] The EB do have various potential applications not only in pharmaceutical industry but in other

Table 1. The types of endophytic bacteria associated with *Lantana camara* as revealed by identification of isolates based on their 16S rRNA gene sequence.

No.	Species	GBN#
1	*Bacillus aerophilus*	JN835561
2	*Bacillus amyloliquefaciens*	JN835555
3	*Bacillus aryabhattai*	JN835568
4	*Bacillus axarquiensis*	JN835538
5	*Bacillus cereus*	JN835564
6	*Bacillus megaterium*	JN835567
7	*Bacillus methylotrophicus*	JN835539
8	*Bacillus pumilus*	JN835565
9	*Bacillus subtilis*	JN835563
10	*Bacillus tequilensis*	JN835548
11	*Bacillus vallismortis*	JN835547
12	*Chryseobacterium daejeonense*	JN835522
13	*Chryseobacterium taeanense*	JN835560
14	*Chryseobacterium taichungense*	JN835557
15	*Cronobacter dublinensis*	JN835541
16	*Cronobacter malonaticus*	JN835544
17	*Cronobacter muytjensii*	JN835546
18	*Cronobacter sakazakii*	JN835534
19	*Cronobacter turicensis*	JN835537
20	*Edwardsiella tarda*	JN835528
21	*Enterobacter cancerogenus*	JN835527
22	*Enterobacter cloacae*	JN835530
23	*Enterobacter cowanii*	JN835536
24	*Enterobacter hormaechei*	JN835523
25	*Enterobacter pyrinus*	JN835525
26	*Erwinia amylovora*	JN835559
27	*Escherichia hermannii*	JN835532
28	*Escherichia senegalensis*	JN835533
29	*Klebsiella oxytoca*	JN835535
30	*Klebsiella pneumonia*	JN835542
31	*Pantoea agglomerans*	JN835554
32	*Pantoea ananatis*	JN835524
33	*Pantoea dispersa*	JN835550
34	*Pantoea eucalypti*	JN835569
35	*Pantoea stewartii*	JN835570
36	*Pseudomonas argentinensis*	JN835549
37	*Pseudomonas flavescens*	JN835553
38	*Pseudomonas fulva*	JN835543
39	*Pseudomonas straminea*	JN835529
40	*Raoultella planticola*	JN835545

#GenBank accession numbers of deposited 16S rRNA gene sequence fragment from respective endophytic bacterial isolate (EBI).

sectors also. We isolated and identified 50 EBIs from *L. camara*. Similarly, endophytic bacteria has been reported from various medicinal plants; for instance, *Gynura procumbens*,[11] *Piper nigrum*,[13] *Trifolium repens*[14] and *Artemisia annua*.[15] However, to our knowledge, this study is the first to illustrate diverse types of EB in *L. camara*. The similarity comparison between the 16S rRNA gene fragment sequences from 50 EBIs and the sequences from GenBank/DDBJ/EMBL database using the BLASTN program revealed identity of each isolate.

Bacterial 16S rRNA gene sequence do provide species-specific signature and can be used in bacterial identification.[16] In fact, this approach is commonly used in identification of bacteria.[17] Therefore, we amplified DNA of 16S rRNA encoding gene for the rapid and accurate identification of EBIs.

Soil bacteria such as *Bacillus spp*., *Pseudomonas spp*. and *Azospirillum spp*. are commonly associated with plants as endophytes. But, we did not find any *Azospirillum spp*. in 50 isolates. Perhaps, the growth medium used might be directly affecting the number and type of EB that can be isolated from the plant tissues. It has been reported that a seasonal fluctuation of the endophytes does occur in plants;[18,19] hence, it is likely that various other types of EB might be colonizing the *L. camara*. We have used 4 different types of tissues (leaf, petiole, stem and fruit) from different plants of *L. camara*; and diverse types of EB were found in analysed 50 EBIs. Most recently, it has been reported that soil type is a major factor that determines the diversity of EB in plants.[20] Plants are also known to harbour endophytic fungi; and most probably, *L. camara* might be harbouring some unique entophytic fungal community like other medicinal plants.[21]

The EB can produce the same metabolites as their hosts;[9] and therefore, EB may serve as one of the potential sources of the natural products and novel antibiotics. The antibacterial, antifungal and antiviral activities of some EB are reported by other researchers.[22,23,24,25,26,27,28] The EB from plants with antimicrobial activities are likely to serve as the potential candidates that may produce novel antibiotics and could help in combating drug resistant microorganisms and pathogens.[29,30,31]

Based on the results, we conclude that *L. camara* does contain diverse types of cultivable EB. This study is the first of its kind to report the endophytic bacterial community associated with *L. camara*. However, the benefits derived by *L. camara* from these EB are not clearly understood yet. We hypothesize that in *L. camara*, these EB might be playing an important role in producing medicinally important bioactive compounds. Nevertheless, our research findings could serve as foundation for further research on *L. camara* and role of its EB in producing therapeutic compounds.

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Chemical constituents of *Aglaia loheri*

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ABSTRACT

The dichloromethane extract of the leaves of *Aglaia loheri* afforded spinasterol (**1**), trilinolein (**2**) and phytyl fatty acid ester (**3**). The structures of 1-2 were identified by comparison of their ^{13}C NMR data with those reported in the literature. Compounds **1**, **2** and **3** were evaluated for cytotoxicity against the colon carcinoma (HCT 116) using the MTT assay. They exhibited moderate cytotoxicity against HCT 116 with IC_{50} values of 40.52, 46.73 and 40.06, respectively.

Keywords: *Aglaia loheri*, Meliaceae, spinasterol, trilinolein, phytyl fatty acid ester, cytotoxic.

INTRODUCTION

Aglaia loheri is an endemic Philippine tree. An earlier study reported that the crude ethanolic extracts of the leaves of *A. loheri* exhibited high cytotoxicity against two human cancer cell lines: lung non-small cell adenocarcinoma (A549) and colon carcinoma (HCT 116) with IC_{50} values below 20 µg/ml. The extract was portioned into ethyl acetate and hexane fractions and tested for cytotoxicity. Lower IC_{50} values were obtained on the ethyl acetate fraction.[1] Another study showed that the methanol extracts of *Aglaia loheri* have teratogenic activity against maternal mice. Mice orally administered with 5 mg/ml and 10 mg/mL concentrations of *Aglaia loheri* extracts resulted in 75% and 100% resorption, respectively.[2] A previous study showed that the extracts of *Aglaia loheri* have angiosuppressive activity on duck embryo. Extracts of *Aglaia loheri* reduced CAM vascular density of treated embryos suggesting antiangiogenic activity.[3]

We report here the isolation, identification and cytotoxicity test results of 1–3 (Figure 1) against the colon carcinoma (HCT 116) using the MTT assay. To the best of our knowledge this is the first report on the isolation of these compounds from *Aglaia loheri*.

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MATERIALS AND METHODS

General Experimental Procedures

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^1H-NMR and 150 MHz for ^{13}C-NMR spectra. Column chromatography was performed with silica gel 60 (70–230 mesh), while the TLC was performed with plastic backed plates coated with silica gel F_{254}. The plates were visualized with vanillin-H_2SO_4 and warming.

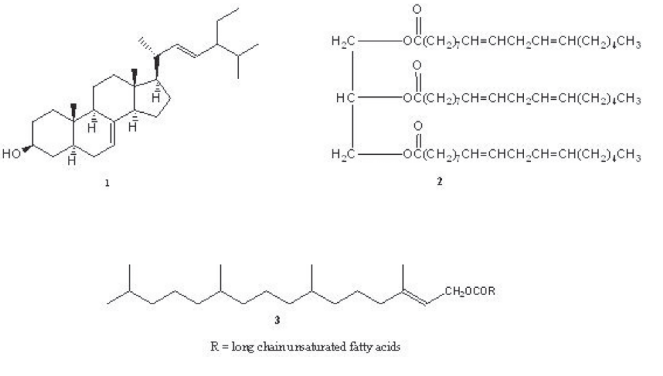

Figure 1. Chemical constituents of *A. loheri*: spinasterol (**1**), trilinolein (**2**) and phytyl fatty acid ester (**3**).

Plant Material

Aglaia loheri leaves were collected from Kanawan, Morong, Bataan Province, Philippines in March 2012. Specimens of the plants were authenticated at the Institute of Biology, University of the Philippines, Diliman.

Extraction and Isolation

The air-dried leaves (550 g) of *A. loheri* were ground in an osterizer, soaked in CH_2Cl_2 for three days, and then filtered. The filtrate was concentrated by evaporation under vacuum to afford a crude extract (5.8 g) which was chromatographed in increasing proportions of acetone in CH_2Cl_2 at 10% increment by volume. The CH_2Cl_2 fraction was rechromatographed (3x) in petroleum ether to afford **3** (5 mg). The 10% acetone in CH_2Cl_2 fraction was rechromatographed (5x) in 5% EtOAc in petroleum ether to afford **1** (12 mg) after washing with petroleum ether. The 20% acetone in CH_2Cl_2 fraction was rechromatographed (4x) in 10% EtOAc in petroleum ether to afford **3** (25 mg).

Spinasterol (**1**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 37.12 (C-1), 31.45 (C-2), 71.05 (C-3), 37.97 (C-4), 40.24 (C-5), 29.62 (C-6), 117.45 (C-7), 139.56 (C-8), 49.43 (C-9), 34.20 (C-10), 21.53 (C-11), 39.44 (C-12), 43.27 (C-13), 55.11 (C-14), 23.01 (C-15), 28.50 (C-16), 55.88 (C-17), 12.04 (C-18), 13.04 (C-19), 40.82 (C-20), 21.36 (C-21), 138.2 (C-22), 129.4 (C-23), 51.2 (C-24), 31.9 (C-25), 21.1 (C-26), 19.0 (C-27), 25.4 (C-28), 12.2 (C-29).

Trilinolein (**2**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 62.08 (glyceryl CH_2), 68.86 (glyceryl CH), 173.29 (C=O α), 172.84 (C=O β), 34.04 (C-2α), 34.18 (C-2β), 24.83 (C-3α), 24.87 (C-3β), 29.08 (C-4α), 29.04 (C-4β), 29.19 (C-5α), 29.27 (C-5β), 29.11 (C-6α), 29.17 (C-6β), 29.62 (C-7α), 29.65 (C-7β), 29.19 (both C-8), 130.00 (C-9α), 129.98 (C-9β), 128.05 (C-10α), 128.07 (C-10β), 25.62 (both C-11), 127.88 (C-12α), 127.87 (C-12β), 130.22 (both C-13), 27.19 (both C-14), 29.36 (both C-15), 31.52 (both C-16), 22.57 (both C-17), 14.07 (both C-18).

BIOASSAY

Cytotoxicity Test

Four milligrams each of **1**, **2** and **3** were dissolved in 1 milliliter each of dimethyl sulfoxide (DMSO) to make 4 mg/mL solutions. Compound **1**, **2** and **3** were tested for cytotoxic activity against a human cancer cell line, colon carcinoma (HCT116). Doxorubicin, an anticancer drug and DMSO were used as the positive and negative controls, respectively. The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay reported in the literature was employed.[4,5]

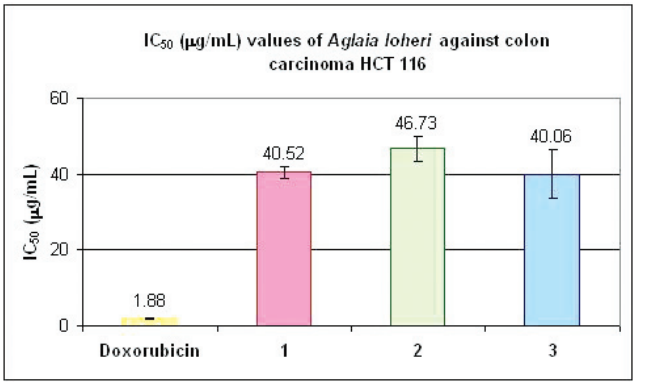

Figure 2. Inhibitory concentrations at 50% (IC_{50}) of doxorubicin and compounds **1**, **2** and **3** from the leaves of *Aglaia loheri* tested against colon carcinoma HCT 116 using the MTT assay. Each value is the mean of three trials with three replicates per trial with standard deviation indicated by bars.

RESULTS AND DISCUSSION

The structures of **1** and **2** were elucidated and confirmed by comparison of their ^{13}C NMR data with those of spinasterol[6] and trilinolein[7] reported in the literature. The structure of **3** was identified by comparison of its ^{1}H NMR data with those reported in the literature for phytyl fatty acid ester.[8]

Cytotoxicity tests on **1**, **2** and **3** were conducted against the human colon cancer cell line, HCT116. Results of the study (Figure 2) indicated that **1**, **2** and **3** exhibited moderate cytotoxicity against HCT 116 with IC_{50} values of 40.52, 46.73 and 40.06, respectively.

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Acetogenins from *Annona muricata*

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ABSTRACT

Silica gel chromatography of the dichloromethane extract of the seeds of *Annona muricata* afforded annoreticuin-9-one (**1**), while the flesh of the fruit yielded *cis*-annoreticuin (**2**) and sabadelin (**3**). The structures of **1**–**3** were elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by mass spectrometry. Acetogenins **1** and **2** were first isolated from *A. reticulata* and *A. montana*, respectively. Acetogenin **1** was reported to exhibit cytotoxic activities against the human pancreatic tumor cell line (PACA-2), human prostate adenocarcinoma (PC-3) and human lung carcinoma (A-549), while **2** was reported to exhibit cytotoxicity against human hepatoma carcinoma cell line (Hep G2).

Keywords: *Annona muricata,* Annonaceae, annoreticuin-9-one, *cis*-annoreticuin, sabadelin

INTRODUCTION

Annona muricata Linn. of the family Annonaceae, commonly known as guyabano is a well known medicinal tree with antibacterial, antiviral and antifungal properties. Recent studies show that extracts from this plant have potential anti-carcinogenic activity. In the Philippines, it is popular for its edible fruit and is used as a folkloric herbal medicine. The juice of the ripe fruit is said to be a diuretic, while a decoction of powdered immature fruits is a dysentery remedy.[1] The ethanolic extract of the stem bark of *Annona muricata* demonstrated inhibitory activity against cytopathic effects of Herpes simplex virus-1 (HSV-1).[2] In another study, the *Annona* sp. methanolic extracts showed activity against the lytic activity of Herpes simplex virus-2 (HSV-2).[3] The dry ethanolic extract of *A. muricata* leaf has molluscicidal properties against both the adult forms and egg masses of *Biomphalaria glabrata*.[4] *A. muricata* aqueous leaf extract treatment has beneficial effects on pancreatic tissues subjected to Streptozotocin-induced oxidative stress. The extract was able to diminish and/or prevent pancreatic oxidative damage produced by Streptozotocin and exhibited antioxidant activity.[5] Aqueous extracts of the skin of *A. muricata* at 50, 100, 150 and 200 µL/dish showed antibacterial effect against *Staphylococcus aureus* and *Vibrio cholerae*.[6]

Seven isoquinoline alkaloids: reticuline, coclaurine, coreximine, atherosperminine, stepharine, anomurine and anomuricine have been isolated from the leaves, root and stem barks of *A. muricata*.[7] The essential oil of the fresh fruit pulp of *A. muricata* was found to contain 2-hexenoic acid methyl ester (23.9%), 2-hexenoic acid ethyl ester (8.6%), 2-octenoic acid methyl ester (5.4%), 2-butenoic acid methyl ester (2.4%), β-caryophyllene (12.7%), 1,8-cineole (9.9%), linalool (7.8%), α-terpineol (2.8%), linalyl propionate (2.2%), and calarene (2.2%).[8] The seeds of *A. muricata* afforded annomuricatin A.[9]

Annohexocin, a mono-THF annonaceous acetogenin has been isolated from the leaves of *A. muricata*.[10] The compound showed significant inhibitory effects among six human cancer cell lines: lung, breast, colon, pancreatic, kidney carcinoma with selectivity for the prostate adenocarcinoma, PC-3. The ED_{50} for PC-3 was 0.0195 µg/mL, while for Adriamycin, a known chemotherapeutic drug, the ED_{50} was 0.0310 µg/mL. Muricoreacin and murihexocin acetogenins have also been isolated from the leaves of *A. muricata* and these compounds showed significant cytotoxicities against six human tumor cell lines with selectivities to the prostate adenocarcinoma (PC-3)

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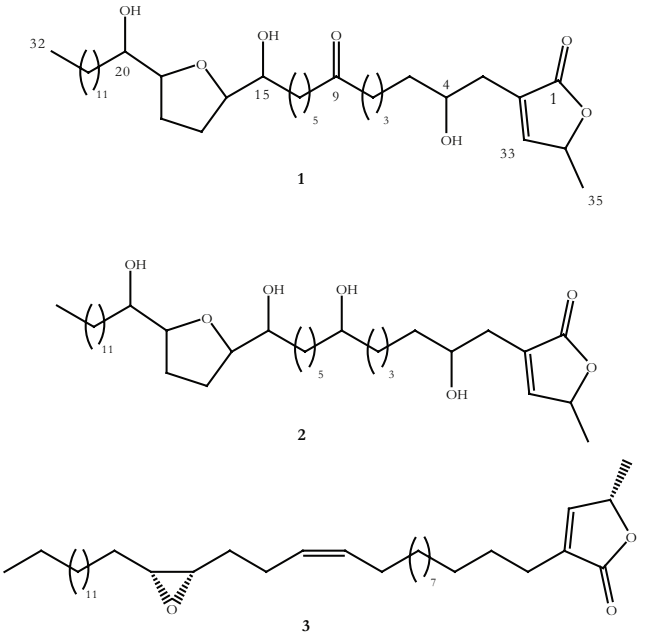

Figure 1. The acetogenins: annoreticuin-9-one (**1**), *cis*-annoreticuin (**2**), and sabadelin (**3**) from *A. muricata*.

and pancreatic carcinoma (PACA-2) cell lines. By the Brine Shrimp Test (BST), the LD_{50} are 19 and 10 µg/mL, respectively.[11]

We report herein the isolation and structure elucidation of the acetogenins, annoreticuin-9-one (**1**) from the seeds and *cis*-annoreticuin (**2**) from the fruit of *A. muricata*. We likewise report the isolation of another acetogenin, sabadelin (**3**) from the fruit of *A. muricata*. To the best of our knowledge this is the first report on the isolation of **1** and **2** from *A. muricata* and the first reported study on the chemical constituents of a local collection of the fruit of the tree.

MATERIALS AND METHODS

General Experimental Procedures

Optical rotation was taken with a Jasco P-2000 digital polarimeter. HRMS was obtained on a Finnigan MAT 95S spectrometer. NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR spectra. Column chromatography was performed with silica gel 60 (70–230 mesh), while the TLC was performed with plastic backed plates coated with silica gel F_{254}. The plates were visualized with vanillin-H_2SO_4 and warming.

Plant Material

Annona muricata fruits were collected from Painaan Rizal, Philippines in September 2010. The specimens of the plant were authenticated at the Bureau of Plant Industry in Quirino, Manila.

Extraction and Isolation

The seeds from the fruit of *A. muricata* were air-dried, ground in an osterizer, and then soaked in CH_2Cl_2 for three days and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (69.5 g), which was chromatographed by gradient elution using 5% EtOAc in petroleum ether, 7.5% EtOAc in petroleum ether, CH_3CN: Et_2O: CH_2Cl_2 (1:1:8, v/v) and 100% acetone. The 100% acetone fraction was triturated with petroleum ether and then rechromatographed (5x) in CH_3CN: Et_2O: CH_2Cl_2 (1.5:1.5:7, v/v) to afford **1** (6 mg).

The flesh of the fruit of *Annona muricata* (1 kg) were freeze-dried and soaked in CH_2Cl_2 for 3 days. The filtrate was concentrated under vacuum to afford a crude extract (7.0 g), which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increments. The 30% acetone in CH_2Cl_2 fraction was rechromatographed in 12.5% EtOAc in petroleum ether to afford **3** (9 mg). The 80% and 90% acetone in CH_2Cl_2 fractions were combined and rechromatographed by gradient elution in CH_3CN: Et_2O: CH_2Cl_2 (0.5:0.5:9, v/v), followed by CH_3CN: Et_2O: CH_2Cl_2 (1:1:8, v/v), followed by CH_3CN: Et_2O: CH_2Cl_2 (1.5:1.5:7, v/v) and finally CH_3CN: Et_2O: CH_2Cl_2 (2.5:2.5:5, v/v) to afford **2** (7 mg) after trituration with petroleum ether.

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extract of the freeze-dried fruit of *Annona muricata* afforded annoreticuin-9-one (**1**), *cis*-annoreticuin (**2**) and sabadelin (**3**). The structures of **1** and **2** were elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by mass spectrometry as follows.

The ^{1}H NMR spectrum of **1** (Table 1) gave resonances for a methyl doublet at δ 1.42 (d, J = 6.6 Hz) and a methyl triplet at δ 0.86 (t, J = 7.2 Hz); an oxymethine proton of a lactone at δ 5.04 (dq, J = 1.2, 7.2 Hz) and five oxymethine protons at δ 3.82, 3.78 (2H), 3.39 (2H); a conjugated olefinic proton at δ 7.16 and methylene protons centered at δ 1.23. The ^{13}C NMR data of **1** (Table 1) indicated resonances for carbonyl carbons of a ketone at δ 211.4 and an ester at δ 174.6; six oxymethine carbons at δ 69.8, 74.0 (2C), 78.0 and 82.6 (2C); olefinic carbons at δ 131.2 and 151.9; and overlapping methylene carbons centered at δ 29.7. These are characteristic resonances of acetogenins which are commonly found in the genus *Annona*.

Table 1. 600 MHz ^{1}H NMR and 150 MHz ^{13}C NMR Data of Acetogenins 1 and 2 in $CDCl_3$.

Position	1		2	
	δ C	δ H (*J* Hz)[a]	δ C	δ H (*J* Hz)[a]
1	174.6		174.6	
2	131.2		131.2	
3	33.2	2.38, 2.50	33.2	2.38, 2.52
4	69.8	3.82	69.9	3.82
5	37.1	1.48, 1.48	37.3	1.42, 1.48
6	33.4	1.38, 1.38	25.6	1.48, 1.34
7	23.7	1.57, 1.57	25.2–29.7	1.26, 1.26
8	42.6	2.38, 2.38	33.5	1.40, 1.40
9	211.4		71.6	3.58
10	42.6	2.38, 2.38	33.5	1.40, 1.40
11	23.7	1.57, 1.57	22.5–29.7	1.25–1.45
12–13	22.5–25.7	1.25–1.45	22.5–25.7	1.25–1.45
14	25.2	1.48, 1.48	25.5	1.35, 1.48
15, 20	74.0	3.39	74.0	3.39
16, 19	82.6	3.78	82.6	3.78
17–18	28.7	1.66, 1.96	28.7	1.66, 1.96
21	25.2	1.40, 1.48	25.5	1.35, 1.48
22–29	23.6–33.5	1.23 br s	25.2–29.7	1.35, 1.48
30	22.7	1.26, 1.26	22.7	1.26, 1.26
31	32.0	1.23, 1.23	31.9	1.23, 1.23
32	14.1	0.86 t (7.2)	14.1	0.86 t (7.2)
33	151.9	7.16 d (1.2)	151.9	7.16 d (1.2)
34	78.0	5.04 dq (1.2, 7.2)	78.0	5.04 dq (1.2, 7.2)
35	19.1	1.42 d (6.6)	19.1	1.41 d (6.6)

[a] Multiplet unless otherwise indicated

The HRMS of **1** gave a molecular ion of *m/z* 594.4496 [M^+], corresponding to a molecular formula of $C_{35}H_{62}O_7$. The molecular formula indicated an index of hydrogen deficiency of five. With the two carbonyls and an olefin deduced from the ^{13}C NMR spectrum, the compound is a bicyclic acetogenin.

Five fragments of **1** (Figure 2) were deduced from COSY as follows: H_2-3/H-4/H_2-5/H_2-6/H_2-7/H_2-8; H_2-10/H_2-11; H_2-14/H-15/H_2-16/H_2-17/H_2-18/H-19/H-20/H_2-21; H_2-30/H_2-31/H_3-32; H-33/H-34/H_3-35.

The protons attached to carbons were assigned (Table 1) from HMQC 2D NMR data and the structure of **1** was elucidated by analysis of the HMBC 2D NMR data: key HMBC correlations are shown in Figure 2. Thus, the carbonyl carbon of the lactone was assigned to C-1 on the basis of long-range correlations between H_2-3, H-33 and this carbon. Long-range correlations were also observed between H_3-35, H-33 and C-34. The presence of α,β-unsaturated γ-lactone in **1** was confirmed by the fragment ion at *m/z* 97 (Figure 3). An oxymethine carbon was attributed to C-4 due to long-range correlations between H_2-3, H_2-5, H_2-6 and this carbon. The carbonyl carbon of the ketone was assigned to C-9 since long-range correlations were observed between H_2-7/H_2-11, H_2-8/H_2-10 and this carbon. Fragment ions at *m/z* 111, 151, 165, 225 and 239 indicated the positions of hydroxyl and keto groups at C4 and C9, respectively (Figure 3). The fragment ions at *m/z* 225 and 239 showed cleavage at the carbonyl and alpha carbon positions, respectively. The carbons (C-12 and C-13) and the protons (H_2-12 and H_2-13) were not assigned due to overlapping resonances. Another oxymethine carbon was attributed to C-15 attached to the THF at C-16 based on long-range correlations between H_2-14, H-16, H_2-17 and C-15. A third oxymethine carbon was detected at C-20 since long-range correlations were observed between H_2-21, H-19, H_2-18 and this carbon. The THF located at C-16 was supported by the fragmentation pattern (Figure 3) deduced from the mass spectrum

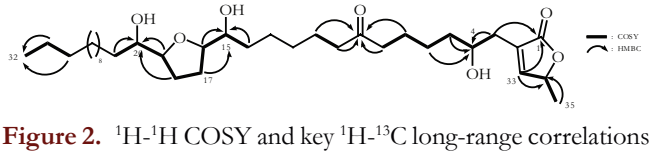

Figure 2. ^{1}H-^{1}H COSY and key ^{1}H-^{13}C long-range correlations of **1**.

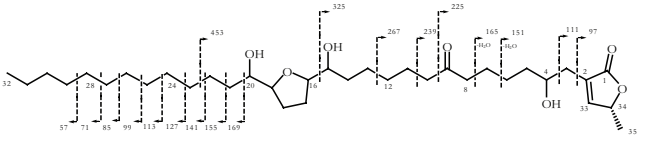

Figure 3. Diagnostic EIMS fragment ions (m/z) of **1**.

of **1**. Fragment ions at *m/z* 325 of the THF carbon and at *m/z* 169 and 155 of the α and β carbons, respectively were observed. The long chain hydrocarbon was located at C-21 to C-32. Resonances for the terminal methyl carbon (C-32) and the methylene carbons (C-31 and C-30) were assigned (Table 1) based on long-range correlations between H_2-30, H_2-31 and C-32. The remaining methylene carbons (C-22 to C-29) were assigned as overlapping resonances. It is noted however, that fragment ions at *m/z* 57, 71, 85, 99, 113, 127, 141, 155 and 169 each resulted from the loss of a methylene. Typical fragments of acetogenins arise from C-C bond cleavage,[12,13] α-cleavage,[11,14,15] hydrogen transfer,[15–18] loss of H_2O,[19] and their combinations.

Literature search revealed that **1** is annoreticuin-9-one as evidenced by similar ^{1}H and ^{13}C NMR data.[20] Annoreticum-9-one (**1**) was first isolated from *A. reticulata*. This acetogenin was also isolated from *A. squamosa* and reported to exhibit cytotoxic activities against the human pancreatic tumor cell line, PACA-2, with $ED_{50} = 2.4 \times 10^{-4}$ µg/mL; human prostate adenocarcinoma, PC-3, with $ED_{50} = 9.8 \times 10^{-3}$ µg/mL; human lung carcinoma, A-549, with $ED_{50} = 2.7 \times 10^{-1}$ µg/mL, and brine shrimp lethality test, BST, with $LC_{50} = 2.4 \times 10^{-4}$ µg/mL.[20]

The ^{1}H NMR data of **2** (Table 1) gave similar resonances to **1**. The only difference was the appearance of the oxymethine proton at δ 3.58 in **2** and the non-appearance of the α-methylene protons at δ 2.38. These suggested that the carbonyl in **1** was reduced to a hydroxyl in **2.** This was supported by the ^{13}C NMR data of **2** (Table 1) which indicated the appearance of an oxymethine carbon at δ 71.6 and the non-appearance of the carbonyl carbon of a ketone at δ 211.4. Fragments of **2** (Figure 4) were deduced from COSY. The protons attached to carbons were assigned (Table 1) from HMQC 2D NMR data and the structure of **2** was elucidated by analysis of the HMBC 2D NMR data: key HMBC correlations are shown in Figure 4.

The structure of **2** was confirmed by HRMS which gave a molecular ion of *m/z* 596.4652 [M^+], corresponding

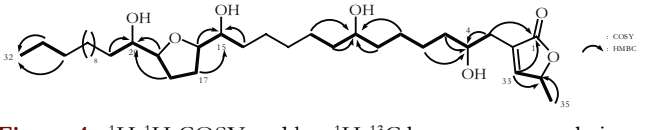

Figure 4. ^{1}H-^{1}H COSY and key ^{1}H-^{13}C long-range correlations of **2**.

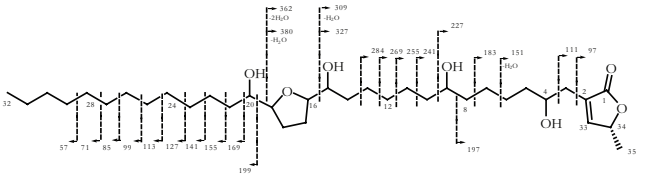

Figure 5. Diagnostic EIMS fragment ions (m/z) of **2**.

to a molecular formula of $C_{35}H_{64}O_7$. In comparison to **1**, acetogenin **2** exhibited similar fragments from the cleavage of C2-C3 and C4-C5 bonds (Figure 5). The respective placements of two alcohols at C4 and C9 were confirmed by *m/z* 151, 183, 197, 227, 241, 255, 269, and 284. Similar to **1**, acetogenin **2** contains another fatty acid chain as confirmed by fragment ions at *m/z* 57, 71, 85, 99, 113, 127, 141, 155, and 169. The fragmentation at C15-C16 (*m/z* 309, 327) and C19-C20 (*m/z* 362, 380) confirmed the location of the THF ring.

Literature search revealed that **2** is *cis*-annoreticuin as evidenced by similar ^{1}H and ^{13}C NMR data.[21] *Cis*-annoreticuin (**2**) was first isolated from *A. montana* and reported to exhibit cytotoxic activity against human hepatoma carcinoma cell line, Hep G2 with $ED_{50} = 2.4 \times 10^{-3}$ µg/mL.[21]

Overall, the fragments obtained by EI-mass spectrometry were consistent with the structures of **1** and **2**. Mass fragmentation analyses revealed that these mono-THF acetoginins have the same location of THF ring. It is noteworthy that no fragmentation corresponding to other possible locations of THF could be detected in all mass spectra. Interestingly, **1** and **2** have similar structural feature with mono-THF acetogenins that have been reported recently.[19] That is, the carbon skeleton has the form C_{13}-THF-C_{18} ($C_{35}H_nO_7$). The subtle structural difference suggests that these natural products probably arise from a common biological precursor.[7]

The structure of **3** was elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of its ^{1}H NMR and ^{13}C NMR data (Table 2) with those reported in the literature for sabadelin,[12] a constituent of the roots of *A. muricata*.

ACKNOWLEDGMENT

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Table 2. Comparison of the 600 MHz ^{1}H NMR and 150 MHz ^{13}C NMR Data of Acetogenin 3 in $CDCl_3$ and 400 MHz ^{1}H NMR and 50 MHz ^{13}C NMR data of Sabadelin in $CDCl_3$.

Position	3		Sabadelin	
	δ C	δ H (*J* Hz)[a]	δ C	δ H (*J* Hz)[a]
1	173.9		173.9	
2	134.3		134.3	
3	25.2	2.30 t (7.2)	25.2	2.26 t (7.3)
4	27.2	1.55	27.2	1.55
5–10	26.6–29.7	1.24–1.29	26.6–29.7	1.25–1.29
11	26.6–29.7	1.32	26.6–29.7	1.32
12	27.4	2.02	27.4	2.05
13	128.3	5.39	128.3	5.41
14	131.0	5.37	131.0	5.39
15	24.3	2.22	24.3	2.22
16	28.0	1.57	28.0	1.58
17	57.3	2.90	57.3	2.93
18	56.8	2.89	56.8	2.91
19	27.9	1.50	27.9	1.50
20–29	26.6–29.7	1.24–1.29	26.6–29.7	1.25–1.29
30	31.9	1.24–1.29	31.9	1.25–1.29
31	22.7	1.24–1.29	22.7	1.25–1.29
32	14.1	0.88 t (7.2)	14.1	0.88 t (6.8)
33	148.8	6.96 d (1.2)	148.8	6.98 d (1.6)
34	77.4	4.98 dq (1.8, 6.6)	77.4	4.99 dq (1.6, 6.8)
35	19.2	1.40 d (6.6)	19.2	1.41 d (6.8)

[a] Multiplet unless otherwise indicated

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In vivo and *in vitro* anti-inflammatory activity of *Harrisonia perforata* root extract

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Introduction: *Harrisonia perforata* root is one of the five medicinal plants in Bencha-Loga-Wichien remedy used for the treatment of fever in Thai traditional medicine. This study was aimed to investigate the anti-inflammatory effect and the action mechanism of the ethnolic extract from the root of *H. perforata in vivo* and *in vitro*. **Methods:** Male Wistar rats were divided into 6 groups and treated with either the ethanolic extract of *H. perforata* at dose 5–400 mg/kg or indomethacin 5 mg/kg or vehicle. Rat paw volume was measured at 1, 2, 3, 4, 5 and 6 h after carrageenan injection. The *in vitro* mechanism of the antiinfammatory response was investigated through the measurement of mRNA expression of proinflammatory cytokines, TNF-α, IL-6 and IL-1β in the macrophage cell. Lipopolysaccharide-stimulated macrophage, J774A.1 cell, were exposed to different concentration of *Harrisonia perforata* root extract (12.5–50 μg/ml). mRNA expression, TNF-α, IL-1β and IL-6, were determined by real-time RT-PCR. **Results:** *H. perforata* root extract 50–400 mg/kg significantly reduced the effect of acute inflammation in rat paw edema by 28.49%–65.05% at 2 hour after carrageenan injection. Cell viability were not significantly altered by the presence of *H. perforata* extract 3.125–50 μg/ml. It was found that the maximum inhibitory effect for TNF-α, IL-1β and IL-6 were 49.83, 47.27 and 32.16% respectively. The concentration of the extract produced maximum effect were 50 μg/ml for both TNF-α and IL-1β but for IL-6, it was 12.5 μg/ml. **Conclusion:** The ethanolic extract from *H. perforata* root possesses anti-inflammatory effect. This may occur via suppression of proinflammatory cytokines.

Keyword: *Harrisonia perforata*, antiinflammation, proinflammatory cytokines.

INTRODUCTION

Inflammation is a process of immune system in response to foreign invader or cells and tissue damage. Inflammation involves the local vascular system, the immune system, inflammatory cells, mediators and cytokines within the injured tissue. It is well established that macrophages play important roles in production of various cytokines, reactive oxygen and nitrogen species, growth factors and chemokines in response to inflammatory trigger substances such as bacteria lipopolysaccharide, chemical mediators.[1] Although modern anti-inflammatory drugs such as steroids and non-steriodal antiinflammatory drugs are effective in the treatment of the inflammatory condition, their use are limited because of the side effect associated with the drug treatment. All non-steroidal anti-inflammatory drugs (NSAIDs) carry a risk of upper gastrointestinal complications.[2] There is a need in searching the new potential bioactive compounds which are as effective as the existing product with the lowest side effect for the treatment of the inflammatory diseases.

Harrisonia perforata (Blanco) Merr. is belonged to Family Simaroubaceae and known in Thailand as Khon Tha. Apart from Thailand, it is found in Myanmar, Philippines, Malaysia, Laos and Indonesia.[3] This plant is used in Vietnam as a folk medicine for the treatment of itching. In Indonesia and Philippines, the root bark is a remedy for diarrhoea, dysentery and cholera[4] *H. perforata* root is one of the five medicinal plants in Bencha-Loga-Wichien remedy used for the treatment of fever in Thai traditional medicine. Pharmacology investigation revealed that *H. perforata* (roots and stem) exhibited a bactericidal effect against *Mycobacterium smegmatis*.[5] Moreover, the extracts of the leaves and the branches demonstrated *in vitro* antimalarial activity against *Plasmodium falciparum*.[6]

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The powder root also showed antipyretic effect.[7] From our previous study, it was found that the ethanolic root extract of *H. perforata* possessed direct inhibitory effect on macrophage activation.[8] Several chromones, peucenin-7-methyl ether, O-methylalloptaeroxylin (perforatin A), perforatin B, perforatic acid, perforatic acid methyl ester and perforatin C-G and perforatinolone, have been isolated from the roots[9,10] leaves[11,12] branches[13] and wood[14] In acute toxicity study, rats fed with the water extract of *H. perforata* at the dose of 5,000 mg/kg did not show any sign of toxicity over 14-day period of observation. Subchronic toxicity study found that the extract at doses up to 1,200 mg/kg body weight caused no significant toxicity to male and female rats over 90 days after the extract feeding including the body and organ weights, hematological and blood clinical chemistry.[15] Although a number of pharmacological activities of this plant have been documented, the supporting evidence for its anti-inflammatory activity is still limited. Therefore this present study is aimed to elucidate the *in vivo* and *in vitro* antiinflammatory activity of the ethanolic root extract from *H. perforata*.

MATERIALS AND METHODS

Plant Material and Preparation of Plant Extract

The ethanolic root extract of *H. perforata* was prepared by Ruangrungsi N, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, the plant was collected from Nongkhai province of Thailand and authenticated. The voucher and number of specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. The roots of *H. perforata* were dried under shade and grinded to coarse powders. The powder of roots was macerated in absolute ethanol in closed conical flask for 24 hours. Then the extracts were evaporated to dryness under vacuum and identified by TLC and stored in a solid stage at –4°C until used.

Preparation of the Extract Solution for In Vitro Study

The ethanolic root extract of *H. perforata* was dissolved in 100% DMSO and stored at –20°C until used. The stock solution was diluted with sterile double distilled to the constant final concentration of DMSO at 0.2%.

Preparation of the Extract Solution for In Vivo Study

The ethanolic roots extract of *H. perforata* was dissolved in 5% Tween 80 solution.

Cells

J774A.1 cells are murine macrophages obtained from ATCC. The cells were maintained in the completed Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, 97% humidity, 5% CO_2. They were subcultured 3 timed weekly during used.

Experimental Animals

Male Wistar rats weighing 100–150 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakhorn Pathom, Thailand. The animals were housed in animal care facility at the Faculty of Medicine, Chulalongkorn University, for at least 7 days before the experiment was performed. They were taken care with standard diet and water in a room under controlled environment; room temperature 25±2°C with 12-h light/dark cycle. All animal handling and experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Ethic Committee, Faculty of Medicine, Chulalongkorn University.

Chemicals and Reagents

The chemicals and reagents used in this study were in the followings; Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA), penicillin/streptomycin (Hyclone, USA), fetal bovine serum (Gibco, USA), sodium bicarbonate (Baker, USA), dimethyl sulfloxide (DMSO) (Sigma, USA), trypan blue dye (Sigma, USA), lipopolysaccharide (LPS) (Sigma, USA), nitric oxide assay kit (Promega, USA), rezasurin (Sigma, USA), TRiZol reagent (Invitrogen, UK), chloroform (Sigma, USA), isopropanol (Bio Basic, Canada), DEPC (Molekula, UK), ImProm-II™ Reverse Transcription system (Promega, USA), agarose gel (Vivantis, Malaysia), primers (Bio Basic, Canada), taq polymerase (Invitrogen, UK), tween 80 (Labchem, New Zealand), indomethacin (Parma, Italy), carrageenan (Sigma, USA), dexamethasone (T.P. drug, Thai).

Carrageenan-induced Paw Edema Test in Rats

The carrageenan-induced paw edema model was used to evaluate anti-inflammatory activity of the extract as the method described by Winter et al.[16] Rats were weighted and marked with black ink at a ground of lateral malleolus for measuring of the paw volume using plethysmometer. The animals were randomly divided into six groups with 6 animals per group. They were intraperitoneally injected with the extract (50, 100, 200, 400 mg/kg), indomethacin

5 mg/kg (positive control group) or 2% tween 80 (negative control group) one hour before carrageenan injection into sub-plantar side of the right hind paw. Paw volume were measured before and at 1, 2, 3, 4, 5 and 6 h after carrageenan injection following the previous studies.[17,18]

The inhibitory activity of *H. perforata* ethanolic root extract was determined as the percentage of paw edema inhibition in the following equation.

$$\% \text{ inhibition} = \left(\frac{(V_t - V_0)_{control} - (V_t - V_0)_{treated}}{(V_t - V_0)_{control}} \right) \times 100$$

Where V_0: basal volume of paw before carrageenan injection.

V_t: volume of edema paw after carrageenan injection at each time point.

Cell Viability

The viability of the cells was assessed in order to evaluate whether the extract possessed cytotoxic effect to J774A.1 murine macrophages cells using the resazurin assay method described by Anoopkumar-Dukie S with some modification.[19] This assay is based on the principle that the enzyme in viable cells can reduce blue color agent resazurin to red color resorufin. The cells were treated with the ethanolic root extract of *H. perforata* at the concentrations 3.125–50 μg/ml. DMSO and 10 μM dexamethasone were used as the negative and positive controls respectively. The cells were stimulated with 100 ng/ml LPS for 24 h at 37°C. Supernatant was removed from the treated cells. The complete DMEM medium containing 50 μg/ml of resazurin 100 μl was added in each well and incubated for 3 h at 37°C.The absorbance of each well was then read at 570 and 600 nm by microplate reader. The optical density of the sample was compared to that of the negative control to obtained the percentage of cell viability as presenting in the following equation.

$$\% \text{ cells viability} = \left(\frac{OD_{0.2\% \text{ DMSO}} - OD_{sample}}{OD_{0.2\% \text{ DMSO}}} \right) \times 100$$

Assay for mRNA expression of inflammatory mediators in LPS-activated acrophages (TNF-α, IL-6 and IL-1β).

Isolation of Total RNA

J774A.1 cells at the density 2×10^5 cell/well in 24-well plate were incubated for 24 h at 37°C, 97% humidity, 5% CO_2. The cells were then pretreated with the ethanolic extract of *H. perforata* root at concentration 12.5–50 μg/ml (3 concentrations) for 24 h at 37°C. 0.2% DMSO and 10 μM dexamethasone were used as negative and positive controls respectively. The treated cells were stimulated with 100 ng/ml LPS for 4 h in order to determine cytokines expression. Total RNA from the cells was isolated by adding 1 ml Trizol reagent to lyse the cells. Then they were homogenized and incubated at room temperature for 15 minutes. Chloroform 0.2 ml was added and centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase was collected and then isopropanol 0.5 ml was added to precipitate total RNA. The mixture was standed for 10 minutes at room temperature and centrifuged at 12,000 × g for 10 minutes at 4°C to obtain total RNA precipitate. RNA pellet was washed with 75% ethanol, centrifuged and the supernatant was then removed. Dissolved total RNA pellet in DEPC-treated water and determined the concentration and contamination of total RNA at 260 and 280 nm by nanodrop. Stored total RNA at –70°C until use.

cDNA Synthesis by Reverse Transcription

cDNA synthesis was performed by adding 1 μl of total RNA of each sample and 1 μl oligo dT15 primer into 0.2 ml PCR tube and mixed. The tubes were heated at 70°C for 5 min and then immediately cooled the tubes on ice for 5 min. Reverse transcription mixture solution was prepared containing 25 mM $MgCl_2$, mixed dNTP, ribonuclease inhibitor, and reverse transcriptase. Added 15 μl of the mixture solution into each tube. Put the tubes in a thermocycler machine and set up the following condition to generate cDNA, setting the temperature at 25°C for 5 min, then 42°C for 1 hour and 30 min, and finally 70°C for 15 min. Stored cDNA samples at –20°C until use.

Inflammatory Mediator Genes Amplification by Polymerase Chain Reaction (PCR)

Each inflammatory mediator gene was prepared by adding 1 μl cDNA of each sample and 24 μl PCR mixture solution containing the corresponding primer for TNF-α, IL-6 and IL-1β gene. Adding dNTP, Taq polymerase and buffer in 0.2 PCR tube and mixed. PCR product on the gel was stained with 0.5 μg/ml ethidium bromide solution. Determined density of the PCR product by gel documentation.[20,21]

Statistical Analysis

All data were presented as means ± S.E.M. Data analysis was performed on SPSS 17.0 One-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test was used to determine the statistical significance analysis. P-value <0.05 was considered as statistically significance.

RESULTS

Carrageenan-induced Paw Edema Test in Rats

H. perforata ethanolic root extract at concentration 50–400 mg/kg and indomethacin 5 mg/kg given by intraperitoneal injection one hour before carrageenan-induced paw edema produced significant inhibitory effect on rat paw edema at every time point of the study time period. The peak inhibitory response to carrageenan occurred at the second hour after *H. perforata* 50, 100, 200, 400 mg/kg administration with the percentage of inhibition of 28.49, 31.18, 47.85 and 65.05 respectively, while indomethacin 5 mg/kg caused 37.10% inhibition of rat paw edema at the corresponding time. The highest inhibition of indomethacin (47.44%) was seen at 5 hour after carrageenan injection. (Table 1, Figure 1)

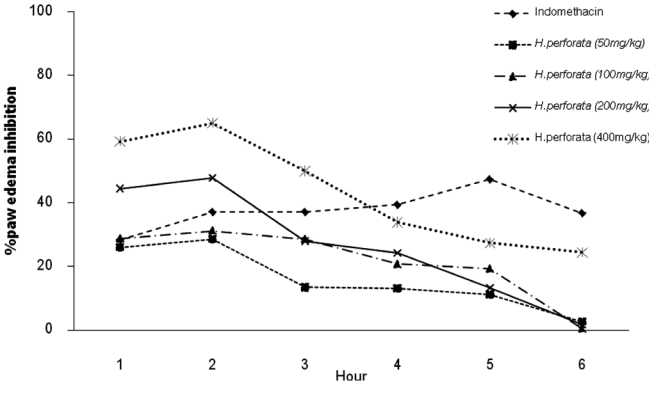

Figure 1. Effects of *H. perforata* ethnolic root extract 50–400 mg/kg and indomethacin 5 mg/kg on carrageenan-induced rat paw edema. Results are presented as the mean ± S.E.M from six rats (n=6). *$P < 0.05$ compared to the control group at the corresponding time.

Cell Viability

Cell viability were not significantly altered by the presence of 0.2% DMSO, 10 μM dexamethasone and *H. perforata* extract at the concentration used (3.125–50 μg/ml) (Figure 2). Thus the value of non-toxic concentration obtained were used in proinflammatory cytokines determination.

Effects of *H. perforata* on Pro-inflammatory Cytokine mRNA Expression

Stimulation of J774A.1 with LPS caused the generation of mRNA expression of pro-inflammatory cytokines. Pre-treated the cells with *H. perforata* 12.5–50 μg/ml showed the inhibitory activity on mRNA expression of TNF-α and IL-1β in dose dependent manner. The

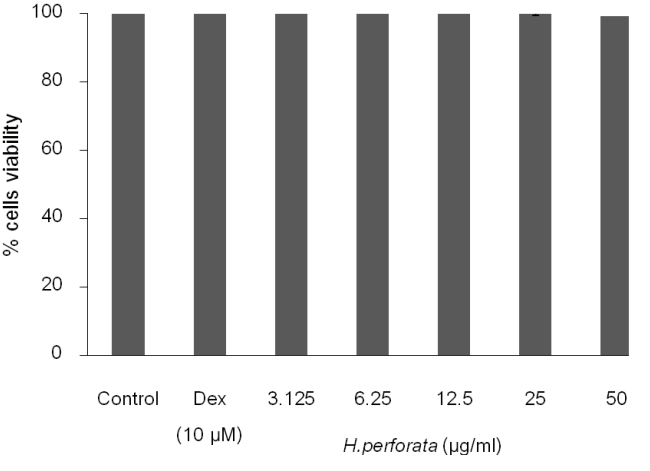

Figure 2. Effect of *H. perforata* ethnolic root extract 3.125–50 μg/ml and 10 μM dexamethasone (Dex) on cells viability in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 4 independent experiments (n=4). *$P < 0.05$ compared to untreated cells.

Table 1. Effects of the ehanolic root extract of *H. perforata* 50–400 mg/kg and indomethacin 5 mg/kg on carrageenan-induced rats paw edema. Results are presented as the mean ± S.E.M from six rats. (n=6). *$P < 0.05$ compared to the control group at the corresponding time.

Time (h)	% inhibition (paw volume)					
	Control (2% ween 80)	Indomethacin (5 mg/kg)	*H. perforata* (mg/kg)			
			50	100	200	400
1	0.36±0.04	28.24* (0.26±0.05)	25.93* (0.27±0.04)	28.70* (0.26±0.04)	44.44* (0.20±0.01)	59.26* (0.15±0.01)
2	0.31±0.04	37.10* (0.20±0.04)	28.49* (0.22±0.03)	31.18* (0.21±0.03)	47.85* (0.16±0.01)	65.05* (0.11±0.01)
3	0.31±0.01	37.10* (0.20±0.04)	13.44* (0.27±0.05)	28.49* (0.22±0.05)	27.96* (0.22±0.03)	50.00* (0.16±0.03)
4	0.33±0.03	39.39* (0.20±0.05)	13.13* (0.29±0.06)	20.71* (0.26±0.06)	24.24* (0.25±0.03)	33.84* (0.22±0.02)
5	0.39±0.03	47.44* (0.21±0.04)	11.11* (0.35±0.05)	19.23* (0.32±0.06)	13.25* (0.34±0.04)	27.35* (0.28±0.03)
6	0.39±0.03	36.75* (0.25±0.04)	2.56* (0.38±0.06)	0.43* (0.39±0.05)	1.71* (0.38±0.03)	24.36* (0.30±0.04)

highest inhibition was found to be 49.83±2.71% and 47.27±3.77%, for TNF-α and IL-1β respectively at the concentration 50 µg/ml (Figures 3, 4, and Table 2). In contrast, it was found that *H. perforata* 50 µg/ml significantly increased mRNA expression of IL-6 by 43.93±5.65%, however the expression of IL-6 was inhibited at the lowers concentration (12.5 and 25 µg/ml). Dexamethasone 10 µM also inhibited TNF-α, IL-1β and IL-6 mRNA expression by 30.06±4.09, 77.96±2.09, 89.44±0.54 respectively (Figure 5, Table 2).

DISCUSSION

As previously described , *H. perforata* root is one component of a Thai traditonnal medicine known as Bencha-Logawichien used for relieving of fever. The extract obtained from various parts of this plant demonstrated antibacterial, antiplasmodiun falciparum,[5,6] antipyretics effect.[7] In addition, the ehanolic root extract posed direct inhibitory effect on macrophage activation by inhibition of mRNA expression of cyclooxygenase (COX) and iNOS.[8] Edematous formation due to carrageenan-induced paw edema in rat used in this model is biphaic phase.[22–24] The first phase begins immediately after carrageenan injection and diminishes in two hours. the second phase begins at the end of the first phase and lasts for three to five hours. In this present study, all dose of the extract (50–40 mg/kg) effectively inhibited rat paw edema at every time point measured. The peak time of inhibition was seen at the second hour after carrageenan injection. The dose that produced maximum inhibition was 400 mg/kg (65.05%) while indomethacin 5 mg/ml

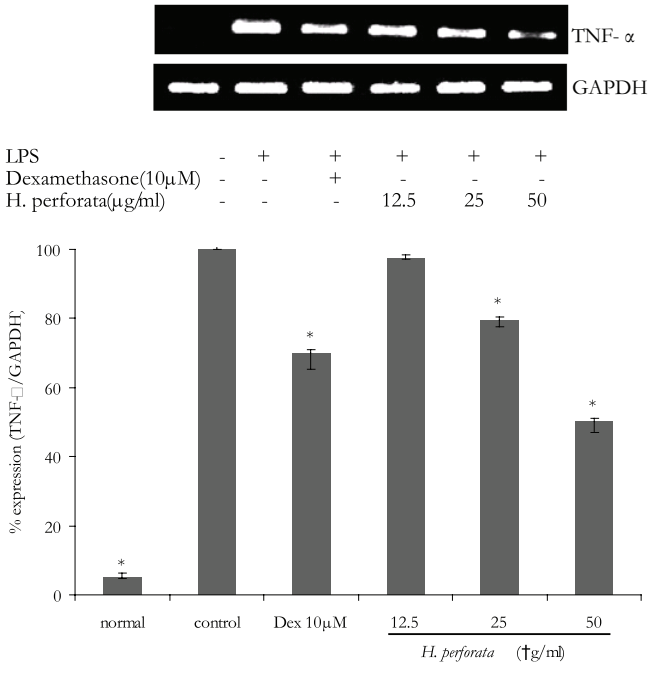

Figure 3. Inhibitory effect of *H. perforata* ethanolic root extract at the concentrations 12.5–50 µg/ml and 10 µM dexamethasone (Dex) on IL-1β mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

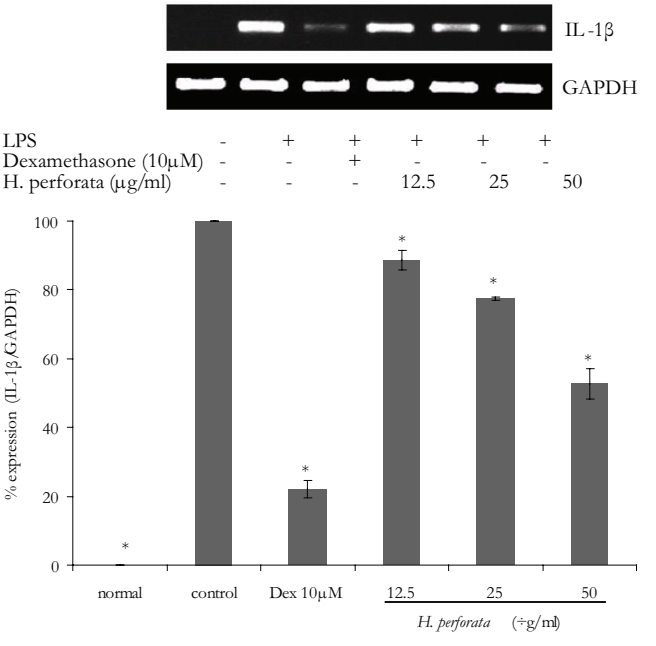

Figure 4. Inhibitory effect of *H. perforata* ethanolic root extract at the concentrations 12.5–50 µg/ml and 10 µM dexamethasone (Dex) on IL-1β mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

Table 2. Inhibitory effect of *H. perforata* ethnolic root extract 12.5–50 µg/ml and 10 µM dexamethasone on pro-inflammatory cytokine mRNA expression in LPS-stimulated J774A.1 cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells.

	Final concentration	% Inhibition		
		TNF-α	IL-1β	IL-6
Dexamethasone	10 µM	30.06±4.09*	77.96±2.09*	89.44±0.54*
H. perforata	12.5 µg/ml	2.54±0.30	11.43±2.53*	32.16±2.82*
	25 µg/ml	20.49±1.61*	22.54±0.41*	18.13±0.52*
	50 µg/ml	49.83±2.71*	47.27±3.77*	–43.93±5.65*

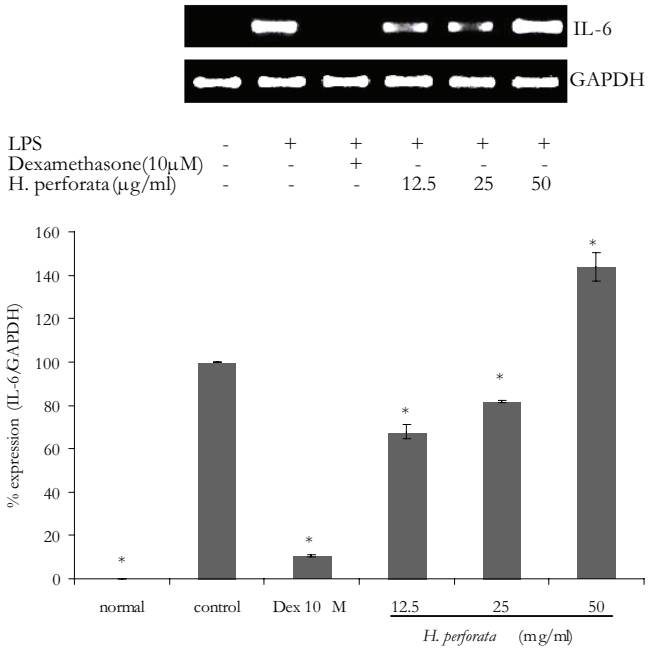

Figure 5. Effects of *H. perforata* ethanolic root extract at the concentrations 12.5–50 μg/ml and 10 μM dexamethasone (Dex) on IL-6 mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

produced 37.10% inhibition at the corresponding time point. This result indicated that *H. perforata* ethanolic root extract was able to attenuate the acute acute inflammatory process in response to carrageenan ant its effect was predominately occurred during the first phase of reaction. On contrary, indomethacin, a non selective cyclooxygenase inhibitor, exhibited the maximum inhibitory effect at the fifth hour after the induction of paw edema (47.44 %). This phenomenon is a well known fact that indomethacin acts by inhibiting prostaglandin synthesis at the late phase of inflammation.

The effect of *H. perforata in vitro* was further investigated for its anti-inflammatory mechanism in stimulated macrophage. As it is already known that macrophages are key innate immune cells contribution to diverse functions including the phagocytosis of foreign substances, expression of reactive oxygen species, production of proteins or enzymes involved in tissue remodeling, expression of chemokines and proinflammatory cytokines. Thus macrophages are involved in modulating the inflammatory process during the pathogenesis and resolution of tissue injury and inflammation.[25] Lipopolysaccharides (LPS) is known to be one of the best – stimuli in macrophage to induce transcription of genes encoding proinflammatory protein and cytokines such as TNF-α, IL-1β, IL-6, increase production of eicosanoids, oxygen and nitric oxide species. Over production of these substances cause acute and chronic inflammatory condition. In this present investigation *H. perforata* root extract significantly reduced TNF-α and IL-1β mRNA expression in LPS-stimulated macrophage J774A.1 cells in dose-dependent manner. Maximum inhibitory concentration were seen at 50 μg/ml of the extract as shown in Table 2, Figures 3, 4. As a result, the extract showed anti-inflammatory activity. However dexamethasone 10 μM exerted more potent inhibitory activity on IL-1β, IL-6 than the extract. Furthermore, it was found that the extract suppressed IL-6 expression both at low and medium concentration (12.5, 25 μg/ml) but not at the high concentration used in this study (50 μg/ml) since it stimulated IL-6 expression. This could be explain at least in part that the different in TNF-α, IL-1β, IL-6 mRNA reduction in LPS-stimulated macrophage were due to the difference in magnitude and time point in response to lipopolysaccharide activation.[26–28] Our previous report also supported this antiinflammatory phenomenon because the extract was able to inhibit nitric oxide production and the expression of iNOS and COX-2.[8] All of these consistent evidences indicated the anti-inflammatory potential of the extract.

In relation to the phytochemical constituents of the extract, it contained several chromones and limonoid compounds, there has been a number of studies report anti-inflammatory action of these phytochemical constituents.[29–31] Therefore, these phytochemical components could be responsible, in part, for the edematous inhibition and the reduction in inflammatory cytokines expression in the *in vivo* and *in vitro* model performed in this present study.

Taken together, it could be concluded that the ethanolic extract from the root of *H. perforata* possessed anti-inflammatory activity in carrageenan-induced paw edema in rat. The ability of the extract to cause edematous inhibiton was partly mediated through inhibition of proinflammatory cytokines production. The antiinflammatory potential of the extract needs further systematic investigation.

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Polyphenolics content and biological activity of *Plectranthus amboinicus* (Lour.) spreng growing in Egypt (Lamiaceae)

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ABSTRACT

Background: Volatile oil, terpenoids, mainly diterpene and polyphenolic compounds including flavonoids and phenolic acids were previously isolated from different *Plectranthus* species. *Plectranthus amboinicus* (Lour.) Spreng growing abroad was subjected to phytochemical study resulted in isolation of several flavonoids, also the plant exhibited antioxidant, diuretic, anti-inflammatory, cytotoxic and antimicrobial activities. **Materials and Methods:** In this study ethyl acetate fraction of *Plectranthus amboinicus* (Lour.) Spreng leaves growing in Egypt was fractionated and chromatographed on silica gel and sephadex to isolate its phenolic constituents. The isolated compounds were identified using UV, ^{1}HNMR and ^{13}CNMR. Total phenolics and tannins content of the leaves, stems and roots of *Plectranthus amboinicus* (Lour.) Spreng were determined using Folin-Ciocalteau and Folin-Denis reagents, respectively. Phenolic compounds of the stems and roots were identified using UPLC-MS analysis. Leaves, stems and roots of this plant were tested for antioxidant, anti-inflammatory, analgesic, diuretic, cytotoxic and antimicrobial activities. **Results:** The isolated compounds were identified as 3-methoxy genkwanin, crisimaritin, *p*-coumaric acid, caffeic acid, taxifolin, rosmarinic acid, apigenin and 5-*O*-methyl-luteolin. The stems showed the highest concentration of the total polyphenolics followed by the leaves then the roots (9.6, 8.4 and 5.4 mg/g of gallic acid equivalents, respectively), while the roots recorded the highest tannins content followed by the leaves then the stems (126, 90 and 81 μg/g of tannic acid equivalents, respectively). UPLC-MS analysis revealed the presence of caffeic acid, rosmarinic acid, coumaric acid and chrysoeriol in the stems and roots, while luteolin, quercitin and eriodyctiol were detected only in the stems. The different extracts of the three organs exhibited antioxidant, anti-inflammatory, analgesic, diuretic, cytotoxic and antimicrobial activities with variable potency.

Keywords: *Plectranthus amboinicus*, Lamiaceae, phenolic compounds, antioxidant, anti-inflammatory, analgesic, diuretic, cytotoxic, antimicrobial

INTRODUCTION

Plectranthus is one of the oil-rich genera belonging to family Lamiaceae.[1] Diterpenoids, usually highly modified abietanoids, are the major group of secondary metabolites in this species.[2] Flavonoids, phenolic acids and phenolic acid esters had been isolated from different *Plectranthus* species.[2–5] Several flavonoids had been isolated from *Plectranthus amboinicus* (Lour.) Spreng growing in South America[6] (synonyms: *Plectranthus aromaticus* Roxb., *Coleus aromaticus* Benth. and *Coleus amboinicus* Lour.).[1] This plant was reported to possess variable biological activities, mainly, antioxidant,[7,8] diuretic,[8,9] anti-inflammatory,[10] cytotoxic[10] and antimicrobial[11] activities. No reports were found on the plant growing in Egypt, so this study was performed to investigate the phenolic content and biological activities of the Egyptian plant. The study includes isolation and identification of the major compounds of the ethyl acetate fraction of the leaves, quantitative determination of the total polyphenolics and tannins content of the leaves, stems and roots and identification of phenolic constituents in the stems and roots using high resolution UPLC-MS analysis.

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The antioxidant, anti-inflammatory, analgesic, diuretic, cytotoxic and antimicrobial activities of the different extracts of the leaves, stems and roots were studied.

MATERIAL AND METHODS

General Experimental

Electro thermal 9100 was used for determination of melting point, UV spectra was determined on Beckman Du-7 and Schimadzu 265 spectrophotometers, ^{1}H-(300 MHz) and ^{13}C-(75 MHz) NMR spectra were recorded on Varian Mercury apparatus at 25°C using TMS as an internal standard and chemical shifts were given in δ values. TLC was performed on precoated silica gel plates 60 F 254 (E-Merck), using solvent systems S_1 [$CHCl_3$: MeOH (98:2)], S_2 [$CHCl_3$: MeOH (95:5)], S_3 [$CHCl_3$: MeOH: Formic acid (90:10:2 drops)], S_4 [$CHCl_3$: MeOH: Formic acid (85:15: 2 drops)], S_5 [$CHCl_3$:MeOH:Formic acid (80:20:2 drops)] and S_6 [$CHCl_3$: MeOH (90:10)]. The chromatograms were visualized under UV light (at λ_{max} 254 and 366 nm) before and after exposure to ammonia vapor, as well as spraying with *p*-anisaldehyde/sulphuric acid spray reagent.

Plant Material

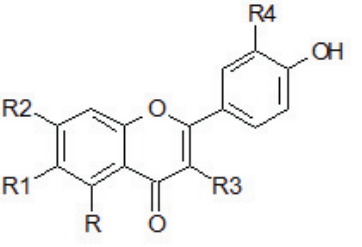
Plant material of *Plectranthus amboinicus* (Lour.) Spreng were collected all over the years (2008–2010) from El-Orman garden. The plant was kindly identified by Dr. Mohamed el Gebaly and Madam Treze (Taxonomist). A voucher specimen was kept in the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Extraction and Isolation

Two kilograms of the air-dried and powdered leaves of *P. amboinicus* were percolated with 70% ethyl alcohol till exhaustion. The hydroalcoholic extract was evaporated under reduced pressure at a temperature not exceeding 60ºC to give 190 g (9.5%) dark green residue. The residue obtained was suspended in water and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The solvent in each case was evaporated under reduced pressure to give *n*-hexane (29 g, 1.45%), chloroform (11.2 g, 0.56%), ethyl acetate (8.5 g, 0.43%) and *n*-butanol (7 g, 0.35%) fractions.

Similarly, the air-dried and powdered stems (1 kg) and roots (200 g) of *P. amboinicus* were extracted with 70% ethyl alcohol to yield 75 g (7.5%) and 25 g (12.5%), respectively, then fractionated to produce *n*-hexane (9 g, 0.9% and 6 g, 3%), chloroform (3 g, 0.3% and 2 g, 1%), ethyl acetate (2 g, 0.2% and 3 g, 1.5%) and *n*-butanol (5 g, 0.5% and 4 g, 2%) fractions of stems and roots, respectively.

Ethyl acetate fraction (7.5 g) of the leaves was fractionated on sephadex LH-20 using 100% methanol as eluent. Fractions of 3 ml each were collected. The obtained fractions were subjected to TLC, similar fractions were pooled and rechromatographed on sephadex LH-20 and/or silica gel 60, which afforded eight compounds (1–8).



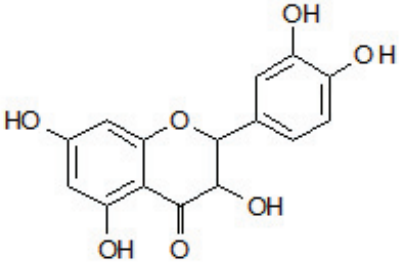
Compound	R	R1	R2	R3	R4
1	OH	H	OCH_3	OCH3	H
2	OH	OCH_3	OCH_3	H	H
7	OH	H	OH	H	H
8	OCH_3	H	OH	H	OH

Compound 1: 240 mg, yellow powder, soluble in chloroform, R_f = 0.56 in S_1, UV λ_{max} nm: MeOH (269–343), $NaOCH_3$ (270–396), $AlCl_3$ (276–387), $AlCl_3$/HCl (277–385), NaOAc (269–352), NaOAc/Boric acid (269–352), ^{1}HNMR (DMSO), δ ppm: 7.59 (2H, d, J = 7.2, H-2' & H-6'), 6.95 (2H, d, J = 8.7 Hz, H-3' & H-5'), 6.80 (1H, br.s, H-8), 6.37 (1H, br.s, H-6), 3.90 (3H, s, 7-OMe), 3.87 (3H, s, 3-OMe), ^{13}C NMR (DMSO), δ ppm: 181.89 (C-4), 165.07 (C-2), 161.10 (C-5), 148.01 (C-9), 120.76 (C-2'), 120.45 (C-6'), 103.31 (C-10), 92.66 (C-8), 55.98 (3 &7- OCH_3).

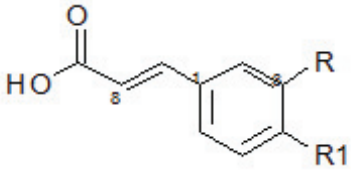
Compound 2: 50 mg, yellow powder, soluble in chloroform, R_f = 0.56 in S_2, UV λ_{max} nm: MeOH (277–335), $NaOCH_3$ (274–376), $AlCl_3$ (301–361), $AlCl_3$ / HCl (300–359), NaOAc (274–336), NaOAc / Boric acid (275–336), ^{1}HNMR (DMSO), δ ppm: 7.98 (2H, d, J= 8.7 Hz, H-2' & H-6'), 6.95 (2H, d, J=8.7 Hz, H-3'& H-5'), 6.93 (1H, s, H-8), 6.84 (1H, s, H-3), 3.93 (3H, s, 7-OCH_3), 3.73 (3H, s, 6-OCH_3), ^{13}C NMR (DMSO), δ ppm: 182.13 (C-4), 164.00 (C-2), 158.53 (C-5), 128.445 (C-2' & C-6'), 115.91 (C-3' & C-5'), 102.61 (C-3), 91.51 (C-8), 59.90 (6-OCH_3), 56.39 (7-OCH_3).

Compound 3: 24 mg, white crystals, soluble in methanol, m.p. 209–213 °C, R_f = 0.64 in S_4, ^{1}HNMR (CD_3OD), δ ppm: 7.57 (1H, d, J = 16.2 Hz, H-7), 7.41 (2H, d, J = 8.4 Hz, H-2, H-6), 6.79 (2H, d, J = 8.4 Hz, H-3, H-5), 6.24 (1H, d, J = 15.9 Hz, H-8), ^{13}C NMR (CD_3OD), δ ppm: 171.05 (C-9), 161.07 (C-4), 146.70 (C-7), 131.08 (C-2, C-6), 127.26 (C-1), 116.82 (C-3, C-5), 115.58 (C-8).

Compound 4: 90 mg, buff powder, soluble in methanol, R_f = 0.72 in S_3, ^{1}HNMR (DMSO), δ ppm: 7.37 (1H, d, J = 15.6Hz, H-8), 7.01 (1H, br. s, H-2), 6.93 (1H, d, J = 8.4 Hz, H-6), 6.74 (1H, d, J = 8.1 Hz, H-5), 6.13 (1H, d, J=16.2 Hz, H-7).^{13}C NMR (DMSO), δ ppm: 167.80 (C-9), 148.05 (C-4), 145.50 (C-3), 144.42 (C-7), 125.64 (C-1), 121.03 (C-6), 115.69 (C-5), 115.12 (C-8), 114.56 (C-2).

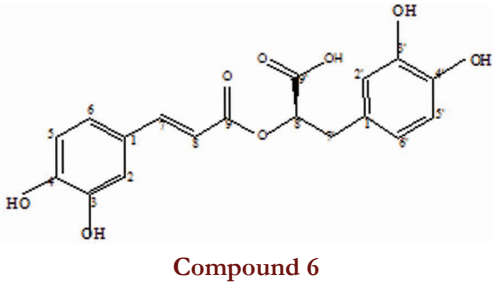


Compund 5



Compound	R	R1
3	H	OH
4	OH	OH

Compound 5: 52 mg, yellow powder, soluble in methanol, R_f = 0.51 in S_4, UV λ_{max} nm: MeOH (292–329), $NaOCH_3$ (247 sh., 328), $AlCl_3$ (280 sh., 312, 375), $AlCl_3$/HCl (312, 375), NaOAc (290 sh., 329), NaOAc/Boric acid (293, 336 sh), ^{1}HNMR (CD_3OD), δ ppm: 6.97 (1H, d, J=2.1 Hz, H-6'), 6.86 (1H, dd, J = 8.5 & 2.1 Hz, H-2'), 6.81 (1H, d, J = 8.4 Hz, H-3'), 5.92 (1H, d, J = 2.1 Hz, H-8), 5.88 (1H, d, J = 2.4 Hz, H-6), 4.93 (d, J = 11.4, H-3), 4.51 (d, J = 11.7 Hz, H-2), ^{13}C NMR (CD_3OD), δ ppm: 198.30 (C-4), 168.79 (C-5), 165.27 (C-7), 164.46 (C-9), 147.10 (C-4'), 146.27 (C-3'), 129.86 (C-1'), 120.89 (C-6'), 116.09 (C-2'), 115.88 (C-5'), 101.79 (C-10), 97.34 (C-6), 96.31 (C-8), 85.08 (C-2), 73.65 (C-3).



Compound 6

Compound 6: 50 mg, colorless amorphous solid, soluble in methanol, R_f = 0.41 in S_5, ^{1}HNMR (DMSO), δ ppm: 7.39 (1H, d, J = 15.9 Hz, H-7), 7.03 (1H, d, J=1.8 Hz, H-2), 6.96 (1H, dd, J=8.1 & 1.8 Hz, H-6), 6.74 (1H, d, J=8.1 Hz, H-5), 6.66 (1H, d, J=1.8 Hz, H-2'), 6.07 (1H, d, J= 8.1 Hz, H-5'), 6.51 (1H, dd, J=8.1 & 1.8 Hz, H-6'), 6.18 (1H, d, J=15.9 Hz, H-8), 4.94 (1H, dd, J=8.7 & 3.9 Hz, H-8'), 3.02 (1H, dd, J=15 & 3.9 Hz, H-7a'), 2.88 (1H, dd, J= 13.5 & 9.3 Hz, H-7b').

Compound 7: 170 mg, yellow powder, soluble in methanol, R_f = 0.57 in S_6, UV λ_{max} nm: MeOH (266, 338), $NaOCH_3$ (277, 323, 390), $AlCl_3$ (277, 386), $AlCl_3$/HCl (277, 385), NaOAc (272, 376), NaOAc / Boric acid (269, 338), ^{1}HNMR (DMSO), δ ppm: 7.87 (2H, d, J = 7.5 Hz, H-2' & H-6'), 6.89 (2H, d, J = 7.8 Hz, H-3' & H-5'), 6.70 (1H, br.s., H-8), 6.42 (1H, s, H-3), 6.13 (1H, br. s, H-6).

Compound 8: 80 mg, yellow powder, soluble in methanol, R_f = 0.45 in S_6, UV λ_{max} nm: MeOH (256, 268 sh, 349), $NaOCH_3$ (274, 405), $AlCl_3$ (274, 310 sh, 421), $AlCl_3$/HCl (270, 381), NaOAc (273, 376), NaOAc/Boric acid (264, 301, 373), ^{1}HNMR (CD_3OD), δ ppm: 7.37 (2H, m, H-2' & H-6'), 6.89 (1H, d, J=9.3 Hz, H-5'), 6.54 (1H, s, H-3), 6.43 (1H, d, J=2.1 Hz, H-8), 6.20 (1H, d, J=1.8 Hz, H- 6).

Quantitative Determination of the Total Polyphenolics Content

Total phenolics content of the leaves, stems and roots of *Plectranthus amboinicus* (Lour.) Spreng were determined by Folin-Ciocalteau reagent, using gallic acid as standard.[12]

Quantitative Determination of the Tannins Content

Tannins content of the leaves stems and roots of *Plectranthus amboinicus* (Lour.) Spreng was determined by Folin-Denis reagent using tannic acid as standard.[13]

High Resolution UPLC-MS Analysis

The ethyl acetate fraction of the stems and roots of *Plectranthus amboinicus* (Lour.) Spreng were subjected to LC/ESI-MS, to investigate the major fingerprint ions. Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with a HSS T3 column (100 × 1.0 mm, particle size 1.8 μm; Waters) applying the following binary gradient, at a flow rate of 150 μL min^{-1}: 0 to 1 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9/0.1 [v/v]); 1 to 16 min, linear from 5 to 95% B; 16 to 18 min, isocratic 95% B; 18 to 20 min, isocratic 5% B. The injection volume was 3.1 μL (full loop injection). Eluted compounds were detected from m/z 100 to 1000 using a MicrOTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in positive and negative

ion modes using the following instrument settings: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 litres min^{-1}, 190 °C; capillary, –5500 V (+4000 V); end plate offset, –500 V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; in-source CID energy, 0 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, argon; collision energy, 10 eV; collision RF 200/400 Vpp (timing 50/50); transfer time, 70 µs; prepulse storage, 5 µs; pulser frequency, 10 kHz; spectra rate, 3 Hz. Internal mass calibration of each analysis was performed by infusion of 20 µL 10 mM lithium formiate in isopropanol/water, 1/1 (v/v), at a gradient time of 18 min using a diverter valve. Identification of phenolic compounds was carried out by comparing retention times and mass spectra with those of authentic standards and/or based on accurate mass of pseudomolecular [M – H] or [M + H] ions. The major peaks observed have been tabulated in Table 1.

Plant Extracts for Biological Study

Alcoholic extracts of the leaves, stems and roots were prepared by macerating 100 g of each organ, separately, in 70% ethyl alcohol till exhaustion. The hydroalcoholic extract in each case was evaporated under reduced pressure to obtain a semisolid residue.

Aqueous extracts were prepared by boiling 100 g of the powdered leaves, stems and roots, separately, with bidistilled water. The aqueous extracts were dried by lyophilization.

Lyophilized juice was prepared by mixing about 500 g of fresh leaves with distilled water (least amount), with the help of mixer. The fresh juice was filtered and concentrated to a dry residue using lyophilizer.

The residues of different extracts were dissolved in tween 80 in selected doses based on their respective LD_{50} or dissolved in dimethyl sulphoxide at a concentration of 200 mg/ ml then 50 µl (containing 10 mg of each extract) were screened for the antimicrobial activity.

Experimental Animals

Albino mice 25–30 g body weight was used for the toxicity study and for analgesic effect.

Adult male albino rats of Sprague Dawely Strain weighing 130–150 g were used for the determination of antioxidant, anti-inflammatory and diuretic activities (according to the ethics of national research center). The rats were kept on standard laboratory diet under hygienic conditions. Water was supplied *ad lib.*

Cancer cell lines: Hepatocellular carcinoma cell line (HEPG2) and breast carcinoma cell line (MCF7), obtained from National Cancer Institute, Kasr El Ainy, Cairo, Egypt.

Micro-organisms: Gram positive bacteria [*Streptococcus mutans* (clinical isolates), *Lactobacillus acidophilus* (clinical isolates), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 6538) and methicillin resistance *staphylococcus aureus* (MRSA) (ATCC 12692)], Gram negative bacteria [*Klebsiella pneumonia* (ATCC 4352), *Pseudomonas aeruginosa* (ATCC 9027) and *E. coli* (ATCC 8739)], filamentous fungi [*Aspergillus flavus* (ATCC 15517) and *Aspergillus niger* (ATCC 16404)], and yeast [*Candida albicans* (ATCC 10231) and *Candida parapsilosis* (ATCC 22019)], available in stock culture of the Microbiology Department, Faculty of Pharmacy, Al-Azhar university, were used for antibiotic sensitivity testing.

Reference Drugs and Kits

Vitamin E: Pharco Pharmaceutical Co.

Indomethacin: Epico, Egyptian Int. Pharmaceutical industries Co., A.R.E. Carrageenan: Sigma Co.

Biodiagnostic glutathione kit.

Table 1. Identified Peaks in the Ethyl Acetate Fractions of Stems and Roots of *P. Amboinicus* by UPLC-MS.

	Polyphenols	Rt Min	M/Z	Ion	Formula	Error ppm	RDB	Organ
1	Caffeic acid	5.85	179.0342	[M–1]	$C_9H_7O_4$	4.4	6.5	S, R
2	Eriodyctiol	6.15	287.1491	[M–1]	$C_{14}H_{23}O_6$	3.5	3.5	S
3	Rosmarinic acid	6.22	359.0781	[M–1]	$C_{18}H_{15}O_8$	–2.5	11.5	S, R
4	Coumaric acid	6.27	163.0398	[M+1]	$C_9H_7O_3$	–5.2	6.5	S, R
5	Luteolin	6.52	285.0407	[M–1]	$C_{15}H_9O_6$	-8.5	11.5	S
6	Chrysoeriol	9.61	301.1406	[M+1]	$C_{18}H_{21}O_4$	9.3	8.5	S, R
7	Quercetin	9.48	303.1555	[M+1]	$C_{15}H_9O_7$	–	–	S

S: Stems, R: Root, -: not determined

Moduretic drug (hydrochlorothiazide): Kahira Pharma and Chemical Ind. Co.

Doxorubicin®: Sigma-Aldrich Co., US.

Discs of ceftriaxon and clotrimazole: 5 µg/disc, Oxoid Chemical Co., UK

Toxicity Study

The LD_{50} of the different extracts was estimated following Karber's procedure.[14]

Antioxidant Activity

The antioxidant activity was calculated by the determination of glutathione in blood of alloxan- induced diabetic rats adopting the method of Beutler *et. al.*,[15] using vitamin E as a positive control.

The animals were divided into 11 groups (6 animals each). One group was kept as a negative control while for the other groups, diabetes mellitus was induced according to the method described by Eliasson and Samet[16] in which a single dose of 150 mg alloxan / kg b.wt. was injected intraperitoneal in each animal followed by an overnight fasting.

A group of diabetic rats was kept non- treated, another group received daily the reference drug (Vitamin E) and the other groups received the tested samples daily in the given doses (see Table 2). Blood samples were taken after a week for the determination of glutathione. The results obtained were recorded in Table 2.

Anti-inflammatory Activity

It was carried out according to the rat paw oedema method.[17] Ten groups of male albino rats were used (6 animals each). The first group received 1 ml saline orally (negative control). The second group was given indomethacin orally (positive control). The other groups received the tested samples in the dose given in Table 3. One hour later, oedema was induced in the right hind paw by a sub planter injection of 0.1 ml of 1% carrageenan solution in saline while 0.1 ml saline was injected in the left hind paw. Three hours after the induction of inflammation, the rats were sacrificed. Both paws were excised and weighed separately using an electric balance. The mean response (increase in the paw oedema) after acute inflammation and the percentage of inhibition were calculated. The results obtained were recorded in Table 3.

Analgesic Activity

Swiss male albino mice were divided into ten groups of six animals each. First group of the animals received 1 ml saline and served as control. Second group served as positive control received indomethacin, while the rest groups received the tested samples. All the samples were administered orally 30 minutes prior to the administration of acetic

Table 2. Antioxidant Activity of the Different Extracts of *P. Amboinicus* Leaves, Stems and Roots.

Groups		Blood glutathione (mg %)	% change from control	% of relative Potency**
Diabetic	Control (1 ml saline)	36.2±1.4	–	–
treated with	Diabetic non treated	21.2± 0.4*	41.44	–
Leaves	Alcoholic 100 mg/kg b. wt	34.6 ±1.3	4.42	96.64
	Aqueous 100 mg/kg b. wt	34.1 ±1.1	5.80	95.25
Stems	Alcoholic 100 mg/kg b. wt	31.8 ±1.2	12.15	88.82
	Aqueous 100 mg/kg b. wt	30.9 ±0.8	14.64	86.31
Roots	Alcoholic 100 mg/kg b. wt	29.7± 0.8*	17.96	82.96
	Aqueous 100 mg/kg b. wt	29.2 ±0.7*	19.34	81.56
Lyophilized juice of leaves 50 mg/kg b. wt		34.1± 0.6	5.80	95.25
Ethyl acetate fraction of leaves 100 mg/kg b. wt		35.4 ±1.5	2.20	98.88
Diabetic treated with 7.5 mg/ kg b. wt vitamin E		35.8 ±0.9	1.10	100.00

* Statistically significant different from control group at $p < 0.01$.
** % of potency as compared to vitamin E.

Table 3. Acute Anti-inflammatory Effect of the Different Extracts of *P. Amboinicus* Leaves, Stems and Roots.

Groups		Dose	% oedema		% of relative potency**
			Mean ± S.E.	% of change	
Control		1 ml saline	61.4± 1.7	–	–
Leaves	Alcoholic	100 mg/kg b.wt	28.3 ±0.4*	53.91	83.37
	Aqueous	100 mg/kg b.wt	32.1 ±1.1*	47.72	73.80
Stems	Alcoholic	100 mg/kg b.wt	36.9 ±1.4*	39.90	61.71
	Aqueous	100 mg/kg b.wt	39.4 ±1.8*	35.83	55.41
Roots	Alcoholic	100 mg/kg b.wt	43.8 ±2.1*	28.66	44.32
	Aqueous	100 mg/kg b.wt	47.8 ±1.6*	22.15	34.26
Lyophilized juice of leaves		50 mg/kg b.wt	37.2 ±1.3*	39.41	60.95
Ethyl acetate fraction of leaves		100 mg/kg b.wt	26.2 ±0.3*	57.33	88.66
Indomethacin		20 mg/kg b.wt	21.7 ±0.3*	64.66	100.00

* Statistically significant from control group at $p < 0.01$
** % of potency as compared to indomethacin.

acid injection (0.2 ml of 0.6% v/v, interperitoneal).[18] Each mice was then placed in an individual clear plastic observation chamber and the total number of writhes/30 minutes was counted for each mice and the percentage protection was calculated for analgesic activity. The results are given in Table 4.

Diuretic effect

The animals were held into metabolic cages, fasted for 18 hours prior to experiment allowing only water during the fasting period. After completion of the fasting period, the first group received 1ml saline on the day of the experiment and kept as negative control. The last group received moduretic drug as a positive control. The other groups received the tested samples, in the doses given in Table 5.

After treatment, the urine was collected in measuring cylinder and measured at 2, 4 and 24 hours after the dose was administered. The collected urine volume of the respective test groups was compared with the standard group. The sodium and potassium concentrations were measured.[19] The results are shown in Table 6.

Table 4. Analgesic Activity of the Different Extracts of *P. Amboinicus* Leaves, Stems and Roots.

Groups		Dose	Number of abdominal constrictions	% of inhibition	% of relative potency**
Control		1 ml saline	48.1 ± 1.3	–	–
Leaves	Alcoholic	100 mg/kg b.wt.	19.2 ± 0.4*	60.08	90.33
	Aqueous	100 mg/kg b.wt.	25.7 ± 0.6*	46.57	70.01
Stems	Alcoholic	100 mg/kg b.wt.	38.6 ± 0.9*	19.75	29.69
	Aqueous	100 mg/kg b.wt.	35.8 ±1.2*	25.57	38.44
Roots	Alcoholic	100 mg/kg b.wt.	31.4 ± 1.1*	34.72	52.20
	Aqueous	100 mg/kg b.wt.	34.1 ± 0.8*	29.11	43.77
Lyophilized juice		50 mg/kg b.wt.	28.7 ± 0.6*	40.33	60.64
Ethyl acetate fraction of leaves		100 mg/kg b.wt.	22.3 ± 0.3*	53.64	80.65
Indomethacin		20 mg/kg b.wt.	16.1 ± 0.2*	66.51	100.00

* Statistically significant from control group at $P < 0.01$
** % of potency as compared to indomethacin

Table 5. Diuretic Effect of the Different Extracts of *P. Amboinicus* Leaves, Stems and Roots.

Groups		Dose	Volume of urine in ml		
			2 hrs	4hrs	24hrs
Control		1 ml saline	0.8 ± 0.01	1.8 ± 0.1	7.2 ± 0.3
Leaves	Alcoholic	250 mg/kg b.wt.	2.6 ± 0.1	4.3 ± 0.1*	12.3 ± 0.9*
	Aqueous	250 mg/kg b.wt.	2.2 ± 0.02	2.6 ± 0.3	10.6 ± 0.8*
Stems	Alcoholic	250 mg/kg b.wt.	1.8 ± 0.01	3.1 ± 0.1	8.1 ± 0.4*
	Aqueous	250 mg/kg b.wt.	1.5 ± 0.01	2.3 ± 0.7	7.7 ± 0.2*
Roots	Alcoholic	250 mg/kg b.wt.	1.4 ± 0.01	1.9 ± 0.4	8.3 ± 0.3*
	Aqueous	250 mg/kg b.wt.	1.1 ± 0.03	1.9 ± 0.3	7.9 ± 0.3*
Lyophilized juice of leaves		250 mg/kg b.wt.	1.3 ± 0.02	2.9 ± 0.6*	9.7 ± 0.5*
Ethyl acetate fraction of leaves		250 mg/kg b.wt.	2.1 ± 0.1	3.8 ± 0.2*	11.7 ± 0.9*
Moduretic drug		5 mg/kg b.wt	3.9 ± 0.6	6.4 ± 1.2*	16.4 ± 1.8*

* Statistically significant from control group at $P < 0.01$

Table 6. The Effect of the Different Extracts of *P. Amboinicus* Leaves, Stems and Roots on Serum Concentrations of Sodium and Potassium.

Groups		Serum electrolytes concentration	
		K^+ mmol/L	Na^+ mmol/L
Control		4.3 ± 0.2	169.2 ± 3.1
Leaves	Alcoholic	4.1 ± 0.1	121.9 ± 2.8*
	Aqueous	4.5 ± 0.2	139.9 ± 3.7*
Stems	Alcoholic	3.9 ± 0.1	138.2 ± 3.6*
	Aqueous	3.7 ± 0.1	141.1 ± 4.1
Roots	Alcoholic	4.2 ± 0.1	143.7 ± 2.8
	Aqueous	3.8 ± 0.1	132.3 ± 4.7*
Lyophilized juice		3.8 ± 0.3	133.5 ± 2.8
Ethyl acetate extract		4.4 ± 0.2	156.7 ± 5.2
Moduretic drug		5.4 ± 0.5	151.1 ± 2.6

* Statistically significant from control group at $p < 0.01$

Statistical Analysis

The data obtained were statistically analyzed using the Student's t- test.[20] The data was presented as mean ± standard error.

In vitro Cytotoxic Activity against Human Cell Lines

The potential cytotoxicity agaist hepatocellular and breast carcinoma human cell lines was tested by Sulphorhodamine B assay (SRB)[21] using doxorubicin as standard and IC_{50} were determined (see Table 7).

Antimicrobial Activity

The agar diffusion method[22] was applied using trypticase Soy agar (Difco) medium inoculated with the bacterial or fungal suspension of the test organisms.

Table 7. Results of Cytotoxic Activity of the Different Extracts of *P. Amboinicus* Leaves, Stems and Roots.

Sample		HEPG2 IC_{50} µg/ml	MCF7 IC_{50} µg/ml
Doxorubicin®		0.9	0.7
Leaves	Alcoholic	24.5	22.5
	Aqueous	25.8	18.2
Stems	Alcoholic	21.9	20.8
	Aqueous	29.1	17.5
Roots	Alcoholic	24.2	11.5
	Aqueous	23.6	15.9
Lyophilized juice of leaves		10.1	6.8

Discs (5 mm) were impregnated with the tested samples or dimethyl sulphoxide (50 µl) as a negative control and discs of ceftriaxon and clotrimazole were used as reference standards for the antibacterial and antifungal activities, respectively. Then, the discs were placed onto the surface of the culture medium. The plates were incubated at 35–37°C for 24–48 hours in case of bacteria and at 25°C for 48 hours in case of filamentous fungi, while yeasts were incubated at 30°C for 24–48 hours. After incubation, the diameters of inhibition zones were recorded in mm and the results were compiled in Tables 8 and 10. The minimum inhibitory concentrations (µg/ml) of the different samples against the oral pathogens *Lactobacillus acidophilus* and *Streptococcus mutans* were also determined by microdilution method,[23] Table 9.

RESULTS AND DISCUSSION

The isolated compounds (1–8) were identified, based on their physical and spectral data and by

Table 8. Antibacterial Activity of the Different Extracts of *P. Amboinicus* Leaves, Stems and Roots.

Microorganisms	Inhibition zone (mm) (potency %)							
	Alcoholic extracts of			Aqueous extracts of			Lyophilized juice	Reference standard Ceftriaxon
	Leaves	Stems	Roots	Leaves	Stems	Roots		
Streptococcus mutans	9 (128%)	9 (128%)	20 (286%)	20 (286%)	23 (329%)	27 (386%)	25 (357%)	7 (100%)
Lactobacillus acidophilus	10 (125%)	8 (100%)	13 (163%)	11 (138%)	8 (100%)	7 (88%)	8 (100%)	8 (100%)
Bacillus subtilis	11 (122%)	7 (77%)	7 (77%)	R	R	R	12 (133%)	9 (100%)
Staphylococcus aureus	10 (142%)	9 (128%)	11 (157%)	13 (186%)	8 (114%)	9 (129%)	7 (100%)	7 (100%)
MRSA	13 (186%)	10 (143%)	12 (171%)	14 (200%)	8 (114%)	7 (100%)	7 (100%)	7 (100%)
E. coli	R	R	R	11 (183%)	8 (133%)	7 (116%)	R	6 (100%)
Klebsiella pneumonia	R	R	R	R	R	R	R	7 (100%)
Pseudomonas aeruginosa	R	R	R	R	R	R	R	6 (100%)

R = resistant

*The results are the mean of 3 readings.

Table 9. The Minimum Inhibitory Concentrations (MIC) of the Different Extracts of *P. Amboinicus* Leaves, Stems and Roots against *Lactobacillus Acidophilus* and *Streptococcus Mutans.*

Sample		MIC (µg/ml)	
		Lactobacillus acidophilus	Streptococcus mutans
Alcoholic	Leaves	12.5	3.85
	Stems	100	3.85
	Roots	100	3.85
Aqueous	Leaves	100	50
	Stems	50	6.25
	Roots	100	50
Lyophilized juice of the leaves		50	50

comparing these data with the published one, as 5,4'-dihydroxy-3,7-dimethoxy flavone (3-methoxy genkwanin),[24,25] 5,4'dihydroxy-6,7-dimethoxy flavone (crisimaritin),[24,25,26] *p*- coumaric acid (hydroxy cinnamic acid),[27] caffeic acid,[1] 3,5,7,3',4' pentahydroxy flavanone (taxifolin),[26] rosmarinic acid,[1] apigenin[26] and 5-*O*-methyl-luteolin.[24,25] These compounds were isolated for the first time from the leaves of *Plectranthus amboinicus* (Lour.) Spreng cultivated in Egypt, while, 3-methoxy genkwanin, *p*-coumaric acid and 5-*O*-methyl-luteolin were isolated for the first time from this plant.

Quantitative Determination of Total Polyphenolics Content

The stems showed the highest concentration of total polyphenolic compounds followed by the leaves then the roots (9.6, 8.4 and 5.4 mg/g, expressed in gallic acid equivalents, respectively).

Quantitative Determination of the Tannins Content

The concentration of tannins was found highest in the roots, followed by the leaves then the stems (126, 90 and 81 μg/g of tannic acid equivalents, respectively).

Identification of Phenolic Constituents in *Plectranthus Amboinicus* (Lour.) Spreng Stems and Roots using High Resolution UPLC-MS Analysis

The LC-MS analysis of the ethyl acetate fractions of the stems and roots of *Plectranthus amboinicus* (Lour.) Spreng revealed the presence of caffeic acid, rosmarinic acid and coumaric acid in the stems and roots which were isolated from the leaves in the present study. Chrysoeriol was also detected in the two organs under investigation, while luteolin, quercitin and eriodyctiol were detected only in the stems.

Determination of Median Lethal Dose (LD_{50})

The LD_{50} of the tested extracts was up to 5 g/kg b. wt. so, they could be considered safe according to Buck, *e.t al.*.[28] This may explain its extensive utilization in traditional medicine.

Antioxidant Activity

The reduced level of blood glutathione in diabetic rats was greatly restored by the different tested samples relative to vitamin E. The ethyl acetate fraction of the leaves showed the highest antioxidant power with potency 98.88% as compared to vitamin E. The alcoholic and aqueous extracts and the lyophilized juice of the leaves, also possess high antioxidant activity, with potency 96.64%, 95.25% and 95.25%, respectively.

Anti-inflammatory Activity

The ethyl acetate fraction and the alcoholic extract of the leaves showed the most potent anti-inflammatory activity with relative potency 88.66% and 83.37%, respectively, as compared to indomethacin. The other tested extracts showed anti-inflammatory activity with moderate potency.

Analgesic Activity

All the tested extracts exhibited analgesia at the tested doses. The alcoholic extract and the ethyl acetate fraction of the leaves

Table 10. Antifungal Activity of the different Extracts of *P. Amboinicus* Leaves, Stems and Roots.

Microorganisms	Inhibition zone (mm) (potency %)							
	Alcoholic extracts of			Aqueous extracts of			Lyophilized juice	Reference standard Clotrimazole
	Leaves	Stems	Roots	Leaves	Stems	Roots		
Candida albicans	R	R	R	15 (75%)	R	R	R	20 (100%)
Candida parapsilosis	R	R	R	16 (84%)	R	R	R	19 (100%)
Aspergillus flavus	R	R	R	R	R	R	R	19 (100%)
Aspergillus niger	R	R	R	R	R	R	R	17 (100%)

R = resistant

*The results are the mean of 3 readings.

showed the highest analgesic activity with potency 90.33% and 80.65%, respectively, as compared to indomethacin.

Diuretic Activity

The alcoholic extract, ethyl acetate fraction and aqueous extract of the leaves showed the highest increase in urine volume as compared to the moduretic drug after 24 hours. Concerning the serum electrolyte level most of the tested extracts showed significant decrease in serum sodium level as compared to the moduretic drug. Meanwhile, no significant effect was observed on K^+ level.

According to the results obtained, the ethyl acetate fraction of the leaves is the best choice as a diuretic with good electrolyte balance.

Cytotoxic Activity

All the tested samples showed high IC_{50} on hepatocellular and breast carcinoma cell lines compared to doxorubicin, so they could be considered as inactive as cytotoxic drugs.

Antibacterial Activity

The results of antibacterial screening revealed that all the tested extracts, exhibited powerful antibacterial activity against Gram positive bacteria, especially the oral pathogens *Streptococcus mutans and Lactobacillus acidophilus*. So, the minimum inhibitory concentration (MIC) of the different extracts against these two micro-organisms was determined. The alcoholic extract of the leaves recorded the lowest MIC against *Streptococcus mutans* and *Lactobacillus acidophilus* (12.5 and 3.85 μg/ml, respectively), this may suggests the incorporation of this extract in toothpaste or mouth wash preparations.

Concerning Gram negative bacteria most of the extracts showed no or moderate activity compared to ceftriaxon except the aqueous extracts of the leaves, stems and roots which showed significant activity against *E. coli*.

Antifungal Activity

Only the aqueous extract of the leaves showed moderate activity against *Candida albicans* (15 mm, 75%) and *Candida parapsilosis* (16 mm, 84%), compared to clotrimazole, while no activity was observed against the tested filamentous fungi. The authors thank Dr. Mohamed Farag, Assistant Professor, Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, for performing the UPLC-MS analysis. Also, we are sincerely gratitude for Dr. Amany Abdallah El-sharif, Department of Microbiology and Immunology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt for performing the antimicrobial study.

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Ethanobotanical, pharmacognostical and physico-chemical studies of stem bark of *Bombax ceiba* L., commonly growing in eastern Uttar Pradesh region of India

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ABSTRACT

Objective: To evaluate the pharmacognostic, physico-chemical characters and ethanobotany of an important medicinal plant, *Bombax ceiba L*. **Methods:** The pharmacognostic studies out in terms of various investigations like organoleptic or morphological characters, microscopic or anatomical studies, physico-chemical evaluations (loss on drying, ash values, extractive values), preliminary phytochemical screening, TLC finger print profiling and fluorescence analysis of powdered crude drug as per WHO recommended guidelines for standardizations. **Results:** The detail microscopy revealed the presence of collapsed phloem, non-collapsed phloem, sieve elements, sieve tubes, companion cells and starch grains. Physiochemical parameters such as percentage of foreign matters, ash values, loss on drying, swelling index extractive values were determined. Preliminary phytochemical screening showed the presence of carbohydrates, terpenoids, glycosides, Flavonoids, tannins and phenolic compounds. **Conclusions:** These studies provided referential information for correct identification and standardization of this plant material. These information will also be helpful to differentiate *Bombax ceiba* from the closely related other species.

Keywords: Ethnoherbological, macromorphology, phytochemical screening, Quality control

INTRODUCTION

Plant material may vary in its phytochemical content and therefore in its therapeutic effect according to different places of collection, with different times in a year for collection and with different environmental factors surrounding the cultivation of a particular medicinal plant.[1] Standardization of natural products is a complex task due to their heterogeneous composition, which is in the form of whole plant, plant parts or extracts obtained thereof. To ensure reproducible quality of herbal products, proper control of starting material is utmost essential. The first step towards ensuring quality of starting material is authentication. Despite the modern techniques, identification of plant drugs by pharmacognostic studies is more reliable. According to the World Health Organization, the macroscopic and microscopic description of a medicinal plant is the first step towards establishing the identity and the degree of purity of such materials and should be carried out before any tests are undertaken.[2]

Bombax ceiba (syn. *Bombax malabaricum* DC. *Salmalia malabarica*) of the family Bombacaceae, is an important medicinal plant of tropical and subtropical India commonly known as Silk Cotton Tree, Indian Red Kapok tree, Semal, Shimul, Shalmali etc.[3] Almost every part of this plant is used as medicine for curing maximum number of ailments. It has beautiful red flowers and large fruits. It yields gum and cotton. It is large and long living tree species which give strength to body, mind and heart. Its bark is mucilaginous, demulcent and emetic, and is used in healing wounds; bark paste is good for skin eruptions.[4,5] Cotton tree and has been used extensively for treatment of some diseases like inflammation,[6] algesia, hepatotoxicity,[7,8] and hypertension, as well as for antiangiogenic and antioxidant activities.[9] Though the plant has been reported for many biological activities, no scientific data available to identify the genuine sample.

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The present investigation of *Bombax ceiba* is therefore taken up to establish quality profile of the stem bark which will help in crude drug identification as well as in standardization. [Figure 1]

MATERIALS AND METHODS

Description of Plant *Bombax ceiba*

The various part of *B. ceiba* such as roots, leaves, seed, stem bark, flower, fruit and gum are documented to possess medicinal properties in ethnobotanical surveys conducted by ethno botanist and in traditional system of medicine such as ayurvedic. **Leaves:** large, spreading, glabrous, digitate, Palmate, to 24 inches long, with rarely 3–4, and commonly 5–7, lanceolate leaflets up to 10 inches long, attached to a long flexible petiole. **Flowers:** Heavy, up to 6 inch wide flower having 5 satiny red or orange petals reflexing strongly after opening. **Fruits:** Capsules, oval woody to 5 inches long filled with small cotton-covered seeds. **Seed:** smooth, black or gray embedded in long white wool. **Bark:** gray or brown covered with hard, sharp, conical prickles. **Gum:** is light brown in colour resembling the galls, and gradually becomes opaque a dark brown.

In February, *B. ceiba* begins dropping all of its leaves. It is time for flowering and what follows is a sensational display of large silky red flowers at the tips of bare branches. In May, white cottony strands, from opened fruits, float downward, settling on the ground, houses and whatever else is in their way. The tree does not begin to releaf until almost all the flowers have fallen. Even without its flowers, *B. ceiba* is still an impressive tree. It displays stout, prickly branches arranged in horizontal tiers emanating from a straight, rough, spiny trunk with buttress roots. The trunks of the oldest specimens lose much of their spines. *B. ceiba* is rare in cultivation and always impresses tourists and first-time viewers. Propagation is by seeds, large cuttings or by air layering.[10]

Figure 1. Exomorphic features of the plant *a-B. ceiba* in full bloom (late Feb), b-flower in early March, c-stem, d-buds with cotton and seed in late April.

Ethnoherbological Properties

Almost every part of the plant is used as medicine. Its young roots are roasted in the fire and eaten like roasted sweet potato while some tribes eat even raw roots during femine or otherwise also.[11] The plant has been found to possess strong anti-inflammatory, antibacterial, antiviral, analgesic, hepatoprotective, antioxidant, oxytocic, hypotensive, hypoglycaemic, antiangiogenic, antimutagenic as well as fibrinolysis enhancing activity.[12] Young roots of the plants have been reported to be useful in diarrhoea, dysentery, urinary troubles, gyneacological problems, bladder disorders, heart diseases, debility, diabetes and impotence.[13,14]

The gum of the kapok tree is a traditional Asian remedy for stomach ailments. The roots of young trees used to be made into a candy that was alleged to have aphrodisiac properties. In the spring, beautiful red flowers appear. Young petals are used in some herbal teas. Cotton which has been used as kapok for stuffing pillows, sleeping bags, life preservers and mattresses.[15] The tree's soft wood is used for making tea boxes and matches. Seeds yield pale yellow oil, which can be used for edible purpose as a substitute for cotton seed oil, for soap making and as an illuminant.[16]

The roots are sweet, cooling, stimulant, tonic and demulcent, and are used in dysentery. The gum is astringent, cooling, stimulant, aphrodisiac, tonic, styptic and demulcent. It is useful in dysentery, haemoptysis of pulmonary tuberculosis, influenza, and menorrhagia, burning sensation, strangury, hemorrhoids, blood impurities and vitiated conditions *of pitta.* Leaves are good for strangury and skin eruptions. Flowers are astringent and are good for skin troubles, splenomegaly and hemorrhoids. Young fruits are useful in calculus affections, chronic inflammations and ulceration of the bladder and kidney. Seeds are useful in treating gonorrhea, chronic cystitis and vitiated conditions of *kaph.*[5]

The plant is among five trees of 'Panchwati' and therefore, has spiritual importance. Ayurvedic scripture 'Rajnighantu' has beautifully described its characteristics and properties. It states that the tree is Yamadruma, Diirghadruma, Kantakdruma, Nirgandhpushpi etc. It is large and long living tree species which give strength to body, mind and heart.[17]

Collection and Authentication

The fresh bark the tree *Bombax ceiba* was collected from the field area of Bahraich, district U.P. India, during the month of March, 2011. For identification and Taxonomic authentication, sample of plant material was given to National Botanical Research Institute (NBRI) Lucknow, India. The plant material was confirmed and authenticated by Dr. A. K. S. Rawat, Scientist and Head, Pharmacognosy and Ethnopharmacology Devision, National Botanical Research Institute Lucknow, India with report no NBRI-SOP-202 Receipt no. and date CIF-RB-2-121, 28-04-2011.

The fresh bark was used for the study of macroscopic and microscopical characters. Whereas collected plant materials were shade-dried and coarsely powdered. This coarse powder was used for the determination of ash values, extractive values, and preliminary phytochemical investigation was studied as per standard methods.

Chemicals and Reagents

All the chemicals and reagents used were of laboratory grade.

Extraction of Plant Materials

100 gm coarse powder of air dried bark of *Bombax ceiba* L. were packed in muslin cloth and subjected to soxhlet extractor for continuous hot extraction with distilled water, ethanol, chloroform and petroleum ether for 8 hrs separately. Then the each extracts were filtered and filtrate was evaporated to dryness. The percentage yield of the water, ethanol, chloroform and petroleum ether extracts was 4.18%, 2.72%, 1.68% and 1.15% respectively.

Macroscopic and Microscopic Studies

The macromorphology of the barks were studied according to standard methods.[18–19] Hand section of the bark was taken, stained and mounted following usual micro-techniques,[20] and representative diagrams were taken with the help of inverted microscope for photodocumentation (Leitz, Japan).

Physicochemical Analysis

Physicochemical analysis i.e. alcohol and water soluble extractive values, fluorescent analysis,[21,22] total ash, acid-insoluble ash, water-soluble ash, swelling index and foreign matter.[23] Calibrated digital pH meter was used to measure the pH of 1 and 10% aqueous extracts and also loss on drying was noted.

Preliminary Phytochemical Screening

Preliminary phytochemical screening was done for the detection of various chemical constituents by using standard procedures described by Harborne[24] and Khandelwal.[25]

Thin Layer Chromatography (TLC)

Thin layer chromatography studies of the different extracts were carried out in various solvents using Silica gel G as adsorbent and the R_f values were determined.[26]

RESULTS

Macroscopic Characters

The pieces of bark of *Bombax ceiba is* are generally curved fragments, 0.5–1 cm thick, freshly collected bark is brownish internally and externally light grey in colour, but on drying they turn from grey to dark brown in colour. Bark is covered with hard, sharp, conical prickles. The eye-catching, spiny trunk of young trees becomes smoother and strongly buttressed with age. The outer surface of the bark having more roughness finely to coarsely rough with vertical and transverse cracks occasionally at places, longitudinally wrinkled and fissured. The inner surface is comparatively fine, brown in color. Fracture is fibrous, texture is also fibrous. The bark is acrid in taste, odorless or characteristic odour and possesses slightly astringent flavour. [Figure 2]

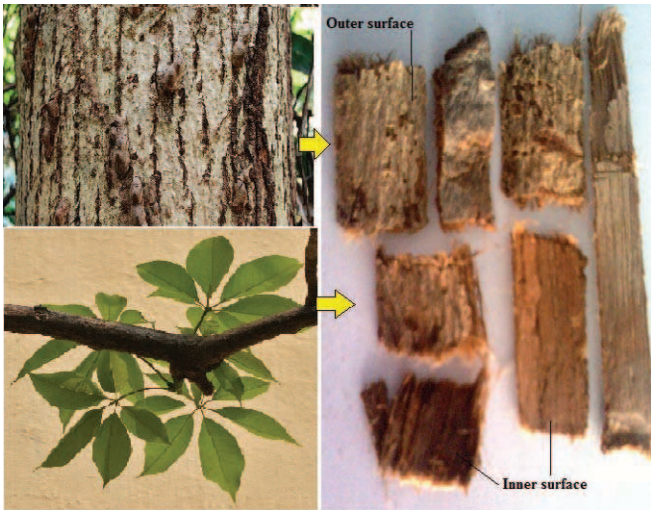

Figure 2. Macroscopical characters of pieces of dried bark with Spiny trunk of young trees.

Microscopical Characters

TS of stem bark

The transverse section of the bark shows the Cork, phellogen, phelloderm, secondary cortex, pericyclic sclerenchyma, mucilage cavities were observed [Figure 3]. Cork is the outer most covering it consists of several layers of cork cells. They occur as regular rows of small slightly thick walled, flat polygonal cells. The cork cambium or Phellogen cells are 3–7 layered, rectangular and flattened. The phelloderm cells are two or more layers of rectangular or oval cells filled with tannin [Figure 4]. The cortex region shows the presence of compact, thin walled, medium sized parenchymatous cells. Scattered stone cells are found in the cortex region. Stone cells were present as singles or in groups. The phloem is characterized by collapse cells structure. Secondary cortex consists of moderately thick-walled, parenchymatous cells. Medullary rays were present which are thin walled, elongated, rectangular and gradually increasing in dimensions towards outer ends. These are bi, tri or multiseriates in the region of inner secondary phloem. [Figure 5]

Powder microscopy of stem bark

Microscopy of bark Powder showed fragments of surface view of cork cells, groups of stone cells and many free stone cells are also observed, parenchymatous cells, starch grains and prismatic crystals of calcium oxalate, phloem fibres and numerous reddish-brown coloured masses.

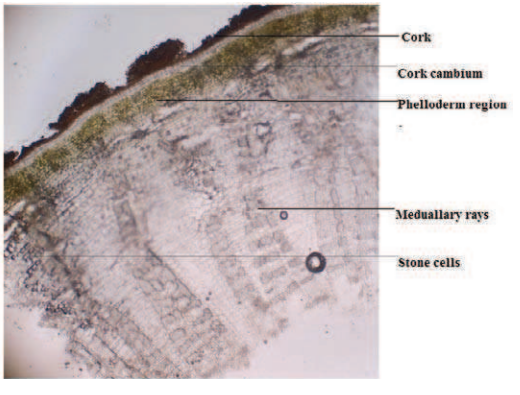

Figure 3. Transverse section of the stem bark of *B. ceiba.*

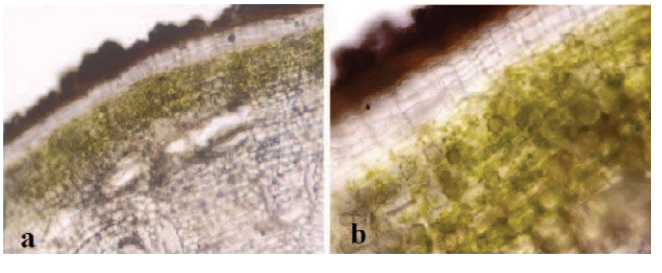

Figure 4. Microscopical characters of the bark a-T.S. of *B. ceiba* showing cork and phelloderm cells; b-T.S. of *B. ceiba* showing phelloderm cells filled with tannins.

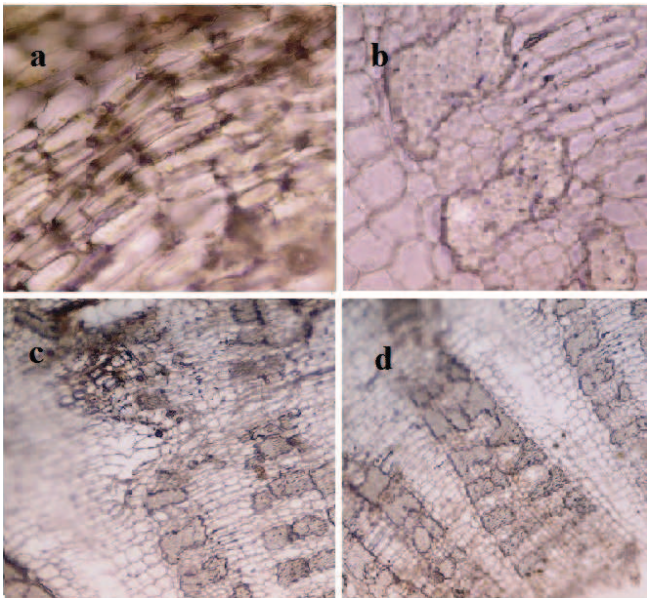

Figure 5. Microscophical characters of the stem bark a-T.S. of *B. ceiba* stem bark showing stone cells in phelloderm region; b-T.S. of *B. ceiba* stem bark showing medullary rays having some stone cells; c and d-T.S. of *B. ceiba* stem bark showing bi, tri and multiseriate medullary rays.

Physicochemical parameters

The physico-chemical characters of powder drug of bark of *Bombax ceiba* such as total alcohol soluble extractive, water soluble extractive, ash value, acid insoluble ash, water-soluble ash, loss on drying and foreign matter are presented in Table 1. The fluorescence analysis of the powder drug of *Bombax ceiba* in various solvents and chemical reagents was performed under normal and Ultra Violet (UV) light Table 2. The pH of 1 and 10% solution of powder drugs of *Bombax ceiba* was noted in Table 3. Result of powder drug reaction was tabulated in Table 5.

Table 1. Physicochemical Parameters of *Bombax ceiba* Bark.

Quantitative parameter	Values obtained (%) w/w
Alcohol soluble extractive	8.4
Water soluble extractive	10.6
Total ash	5.5
Acid insoluble ash	1.70
Water – soluble ash	2.25
Loss on drying	6.5
Foreign matter	1.2

Table 2. Fluorescence Analysis of *Bombax ceiba* Bark.

Solvent used	Day Light	U V light (254 nm)	U V light (366 nm)
Powder as such	Redish brown	Brown	Blackish brown
1N HCl	Brown	Brownish green	Dark brown
50% HCl	Light brown	Brown	Dark brown
50%HNO_3	Dark Brown	Brownish green	Blackish Brown
50%H_2SO_4	Brown	Dark brown	Blackish Brown
1N NaOH	Dark brown	Light green	Golden brown
Alcoholic NaOH	Blackish brown	Darkgreen	Black
Methanol	Light brown	Yellowish brown	Brownish green
Benzene	brown	Light green	Light brown
$FeCl_3$	Brownish yellow	Brown	Greenish brown
1% KOH	Brownish black	Light buff	Dark brown
Lead acetate	White	Brown	Light brown
Distilled water	Brown	Llight brown	Dark brown

Table 3. Determination of pH of Drug.

Sample	pH
pH of 1 % solution	6.56
pH of 10 % solution	5.87

Preliminary phytochemical screening

The preliminary phytochemical investigation of the aqueous, methanol, petroleum ether and chloroform extracts of *Bombax ceiba* L. showed the presence of phytosterols, flavonoids, terpenoid, saponins, carbohydrates, tannins and glycosides. [Table 4]

Table 4. Qualitative Phytochemical Analysis of *Bombax ceiba* Bark Extract.

Constituents	Bark extract
Alkaloids	–
Carbohydrate	+
Glycoside	+
Phenolic compound and tannins	+
Flavonoids	+
Proteins and Amino Acids	–
Saponins	–
Sterols	+
Acidic compound	–
Lipids/ fats	–

Table 5. Powdered Drug Reaction with Different Reagent.

Treatment	Observation
Conc. HCL	Light brown
Conc. HNO_3	Brownish black
Conc. H_2SO_4	Black
Glacial Acetic acid	Yellowish brown
Iodine solution	Dark brown
NaoH in Methanol	Brown

TLC profile of Bombax ceiba

Thin layer chromatography of the petroleum ether, chloroform, methanolic extract and aqueous extracts were carried out separately using Hexane: Ethylacetate (90: 10) for petroleum ether, Hexane: Ethyl acetate (5:2) for chloroform extract, n-butanol: Acetic acid: water (6:1:2) for methanolic extract and n-butanol: Acetic acid: water (6:1:2) for aqueous extract as mobile phase respectively and the R_f values were recorded. [Table 6] The visualizing reagent employed was exposer to iodine vapours to effect visualization of the resolved spots.

DISCUSSION

As a part of standardization study, the macroscopical examination of bark was studied. Macroscopical evaluation is a technique of qualitative evaluation based on the study of morphological and sensory profiles of drugs. The macroscopical characters of the bark of plant can serve as diagnostic parameters. The extractive value, ash value, loss on drying and fluorescent analysis of the bark extracts have been carried out. Percentages of the extractive values were calculated with reference to air-dried drug. The percent extractives in different solvents indicate the quantity and nature of constituents in the extract. The extractive values are also helpful in estimation of specific constituents soluble in particular solvent.

The ash value was determined by three different methods, which measured total ash, acid-insoluble ash, and water-soluble ash and the result were tabulated. The fluorescence analysis of the powdered drug from the bark of *Bombax ceiba* in various solvents was performed under normal and UV light. All the bark extracts are examined in short UV (254 nm) and long UV (366 nm) to detect the fluorescent compounds.

Table 6. Thin Layer Chromatography of *Bombax ceiba* Bark Extracts.

Bark extracts	Solvent system	Number of spots	R_f value
Petroleum ether	Hexane : Ethylacetate (90 : 10)	4	0.26 0.54 0.77 0.84
Chloroform	Hexane: Ethyl acetate (5: 2)	2	0.12 0.88
Methanolic extract	n-butanol : Acetic acid : water (6:1:2)	3	0.46 0.65 0.84
Aqueous extract	n-butanol : Acetic acid : water (6:1:2)	3	0.16 0.36 0.83

Thin layer chromatography (TLC) is particularly valuable for the preliminary separation and determination of plant constituents. The visualizing reagent employed was expose to iodine vapors to effect visualization of the resolved spots. The chromatographic profile may serve as a characteristic finger print for qualitative evaluation of bark.

The preliminary phytochemical investigation of the plant was carried out with standard protocol. Freshly prepared bark extracts were tested for the presence of phytochemical constituents and the results are given in table.

CONCLUSION

After present investigation it can be concluded that the standardization and preliminary phytochemical investigation study of *Bombax ceiba* bark yielded a set of standards that can serve as an important source of information to ascertain the identity and to determine the quality and purity of the plant material in future studies. This study is a substantial step and it further requires a long term study to evaluate therapeutic efficacy and toxicity of bark, to establish as the drug. It would also help in distinguishing the plant material of *Bombax ceiba* from its related species, *Bombax insigne* L.

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Evaluation of aphrodisiac effect of vanillin in male wistar rats

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ABSTRACT

Introduction: Vanillin is one of the primary chemical components of the vanilla bean (*Vanilla planifolia*). An aphrodisiac is defined as any food or drug that arouses the sexual instinct, induces venereal desire and increases sexual pleasure and performance. Unpublished data claim that vanillin, taken under proper guidance, can help relieve problems of impotence, erectile dysfunction, frigidity, loss of libido and promotes arousal. Hence this study was conducted to study the potential aphrodisiac effects of vanillin in rats. **Methods:** Twenty four male rats were divided into four groups which received vehicle, vanillin 100 mg/kg/day, 200 mg/kg/day and 400 mg/kg/day orally, respectively. Female rats were brought to oestrous cycle by the sequential administration of estrogen (10 µg/100 g) and progesterone (0.5 mg/100 g) through intraperitoneal injections, 48 hours and 4 hours (respectively) prior to pairing with the male on day 1 to study the acute effects and on day10 to study the sub-chronic effects. Sexual behaviors were observed for three hours. Serum testosterone levels were estimated. The data was analyzed using one way ANOVA followed by posthoc tests. **Results:** There was a statistically significant increase in the mount frequency and intromission frequency compared to control following both acute and chronic treatment with vanillin (200 mg/kg). The mount latency, intromission latency, ejaculation latency and post-ejaculatory interval decreased in the vanillin treated groups especially at 200 mg/kg. There was no significant difference in the serum testosterone levels among the groups. **Conclusion:** Vanillin in the dose of 200 mg/kg demonstrated aphrodisiac properties in male wistar rats.

Keywords: Vanillin, Aphrodisiac, Sexual dysfunction

INTRODUCTION

An aphrodisiac is defined as any food or drug that arouses the sexual instinct, induces venereal desire and increases pleasure and performance. This word is derived from "Aphrodite" the Greek goddess of love and these substances are derived from plants animals or minerals and since time immemorial they have been the passion of man.[1] There are two main types of aphrodisiacs, psychophysiological stimuli (visual, tactile, olfactory and aural) preparations and internal preparations (food, alcoholic drinks).[2] Based on their mechanism of actions, aphrodisiacs can be divided into three categories which include: a. Aphrodisiacs that simply provide a burst of nutritional value, thereby improving the immediate health or well-being of the consumer and consequently improving sexual performance and libido. This simple improvement in general health can lead to a burst of energy and translate into an increased sexual appetite,[3] b. The second group are those with specific physiological effect. They may affect blood flow; mimic the burning of fire of sex and intercourse and increase the duration of sexual activity,[4] c. The third group of biologically active aphrodisiacs are those that are psychologically active in nature. They actually cross the blood brain barrier and mimic or stimulate some areas of sexual arousal. Examples include hormones and a wide variety of neurotransmitters.[5] Sexual health and function are important determinants of quality of life. Inability to perform this function effectively is a major problem facing the reproductive process. This is known as sexual dysfunction.[6]

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The increasing incidence of male sexual dysfunction is necessitating more and rapid search into plants & plant products with aphrodisiac potentials.

Vanilla (*Vanilla planifolia*), a monocotyledoneous orchid native of Central America, is grown for the attractive aroma produced by its fruit.[7] Because vanilla is so much in demand, and expensive, synthetic vanilla are often used instead of natural vanilla. In fact, 97% of vanilla used as a flavour and fragrance is synthetic. Synthetic vanilla contains only one organic component – vanillin – the flavour and fragrance that we most associate with vanilla. Natural vanilla extract is a mixture of several hundred different compounds in addition to vanillin. Vanillin is one of the primary chemical components of the extract of the vanilla bean. It is a pleasant aromatic compound that occurs naturally in vanilla beans (*Vanilla planifolia*); it is a fine, white to slightly yellow crystal, usually needle-like, having an odour and taste suggestive of vanilla. Synthetic vanillin is used as a flavouring agent in foods, beverages, and pharmaceuticals.[8] Studies on vanillin has demonstrated that it has antimutagenic,[9] antinvasive and antimetastatic.[10,11] It has been claimed in unpublished data that vanillin, taken under proper guidance, can help relieve the problems of impotence, erectile dysfunction, frigidity, loss of libido and promotes arousal.

OBJECTIVES

To study the aphrodisiac effect of the Vanillin in adult male wistar rats.

MATERIALS AND METHODS

Animals

Male albino wistar rats inbred in the central animal house of KMC were used for the study. Rats were housed in clean polypropylene cages, three rats in each cage, in a controlled environment (24–26°C) with a 12 hour light and dark cycle with standard chow containing fat 4.15%, protein 22.15%, carbohydrates 4% (supplied by Amruth laboratory animal feed manufactured by Pranav Agro industries ltd., Sangli) and water *ad libitum*. The rats were allowed to acclimatize to these conditions for one week. Experiment was performed during the light phase of the cycle (10:00–17:00 hrs). The animals were maintained as per the CPCSEA guidelines and regulations. The study was approved by Institutional animal ethics committee.

Study Drug

Vanillin [IUPAC name 4-hydroxy-3-methoxybenzaldehyde, chemical formula $(CH_3O)(OH)C_6H_3CHO$, molecular weight of 152.15] obtained from HiMedia laboratories at Kasturba Medical College, Mangalore.

Study Design

Twenty four male rats were divided into four groups of 6 rats each.

Drug dosage in different groups of rats

Group	Dose
1 (Gum Acacia 2%)	10 mg/kg
2 (Vanillin)	100 mg/kg
3 (Vanillin)	200 mg/kg
4 (Vanillin)	400 mg/kg

Dose Formulation

Required quantity of test item was weighed as per the dose. The weighed test item was suspended in required quantity of gum acacia to get the desired concentration as per the dose. Formulation of the test item was prepared shortly before dosing.

Administration of Study Drug

All male rats were administered increasing doses of the test drug/vehicle orally by gavage using oral feeding tube at the same time of the day for 10 days. The dose volume was approximately 1.5 ml. The dosage volume administered to individual rat was adjusted according to its body weight recorded on the day of dosing.

Methodology

Six rats from each group were monitored for sexual behavior on day 1 (for the acute study) and day 10 (for the subchronic study) after daily doses of the drug. Twenty four female rats were brought to oestrus cycle by the sequential administration of estradiol benzoate (10 µg/100 g) and progesterone (0.5 mg/100 g) through intraperitoneal injections, 48 hours and 4 hours (respectively) prior to pairing. The animals were given a 20 minute adaptation period, after which a primed female was placed in the same cage with the male. Sexual behavior studies were monitored in a separate dark room for 3 hours following the administration the drug.

Sexual Behaviours

Sexual behavior in male rats consists of three distinct phases:

1. **Mount:** the animal assumes the copulatory position, but does not insert its copulatory organ (the penis) into the vagina
2. **Intromission:** the copulatory organ enters the vagina during a mount
3. **Ejaculation**: forceful expulsion of semen. This is characterized by longer, deeper pelvic thrusting and slow dismount followed by a period of inactivity.

Parameters Assessed

- Mount latency: the time interval between the introduction of the female to the first mount by the male.
- Mount frequency (MF): the number of mounts without intromission from the time of introduction of the female until ejaculation.
- Intromission latency: the interval from the time of introduction of the female to the first intromission by the male.
- Intromission frequency (IF): the number of intromissions from the time of introduction of the female until ejaculation.
- Ejaculatory latency: the time interval between the first intromission and ejaculation.
- Post ejaculatory interval: the time interval between ejaculation and the next mount
- Testosterone assay: After observing the sexual behavior the rats were anaesthetized with ether & blood sample (1.5 ml) for assay was collected by cardiac puncture. Then animals were sacrificed by giving sodium pentobarbitone at dose of 100 mg/kg intraperitoneally.

RESULTS

As shown in Table 1, in the acute study the intromission latency, ejaculation latency and post-ejaculatory interval significantly decreased following treatment with vanillin 200 mg and 400 mg/kg compared to the control. Ejaculation latency and post-ejaculatory interval were also significantly low compared to group 2 (vanillin 100 mg/kg). Moreover, the mount frequency and intromission frequency significantly increased following treatment with vanillin at the dose of 200 mg/kg.

As shown in Table 2, in the sub-chronic study the mount latency decreased but ejaculation latency and post-ejaculatory interval significantly increased in the group treated with vanillin 100 mg/kg compared to control. At 200 mg/kg vanillin significantly decreased the mount

Table 1. Acute Study.

Group		Mount latency (sec)	Mount frequency	Intromission latency (sec)	Intromission frequency	Ejaculatory latency (sec)	Post ejaculatory interval (sec)
1	Mean	581	14.67	6401	1.67	9000	9000
	SD	426	13.868	3841	1.528	3118	3118
2	Mean	930	16.00	3920	2.67	8040	7380
	SD	1003	8.544	2547	1.528	4781	5924
3	Mean	401	34.33*#	1440*	6.00#$	2120*#	840*#
	SD	92	4.041	318	1.000	270	104
4	Mean	581	19.00	1601*	1.67€	2400*#	820*#
	SD	92	3.00	92	0.577	120	92

*p<0.05 vs group 1; #p<0.05 vs group 2; $p<0.01 vs group 1; €p<0.01 vs group 3

Table 2. Sub-chronic Study.

Group		Mount latency (sec)	Mount frequency	Intromission latency (sec)	Intromission frequency	Ejaculatory latency (sec)	Postejaculatory interval (sec)
1	Mean	640	15.67	2340	5.33	1520	1700
	SD	151	3.512	360	2.082	510	524
2	Mean	300*	15.33	2081	5.67	7520£	7860£
	SD	60	5.312	819	3.055	94	5092
3	Mean	220*	39.67*#	1060£$	9.33£$	1820$	600$
	SD	92	4.509	92	.577	211	120
4	Mean	400£€	25.67£$€	1640	5.00€	2120$	700$
	SD	35	3.055	250	1.000	125	92

*p<0.01 vs group 1; £p<0.05 vs group 1; #p<0.001 vs group 2; $p<0.05 vs group 2; €p<0.05 vs group 3

latency and intromission latency compared to the control. Also, the mount frequency and intromission frequency increased with vanillin treatment at the dose of 200 mg/kg compared to the control. Vanillin 400 mg/kg significantly decreased mount latency and increased mount frequency compared to control.

DISCUSSION

This study was designed to evaluate the aphrodisiac effects of vanillin at three different doses (100, 200 and 400 mg/kg) in normal wistar rats. Results obtained in the present study reveal that acute administration of vanillin at 200 mg/kg improved sexual behavior as observed by a decrease in intromission and ejaculatory latency and post ejaculatory interval and an increase in mount and intromission frequency. At the dose of 400 mg/kg vanillin only decreased intromission and ejaculatory latency and post ejaculatory interval but did not increase the mount and intromission frequencies. And at 100 mg/kg it did not show any change in sexual behavior.

In the sub-chronic study too, administration of vanillin 200 mg/kg improved sexual behavior as observed by a decrease in mount and intromission latency and increase in mount and intromission frequencies. At the dose of 400 mg/kg, vanillin only decreased mount latency and increased mount frequency; while the other parameters did not show any improvement in sexual behavior. At 100 mg/kg, vanillin decreased the mount latency but other parameters did not show any improvement.

Most plant products that improve sexual behavior have shown to increase serum testosterone levels;[13] however vanillin did not show any significant change in the testosterone levels on sub-chronic administration. Hence the mechanism by which it enhances sexual behavior needs further evaluation.

The results conclude that vanillin has aphrodisiac properties especially at doses of 200 mg/kg in rats and the effects are better on acute administration than sub-chronic administration.

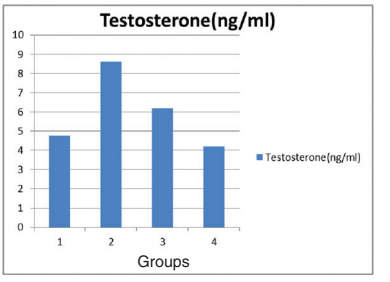

Figure 1. Effect of vanillin on testosterone levels.

There was no significant difference in the testosterone levels in any of the groups.

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Antioxidant and α-glucosidase inhibitory activities of *Murraya koenigii* leaf extracts

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ABSTRACT

Medicinal plants are source for a wide variety of natural antioxidants. Dietary antioxidant consumption may be an important approach for inhibiting or delaying the oxidation of susceptible cellular substrates and thus is relevant to disease prevention in many paradigms. *Murraya koenigii* is one of the important medicinal herb which is used as a food ingredient across India. In this study, we demonstrated the reducing power, total antioxidant potential, radical scavenging capabilities and α-glycosidase inhibitory property of different crude extracts of curry leaves (*Murraya koenigii* L.). The extracts were evaluated for their radical scavenging activities by means of DPPH, NO, •OH, $O2^-$ and anti-lipid peroxidation assays. In addition, extract of *M. koenigii* were tested for α-glycosidase inhibitory property. The extracts of *M. koenigii* scavenged radicals effectively in varied degree. Similarly, the total reducing power of alcohol extract was found higher in both phosphomolybednum and FRAP methods. *In vitro* assay of α-glucosidase activity of MKA and MKW showed an IC_{50} of 174.74 and 287.00 µg/ml respectively, while other two extracts did not show any significant effect. Simultaneously, total phenolic and total flavonoid contents of extracts were studied, where values of MKW were found to be higher than that of other extracts. In present study, we found that MKA and MKW extracts contain effective antioxidant and radical scavenging activities as compared to other extracts. Our study provides a proof for the ethno-medical claims and reported biological activities that curry leaves have significant therapeutic potential.

Keywords: *Murraya koenigii*, antioxidant, free radical, α-glucosidase.

INTRODUCTION

Reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl (•OH) radical are often generated as byproducts of biological reactions or from exogenous. These ROS create homeostatic imbalance which generate oxidative stress and cause cell death and tissue injury.[1] Free radicals and ROS are well known inducers of cellular and pathological processes including diabetes, cell proliferation, inflammatory conditions and many neurodegenerative disorders apart from aging processes.[2,3,4] A potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated diseases.[5] It has been established that antioxidants provide protection to living organisms from damage triggered by uncontrolled production of ROS and associated lipid peroxidation, protein damage and DNA strand breaking.[6] The health promoting effect of antioxidants from plants is thought to arise from their potential effects on the reactive oxygen/nitrogen species. Plant-derived antioxidants could function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors or synergists.[7]

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Most plant species possess tremendous medicinal properties because they are used both to sustain health and to cure illness.[8] Dietary antioxidant intake may be an important approach for inhibiting or delaying the oxidation of susceptible cellular substrates and is thus relevant to disease prevention in many models. Phenolic compounds such as flavonoids, phenolic acids, diterpenes etc. have received attention for their high antioxidative activity.[9,10,11] *In vitro* bioassay systems have been extensively used to monitor biological activities of medicinal plant extracts used in traditional medicines.

Numerous plants used in traditional medicine are effective in treating various ailments caused by oxidative stress, bacterial and/or viral infections. Since ancient times, spices and herbs have been added to different types of food to improve flavours as well as for their antioxidant capacity. Converging evidence from both experimental and epidemiological studies have demonstrated that medicinal plants in particular contain a myriad of phenolic compounds and still present a large source of natural antioxidants that might serve as leads for the development of novel drugs.[12] Therefore, the exploration for natural antioxidants and other preparations of plant origin to achieve this objective has been gained importance over the years.

Murraya koenigii L. Spreng (Rutaceae) is a small and strong smelling perennial shrub or small tree commonly found in forests as undergrowth, cultivated in India for its characteristic flavour and aroma. Leaves of this plant are used as a condiment in the preparation of curry powder, pickle, chutney, sausages and seasonings.[13,14] The flavour and fragrance of leaves is retained even after drying.[15] Leaves relieve nausea, indigestion, vomiting and used as a cure for diarrhoea and dysentery.[13,16]

In this study, we have demonstrated the antioxidant efficiency of different *M. koenigii* using series of *in vitro* assays. In addition, we also evaluated its α-glycosidase inhibitory activity apart from determination of phytochemical constituents.

MATERIALS AND METHODS

Materials

All solvents used in this study were of analytical grade. Methanol, ethyl acetate, hexane, and Folin- Ciocalteau reagent obtained from Merck (Merck, India) while other chemicals were procured from HiMedia chemicals, India.

Plant Material

Curry leaves (*M. koenigii* L.) were purchased from local market in Hyderabad, India in the month of July, 2011. The identity of the plant was confirmed by Dr. VC Gupta, Taxonomist, Central Research Institute of Unani Medicine, Hyderabad. The voucher specimen of the plant was kept for future reference. One hundred grams of curry leaves were dried at ambient temperature for 10–15 days. After drying completely, leaves were grounded to a coarse powder using domestic electric grinder.

Preparation of Extracts

The coarse powder was subjected to successive extraction in a soxhlet apparatus using different solvents such as hexane, ethyl acetate, ethanol and water. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. Furthermore, extracts were filtered through Whatman No.1 paper filter and concentrated to the dry mass with the aid of rotary evaporator. The yield of each extract was measured and residues were stored in dark for further analysis. Different extracts were designated as MKH (for hexane extract), MKE (for ethyl acetate extract), MKA (for ethanol extract) and MKW (for water extract). Dried extracts of 20 mg/ml stock solution were prepared and different concentrations were used in various experiments.

DETERMINATION OF PHYTOCHEMICAL CONSTITUENTS

Total Phenolic Content

Total phenolic content of extracts were determined by Folin-Ciocalteu method[17] with little modification. Briefly, 10 µl of the extracts were taken to which 500 µl of double distilled water was added, followed by 100 µl of Folin Ciocalteu's reagent. After incubating the mixture for 10 min at room temperature, 300 µl of 20% Na_2CO_3 was added, thoroughly vortexed and the volume of the reaction mixture was adjusted to one ml with double distilled water. The mixture was then incubated for 2 hrs in dark and the absorbance was measured at 765 nm against blank. Results were expressed as mg of gallic acid equivalents (GAE)/g dry weight (dw).

Total Flavanoid Content

Total flavonoid content was quantified by following the method of Barreira[18] with minor modifications. Briefly, 20 µl of each extract were mixed with 500 µl double

distilled water and 30 µl of 5% $NaNO_2$ solution. After 5 min of incubation at room temperature, 60 µl of 10% $AlCl_3$ solution was added. Subsequently, 350 µl of 1 M NaOH and 40 µl of double distilled water were added to make the final volume to one ml. Samples were further incubated for 15 min at room temperature and the absorbance of samples was measured at 510 nm. The total flavonoids were determined as qurecetin equivalents (mg QE)/g of dw and the values were expressed as means of triplicate analysis.

Determination of Total Antioxidant Activity

Total antioxidant activities of extracts were evaluated by phosphomolybdenum method.[19] The assay is based on the reduction of Mo^{+6}–Mo^{+5} by the antioxidant compounds and subsequent formation of a green phosphate/Mo^{+5} complex at acidic pH. The reagent solution contains ammonium molybdate (4 mM), disodium hydrogen phosphate (28 mM) and sulfuric acid (0.6 M) mixed with the extracts. Samples were incubated for 60 min at 90°C and the absorbance of the green phosphomolybedenum complex was recorded at 695 nm. Ascorbic acid was used as reference and reducing capacity of the extracts was expressed as the mg ascorbic acid equivalents per gram dry weight.

Determination of Reducing Antioxidant Power (FRAP)

The reducing antioxidant power of the plant extracts was determined according to the method described by Oyaizu[20] with slight modifications. Briefly, 10 µl of each extract was taken and the volumes were made to 250 µl with double distilled water. Further, 250 µl of potassium ferricyanide (1%) was added to the tubes and incubated for 20 min at 50°C. Then 250 µl of trichloroacetic acid (10%) was added to the incubated mixture. Upper part of the mixtures (500 µl) were taken and mixed with 400 µl of double distilled water and 100 µl of ferric chloride (0.1%). The absorbance of the mixture was measured at 700 nm and reducing power of extracts was expressed as mg ascorbic acid equivalents (AAE) per gram (g) of dry weight (dw).

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the plant extracts was determined according to the method described by Braca.[21] The activity was assessed using stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH). DPPH solution (0.004% w/v) was prepared in 95% methanol, mixed with different dilutions of plant extracts and thoroughly vortexed. The reaction mixture was then incubated in dark at room temperature for 45 min and absorbance solution was measured at 517 nm against the blank. Methanol (95%) and ascorbic acid were used as blank and reference compound respectively.

Nitric Oxide Radical Scavenging Activity

The activity was measured according to the modified method of Sreejayan and Rao.[22] To 100 µl of the extract having different concentrations (40–400 µg/ml), 20 µl of sodium nitroprusside (SNP) solution (10 mM) was added and incubated for 15 min under light conditions. After incubation, the mixture was diluted with 300 µl of Griess reagent (1% sulfanilamide in 2% H_3PO_4). The reaction mixture was further incubated for 45 min under light conditions at 30°C followed by addition of 10 µl of 0.1% naphthylethylene diamine dihydrochloride in 2.5% H_3PO_4. Final volume was made to 1 ml with double distilled water. The absorbance of the chromophore was taken immediately at 546 nm and compared to the standard (ascorbic acid).

Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity was measured as per the protocol of Kunchandy and Rao[23] with minor changes by studying the competition between deoxyribose and extracts for hydroxyl radicals generated by Fenton's reaction. Briefly, solution of Fenton's reagent [Fe (III) chloride, ascorbic acid and H_2O_2] was prepared in distilled water just prior to use. To 0.1 mL of Fenton's reagent, thiobarbituric acid (1% w/v) in 25 mM NaOH (1 ml) and tricholoroacetic acid (1 ml, 2.8% w/v) were added and volume was made to 3 ml with distilled water. The mixture was then incubated at 80°C for 90 min and amount of pink chromogen produced was considered as control and was measured spectrophotometrically at 532 nm. The protection of oxidation of D-ribose has been conducted by pre-incubation with extracts in different concentrations and decrease in the formation of pink colour was considered as antioxidant property which was compared to standard (ascorbic acid).

Superoxide Radical Scavenging Activity

The activity was evaluated using nitro blue tetrazolium (NBT) reduction method.[24] The reaction mixture consisted of 0.5 ml of NBT solution (156 µM, 0.5 ml nicotinamide adenine dinucleotide (468 µM, NADH), and extracts of different concentrations (40–400 µg/ml). The reaction was initiated by adding 50 µl of phenazine methosulfate solution (60 µM, PMS) in phosphate buffer

(pH 7.4). The reaction was incubated at 25°C for 20 min and then absorbance was measured at 560 nm against blank. Ascorbic acid was used as the reference.

Determination of Inhibition of Lipid Peroxidation

Lipid peroxidation inhibitory activity of *M. koenigii* extracts and standard (ascorbic acid) were performed as per the protocol given by Halliwell and Gutteridge.[25] The rat liver homogenate was used for induction of lipid peroxidation, mediated by $FeCl_3$ as pro-oxidant. Healthy albino rats of the wister strain (250 g) were sacrificed and perfused the liver with 0.15 M KCl followed by centrifugation of homogenate at 800 g for 15 min at 4°C and supernatant was used for thiobarbutaric acid assay. The extracts at different concentrations (40–400 µg/ml) were mixed with the liver microsome preparation and the mixtures were incubated in presence and absence of fenton's reagent (50 µl of 10 mM $FeCl_3$; 10 µl of 2.5 mM H_2O_2) in phosphate buffer (0.2 M, pH 7.4) and the final volume was made to 1 ml. The reaction mixtures were incubated at 37°C for 30 min. After incubation, 2 ml of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid, 0.5% thiobarbutaric acid, and 0.5% butylated hydroxytoluene (BHT) was added to the reaction mixture followed by heating at 100°C for 10 min. The reaction mixture was put in an ice bath for 5 min for cooling. After that the mixture was centrifuged at 1000 g for 10 min and the extent of lipid peroxidation was subsequently monitored by the formation of thiobarbutaric acid reactive substances (TBARS) as pink chromogen in the presence or absence of extracts and standard (ascorbic acid). The absorbance of supernatant was measured spectrophotometrically at 532 nm and decline in the formation of pink chromogen in the pre-treated reactions was considered as inhibition of lipid peroxidation.

Effects of Extracts on α-glucosidase Activity *In Vitro*

The α-glucosidase inhibitory activity was determined according to the method given by Matsui[26] with slight modifications by measuring the release of 4-nitrophenol from 4-nitrophenyl α-D-glucopyranoside (4-NPGP). Briefly, the enzyme reaction was performed using p-nitrophenyl-α-D-glucoside (PNP-glycoside) as a substrate in 0.1 M phosphate buffer (pH 6.8). PNP-glycoside (10 mM) and 10 µl of GSH (3 mM) was pre-mixed with samples at various concentrations. Each mixture was added to an enzyme solution (0.01 units) to make 1 ml of final volume. The reaction was terminated by adding 5 µl of 100 mM sodium carbonate solution. Enzymatic activity was quantified by measuring the p-nitrophenol released from PNP-glycoside at 400 nm. All reactions were carried out at 37°C for 30 min with three replications. Acarbose was used as a positive control and IC_{50} values were calculated by the graphic method.

Calculations and statistical analysis

The percentage inhibitions of radicals, lipid peroxidation and α-glucosidase inhibitory activities of the extracts were calculated using the formula:

$$\text{Percentage inhibition} = (A_{control} - A_{sample})/A_{control} \times 100.$$

All the experiments were performed in triplicates and experimental results were expressed as mean ± standard deviation of mean (SEM) of three replicates. IC_{50} value (the concentration of the extracts required to scavenge 50% of radicals) was calculated for different extracts of *M. koenigii*. Graphpad prism 5 software was used for statistical analysis and to prepare the graphical representation of results.

RESULTS AND DISCUSSION

Determination of Phytochemical Constituents

The results of total phenolic content of different leaf extracts of *M. koenigii* were significant and found in the range of 2.37 to 28.84 mg GAE/g dw (Table 1). Among the tested extracts, the highest amount of total phenolics was observed in MKW (28.84 mg GAE/g dw) whereas MKH showed least (2.37 mg GAE/g dw) content of phenolics. The flavonoid contents of the extracts in terms of quercetin equivalent were between 8.28 and 39.90 mg QE/g dw (Table 1), highest being in MKW (39.90 mg QE/g dw) and lowest in MKH (8.28 mg QE/g dw). It is well known that plant polyphenols are widely distributed in the plant kingdom and are very important plant constituents.[27] It has been recognised that phenolic compounds such as flavonoids, phenolic acid and tannins possess diverse biological activities like anti-diabetic anti-

Table 1. Total Polyphenol, Flavonoid, Antioxidants and Ferric Reducing Antioxidant Power of *M. koenigii* Extracts.

Extract	Total polyphenol content[a]	Total flavonoid content[b]	Total antioxidant activity[c]	Ferric reducing power[c]
MKH	2.37±0.36	8.28±0.18	6.04±0.25	3.31±0.012
MKE	12.41±1.60	14.93±1.19	21.86±1.04	9.91±0.02
MKA	21.18±1.47	23.52±0.81	23.46±0.52	17.12±0.47
MKW	28.84±0.49	39.90±1.23	25.54±0.72	2.69±0.08

a: gallic acid; b: quercetin; c: ascrobic acid equivalents mg/g dw plant material respectively; Results represented in means ± standard deviation (n = 3).

inflammatory, anti-carcinogenic and anti-atherosclerotic activities and their effects on human nutrition and health are considerable.[28] It is interesting to note that both the polyphenol and flavonoid contents of MKW are higher than other extracts. This may be due to the better solubility of the polyphenol and flavonoid type of constituents in the aqueous solvent than the other medium. According to our study, the high contents of these phytochemicals in *M. koenigii* can explain its high radical scavenging activity.

Determination of Total Antioxidant Activity

Total antioxidant activity of plant extracts was determined by phosphomolybednum method which is based on the reduction of Mo (VI)–Mo (V) by the antioxidant compounds and subsequent formation of green phosphate/Mo (V), is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid (AA) per gram dry weight of extract. All the four extracts possess antioxidant potential (Table 1), but discrepancies were noticed in the extracts of different polarity. The antioxidant activity was in the range of 6.04 to 25.54 mg AAE/g dw in the leaf extracts. MKW showed the greatest value of 25.54 mg AAE/g dw, as was the case of total phenols and flavonoids, whereas the lowest value of 6.04 mg AAE/g dw was found in MKH. In our study, total polyphenol, flavonoid content and antioxidant activity of the plant extracts correlated significantly and could also contribute to the overall antioxidant potential.

Determination of Reducing Antioxidant Power (FRAP)

The reducing ability to convert Fe^{3+} to Fe^{2+} is also an indirect evidence for the antioxidant activity of an extract or a compound.[29] In this assay system, the antioxidants present in the extract causes the reduction of the Fe^{3+}/ferricyanide complex to form Fe^{2+} ions, which was monitored spectrophotometrically by recording the absorbance of the reaction mixture at 700 nm.[30] All the tested samples showed some degree of electron donation capacity (Table 1). The reducing power of MKA was the highest amongst all the tested samples, with 17.12±0.47 mg AAE/g dw, followed by MKE (9.91±0.02 mg AAE/g dw), whereas those of the others were much lower, with a varied range from 2.69±0.08 to 3.31±0.012 mg AAE/g dw. The data presented here indicate that the marked reducing power of *M. koenigii* extracts seem to be attributed to their antioxidant activity.

DPPH Radical Scavenging Activity

Substances that are capable of donating hydrogen or an electron to DPPH$^{\bullet}$, nitrogen centered free-radical are considered as antioxidants and therefore, radical scavengers. The degree of discoloration of violet colour of DPPH, as it gets reduced, indicates the radical scavenging potential of the antioxidant.[31] In this assay, all extracts showed significant dose-dependent DPPH radical scavenging capacity (Figure 1). The IC_{50} values ranged from 22.12±2.97 to 32.55±1.42 and the DPPH radical-scavenging efficiency increased as follows: MKH < MKA < MKW < MKE. MKE was most efficient, with the lowest IC_{50} value, 22.12 µg/ml. The antioxidant(s) in crude extracts neutralized the free radical character of DPPH by transferring either electrons or hydrogen atoms to DPPH[32], thereby changing the colour from purple to the yellow-coloured diphenyl picrylhydrazine. This experiment suggests that the plant extracts could contain more bioactive compounds that may attribute the antioxidant properties of *M. koenigii*.

Nitric Oxide Radical Scavenging Activity

Abnormally high level of NO has been linked with chronic inflammation and may be associated with the etiology and pathology of a number of chronic diseases.[33] Besides its own toxicity, this radical can further react with other species instigating even more toxic radicals, such as peroxynitrite, which results from its reaction with superoxide. We tested *M. koenigii* extracts for their inhibitory effect on nitric oxide production andnitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by the extracts. The extracts at varied concentrations (40–400 µg/ml) showed significant inhibitory effect of nitric oxide radical scavenging activity. The percentage scavenging activity increased with increasing concentration. Among the different extracts, the lower IC_{50} value was observed for MKE (162.27±2.26 µg/ml) followed by MKW (163.06±1.86 µg/ml) and highest IC_{50} was observed for MKA (Table 2; Figure 2).

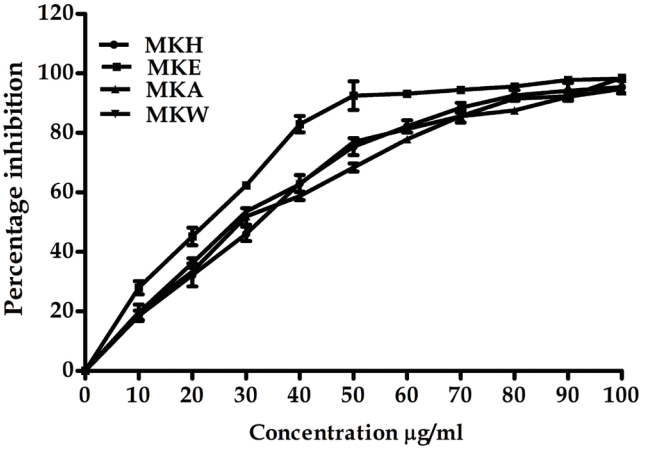

Figure 1. DPPH scavenging activity of different *M. koenigii* leaf extracts.

Table 2. IC_{50} Values of *M. koenigii* Extracts on Tested Radicals.

Extract	DPPH	Nitric oxide	Superoxide	Hydroxyl radical	Lipid peroxidation	-glucosidase inhibition
MKH	32.55±1.42	188.82±0.78	90.38±0.64	380.44±0.38	281.12±0.47	ND
MKE	22.12±2.97	162.27±2.26	129.73±0.42	535.90±0.36	31.67±1.65	ND
MKA	28.88±2.76	267.20±2.035	141.76±0.53	177.52±0.28	72.25±1.9	287.00±0.49
MKW	28.00±0.72	163.06±1.86	203.56±1.13	82.23±0.33	260.41±0.53	174.74±0.72

(Values expressed in µg/ml; Results represented in means ± standard deviation (n = 3); NA: No activity.

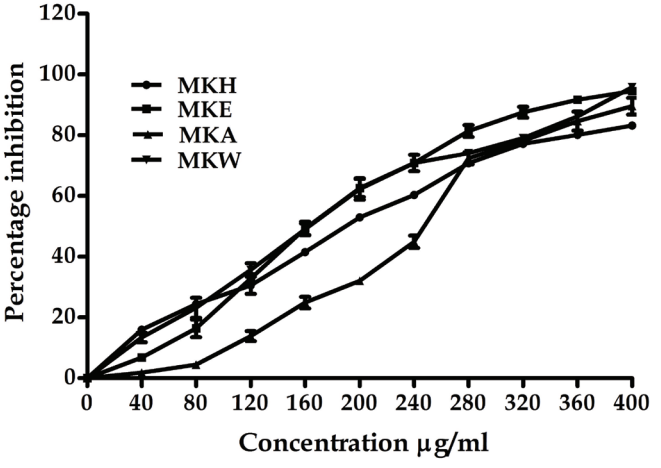

Figure 2. Nitric oxide scavenging activity of different *M. koenigii* leaf extracts.

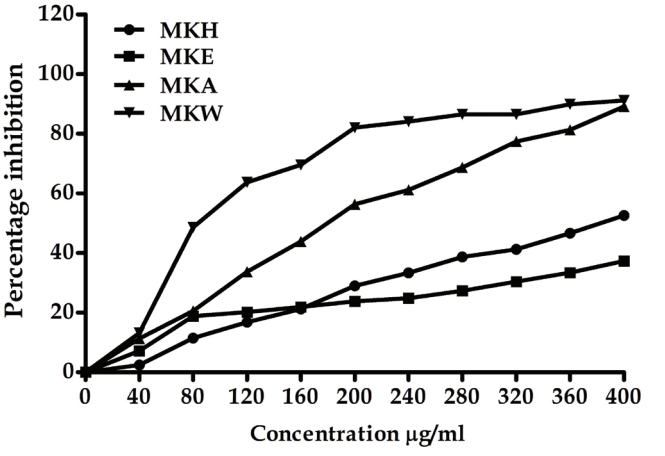

Figure 3. Superoxide scavenging activity of different *M. koenigii* leaf extracts.

Superoxide Radical Scavenging Activity

Superoxide anion is a free radical generated from the normal energy process of energy generation in the human body. It is toxic to cells and tissues and can act as precursors to other reactive oxygen species.[34] It was found that the superoxide-scavenging activities of different extracts of *M. koenigii* were increased markedly with increasing concentrations (Figure 3). The inhibitory activity of MKH was significantly higher than that of other extracts (Figure 3). The IC_{50} values of extracts was found to be in the order of MKW > MKA > MKE > MKH (Table 2). These results imply that water extract is better superoxide scavenger and its capacity to scavenge superoxide may contribute to its antioxidant activity.

Hydroxyl Radical Scavenging Activity

Hydroxyl radicals are the major reactive oxygen species that are responsible for oxidation of lipids and massive biological damage.[35,36] The evaluation of radical scavenging activity was based on the generation of •OH by Fenton reaction. The percentage inhibitions against the hydroxyl radical of different extracts of *M. koenigii* are presented in Figure 4. MKW was found to be efficient scavengers of hydroxyl radicals with least IC_{50} value (82.23±0.33 µg/ml), while other extracts were found to be less efficient scavengers. By comparing the IC_{50} values of all extracts, we can point out that the aqueous extracts of this plant (MKW) was more efficient hydroxyl radical scavengers than its other counterparts.

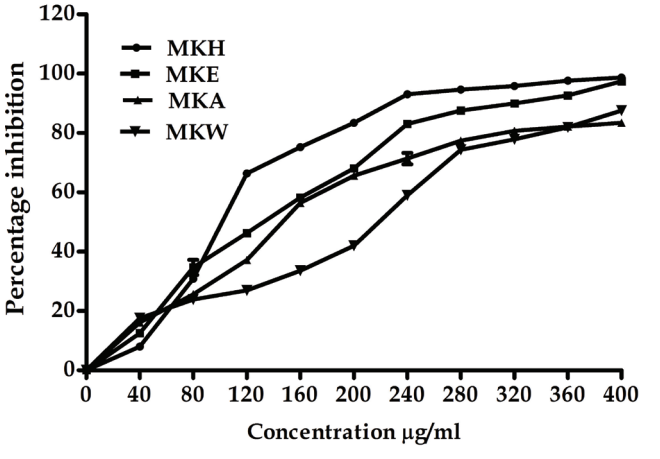

Figure 4. Hydroxyl radical scavenging activity of different *M. koenigii* leaf extracts.

Determination of Inhibition of Lipid Peroxidation

In biological systems, lipid peroxidation generates a number of degradation products such as malondialdehyde which is one of the causes of cell membrane destruction

and cell damage. In order to determine if the *M. koenigii* extracts were capable of reducing *in vitro* oxidative stress, lipid peroxidation was assessed by means of an assay that determines the production of malondialdehyde and related compounds in rat liver homogenates. TBARS are produced as by-products of lipid peroxidation that occurs in the hydrophobic core of biomembranes. Our results clearly indicate that extracts in rat liver homogenate were capable of quenching the extent of lipid peroxidation caused by a Fe^{2+}/ascorbate system (Figure 5). The IC_{50} value was found to be least for MKE (31.67±1.65 µg/ml) followed by MKA (72.25±1.9 µg/ml). The IC_{50} of other two extracts MKH and MKW were much higher (Table 2). This inhibition of lipid peroxidation may have been either due to chelation of Fe^{2+} ions or by trapping of free radicals produced by Fe^{2+}/ascorbate in the reaction system.

Effects of the Extracts on α-glucosidase Activity *In Vitro*

Natural products are still the most readily available source of α-glucosidase inhibitors.[37] In recent reports from other traditional plants, polyphenols were observed to contribute to strong α-glucosidase inhibition.[38] Therefore, we investigated the activity in different extracts. It was found that out of four extracts tested for α-glucosidase inhibitory activity, only MKA and MKW showed significant inhibition property (Figure 6), whereas other extracts (MKH and MKE) did not inhibit α-glucosidase at all. The percentage inhibition of α-glucosidase by MKA and MKW exhibited significant inhibitory activity at dose-dependent acceleration suggesting a competitive type of inhibition. MKW (IC_{50}=174.74 µg/ml) exerted the most powerful

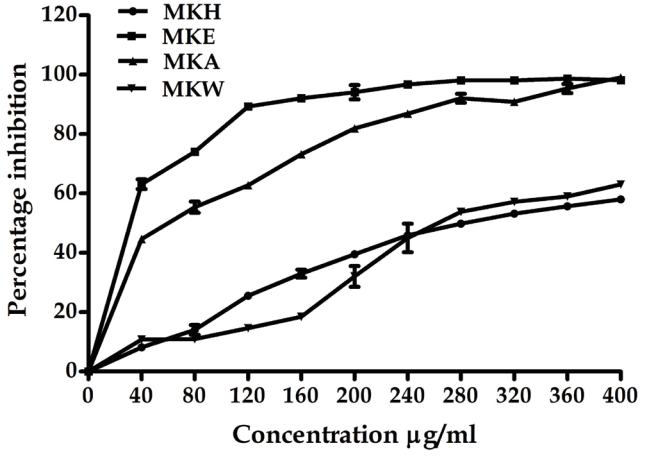

Figure 5. Inhibition of lipid peroxidation of different *M. koenigii* leaf extracts.

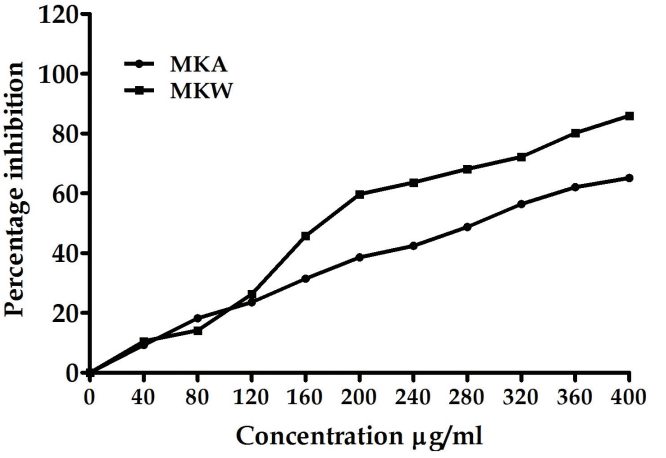

Figure 6. α-glycosidase inhibitory activity of different *M. koenigii* leaf extracts.

inhibitory activity. Acarabose, an antidiabetic drug exerts almost similar effects (IC_{50}=128 µg/ml) under our assay conditions. The IC_{50} value for MKA was found to be 287.00 µg/ml. Based on our results presented here, we can say that *M. koenigii* exert inhibitory effect on α-glucosidases, with MKW being the most effective. With these results, we can further support the traditional use of the plants for its wide medicinal applications.

CONCLUSION

In conclusion, the results of this study clearly indicate that *M. Koenigii* has powerful antioxidant activity against various oxidative systems *in vitro*. Various antioxidant properties of this potent medicinal plant may be attributed to its components effectiveness as scavengers of free radicals, reductive capacity, and metal chelating ability, as well as lipid peroxidation inhibition. The free radical-scavenging property may be one of the mechanisms by which this plant is attributed as useful for foodstuff as well as traditional medicine. Thus, our results support *M. koenigii* as an accessible source of natural antioxidants and a food supplement.

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Antiobesity actions of *Embelia ribes*

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ABSTRACT

Embelia ribes has been used as a folk medicine whole world. An aqueous extract of *Embelia ribes* have visceral fat accumulation and improved insulin resistance in rat model with HFD induced obesity may possibly be through down regulation of leptin, TNF-α, SREBP1γ, and PPARγ2 gene expression. The leptin might contribute to hepatic steatosis by promoting insulin resistance and also by altering insulin signaling in hepatocytes, which consequently promote increased intracellular fatty acid. Hence, drug treatment to rats with HFD prevents increase of these levels due to decrease of the body fat content. Therefore we investigated the anti-obesity effects of the aqueous extract of *Embelia ribes* by feeding a high-fat diet to rats for 42 weeks. Body weights at 2–6 weeks and parametrial adipose tissue weights were significantly lower in rat fed the high-fat diet containing aqueous extract of *Embelia ribes* than in the controls fed the high-fat diet. Our data suggest that the antiobesity effects of aqueous extract of *Embelia ribes* in rat fed a high-fat diet may be through down regulation of leptin, TNF-α, SREBP1γ, and PPARγ2 gene expression.

Keywords: *Embelia ribes*; Leptin; TNF-α; SREBP1γ; PPARγ2; high-fat diet; rat

INTRODUCTION

Obesity is a condition of abnormal body weight resulting from an accumulation of extra adipose tissue, generally in response to a state of positive energy balance that occurs when intake exceeds energy expenditure. It is a growing global health problem in the present era. Obesity is a chronic relapsing, stigmatized neurochemical disease that is more prevalent in developing and developed countries and leading to much comorbidities.[1] Obese individuals are at increased risk for developing a myriad of medical, psychological and behavioral problems. The disorder is a well established risk factor for hypertension, hyperlipidemia, Diabetes-2 coronary heart disease, stroke, asthma, obstructive sleep apnoea. Actually, obesity is not a disease, it is a disorder, but, has been recognized as a chronic disease by National Institute of health consensus conference in 1985 due to the increasing prevalence and associated risks.[2] This situation currently prevailing widely, anti-obesity drugs expanding rapidly in the market, but drugs are sparse in relation to need. Hence, novel anti-obesity drug is required to handle the problem.

Plants and natural products are long been recognized as an important sources for the therapeutically effective medicines. Approximately 80% of the world's population relies on plants for the natural products and medication. Of the 520 new drugs approved between 1983 and 1994, 39% were of natural origin and 60–80% of antibacterial and anticancer drugs were derived from natural products. Infact several Indian medicinal plants or extracts have been used as anti-obesity plant.[3]

Embelia ribes, commonly known as Vidanga, False Black Pepper or White-flowered (family Myrsinaceae).E. ribes contain quinine derivative Embelin, 2, 5-dihydroxy-3-undecyl-p-benzoquinone, (Figure 1), quercitol and fatty ingredients; an alkaloid, christembine. Among them embelin has various pharmacological and medicinal properties like analgesic property. The fruit cure tumors, ascites, bronchitis, jaundice and mental disorders (Figure 2). *E. ribes* Burm have antidiabetic, antidyslipidemic and antioxidant activity in streptozotocin-induced diabetes in rats, using gliclazide as the positive control

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Figure 1. Dried fruits of *Embelia ribes*.

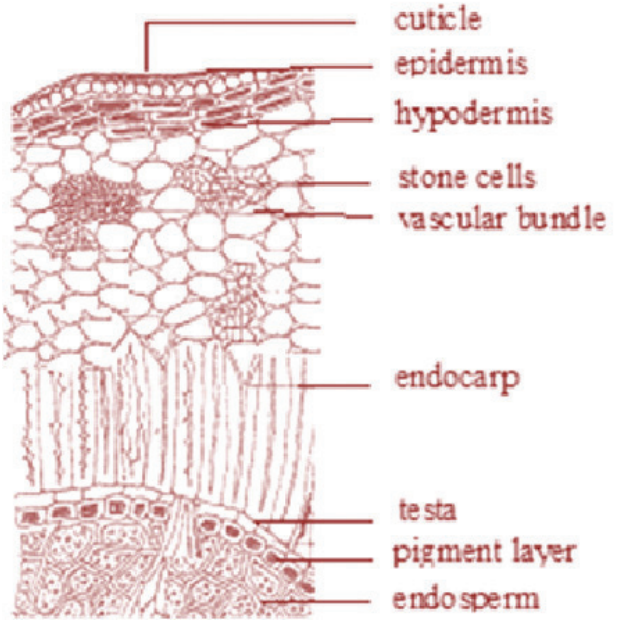


Figure 2. TS of fruit.

drug.[4] It is recently reported that aqueous extract of *E. ribes* have cardioprotective activity in isoproterenol induced myocardial infarction in albino rats.[5,6]

However, *E. ribes* has not been investigated for its anti-obesity potential so far. Hence, this study was carried out to test the hypothesis that aqueous extract of *E. ribes* may have anti-obesity activity in high fat diet (HFD)- induced obesity in Wistar rats.

MATERIALS AND METHODS

Plant material

Dried *E. ribes* fruits were purchased from Local market Kharibaoli, Delhi and authenticated by Dr. HB. Singh, expert taxonomist, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India.

Preparation of Aqueous *E. ribes* Extract

Fruits material of *E. ribes* was washed; shade dried and powdered, then extracted with water in a soxhlet apparatus for 72 hours. The solvent was removed under reduced pressure to give a dry extract, 5% yield w/w with respect to the crude material and stored at - 20°C till experiment was not started. The weighed amount of aqueous *E. ribes* extract was suspended in 1% gum acacia in normal saline for pharmacological activity. This extract was used for phytochemical analysis for standardization.

Macroscopical Studies

The fresh fruits of *Embelia ribes* were subjected to macroscopical studies which comprised of organoleptic characteristics of the drug viz., size, colour, odour, taste, smell, texture, fracture etc.

Microscopical Studies

Qualitative microscopic evaluation was carried out by taking transverse sections of fruit of *Embelia ribes*. Fruit was boiled with chloral hydrate to remove all the coloring matter and then stained with phloroglucinol and hydrochloric acid. The sections were transferred (glycerin) on a slide and a cover slip was placed over it. For powder microscopic study, 5 g of the drug sample was taken, powdered. The powdered material was taken on an 85 mesh sieve and allowed in slow running water for washing away the minerals. The materials were cleared in chloral hydrate, wash with distilled water and mounted in glycerin, then observed characters.[7]

Physico-chemical Standardization

Different physico-chemical values such as pH values; foaming index; hot extractive values using water, ethanol, petroleum ether, chloroform, acetone, and methanol; percentage of total ash, acid-insoluble ash and water-soluble ash of aqueous *E. ribes* extract was determined as per method described in Indian Pharmacopoeia.[7]

Preliminary phytochemical screening

The presence or absence of different phytoconstituents like alkaloids, carbohydrates, glycosides, phenolics, flavonoids, proteins, saponins, sterols, resins, and lipids/fats were evaluated by standard qualitative and quantitative methods.[8]

Animals and Diets

The female Wistar rats (100–150 g) were procured from central animal house facility of Hamdard University, New Delhi, India. The animals were housed in standard

polypropylene cages and maintained under controlled room temperature (22 ± 2°C) and humidity (55 ± 5%) with 12 h light and 12 h dark cycle. All the rats in Normal group were provided with commercially available rodent chow diet (Amrut rat feed, Nav Maharastra Chakan Oil Mills Ltd, Delhi, India) and tap water *ad libitum*. The guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India were followed and prior permission was sought from the Institutional Animal Ethics Committee, Jamia Hamdard, New Delhi for conducting the study.

HFD-Induced Obesity

Rats were fed a HFD (20 g/rat/day) in a pellet form and water *ad libitum* for the period of 6 weeks. HFD was purchased from National Centre for Laboratory Animal Science (NCLAS), National Institute of Nutrition (NIN), Hyderabad, India. Composition of HFD is shown in (Table 1).

Experimental Design

In this study, a total of 50 rats were used and divided into five groups of 6 rats each. Group 0020I: normal healthy control rats fed with rodent chow diet and administered 1% gum acacia (1 ml/kg, body weight (bw)) for the period of 42 days (NC); Group II: obesity control rats fed with HFD (20 g/rat/day) for the period of 42 days (HFD); Group III: rats fed with HFD (20 g/rat/day) for the period of 42 days , treated with aqueous *E. ribes* extract (20 mg/kg, bw) for period of 42 days (HFD+ER); Group IV: rats fed with HFD (20 g/rat/day) for the period of 42 days, treated with Orlistate (5 mg/kg, bw) for period of 42 days (HFD+ ORL); Group V: rats fed with rodent chow diet for the period of 42 days + treated with aqueous *E. ribes* extract (20 mg/kg, bw) for period of 42 days (ER *per se*). All the drugs were administered orally via a standard orogastric cannula (Table 2).

Table 1. Compositions of the HFD (g/kg diet).

Ingredients	Quantity
Casein	342.0
L-Cystine	3.0
Starch	172.0
Sucrose	172.0
Cellulose	50.0
Ground nut oil	25.0
Tallow	190.0
AIN Salt mix	35.0
AIN Vitamin mix	10.0
Total (g)	999.0

Table 2. Experimental Design.

S.No	Groups	No. of animals
1	Normal control	6
2	Obese control (HFD)	6
3	HFD + *Embelia ribes* (20.0 mg/kg)	6
4	HFD + Orlistat std (5 mg/kg)	6
5	ER *per se*	6
	Total	30

Food and water intakes were measured daily for the period of 42 days at the same time. Food intake and water intake were measured on per cage basis (6 rats per cage) and the average food and water consumed were calculated. At the end of the experimental period (on 43th day), the animals were anesthetized with ether, following overnight fasting. Blood was drawn by retro-orbital method into a tube and the serum was obtained by centrifugation. After collection of blood, rats were sacrificed; heart and perirenal, epididymal, mesenteric fat pads were excised immediately, rinsed with phosphate buffer saline and weighed. The serum and heart samples were stored at -70° C until analysis.

Anthropometric Measurements

The body weights were determined once a week. Body mass index (BMI) was calculated from formula:

$$BMI = \text{body weight (g)} / \text{length}^2 (\text{cm}^2)$$

Biochemical Serum Analyses

Serum glucose, triglyceride (TG) and total cholesterol (TC) levels were determined by enzymatic methods using commercial assay kits (SPAN Diagnostics Ltd, Surat, India) according to the manufacturer's protocols. Serum High-density lipoprotein cholesterol (HDL-C) levels were determined using HDL Cholesterol Test Kit (Reckon Diagnostics P. Ltd, Baroda, India). Serum leptin and insulin levels were measured by immunoassays using a commercially available ELISA assay kit from Ray Biotech, Inc (Norcrcoss, US) and Crystal Chem Inc (Downers Grove, IL 60515, USA) respectively. Low-density lipoprotein cholesterol (LDL-C) levels were calculated using Friedewald formula.[9] Serum very low density lipoprotein cholesterol fraction (VLDL-C) concentration was calculated by deduction of the sum of HDL-C and LDL-C concentrations from that of TC.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). The statistical significance of difference between the mean values for the treatment groups was analysed by ANOVA (analysis of variance) followed by Dunnett's t- test using Graph pad InStat® version

3.06 (Graph Pad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered significant.

RESULTS

Macroscopical Studies

The *Embelia ribes* fruits are blackish brown colored with 2.4-4mm size, sub globular shape, odour distinct, astringent taste and wrinkled textured shown in (Figure 1). The organoleptic evaluation of *Embelia ribes* fruit were showed in (Table 3).

Microscopical Studies

Epicarp of the fruit consist of dome shaped, 3 sides cells covered with cuticle;followed by thick layer of hypodermis which is filled with brown contents; thick walled stone cells and vascular strands embedded in parenchymatous cells of the mesocarp; underneath this lie cells of endocarp. Testa cells composed of narrow, rectangular underneath this lies pigment layer and endosperm cells filled with aleurone grains and fixed oil (Figure 2). Powder characters are shown in (Figure 3).

Physico-chemical Standardization, Preliminary Phytochemical Screening

The physico-chemical values such as extractive values, ash values, pH values and foaming index of aqueous *E. ribes* extract was determined and tabulated in (Table 4). Phytochemical screening of aqueous *E. ribes* extract showed positive results for alkaloids, carbohydrates, phenolics, and saponins.

Table 3. Oraganoleptic Evaluation of Fruit.

Size	2-4mm
Colour	Blackish
Shape	Sub globular
Odour	Distinct
Texture	Wrinkled
Taste	Astringent

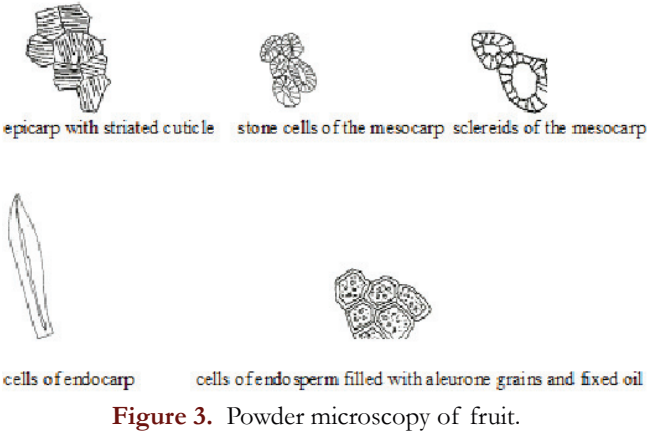


Figure 3. Powder microscopy of fruit.

Table 4. Physico-chemical Constant of Aqueous *E. ribes* Extract.

Sr. No	Parameters	Mean ± SEM
1	**% Extractive value (Hot extraction)**	
1a.	Water	4.061 ± 0.003
1b.	Ethanol	7.605 ± 0.001
1c.	Petroleum ether	5.685 ± 0.002
1d.	Chloroform	2.569 ± 0.009
1e.	Acetone	5.005 ± 0.007
1f.	Methanol	4.309 ± 0.004
2	**Ash values**	
2a.	Total ash	4.78 ± 0.003
2b.	Acid insoluble ash	1.098 ± 0.002
2c.	Water soluble ash	2.006 ± 0.004
3	**pH determination**	
3a.	1% solution	5.587 ± 0.003
3b.	10% solution	5.089 ± 0.006
4	Foaming Index	555.586 ± 0.024

Effect on Food and Water Intake

Negligible ($p > 0.05$) change in food and water intake was observed in HFD fed group as compared to the NC group. Administration of aqueous *E. ribes* extract (20 mg/kg) along with HFD (HFD + ER group) did not produce significant change in the food and water intake as compared to HFD group (Table 5).

Effect on Anthropometric Parameters

In the HFD group body weight gain of rats was greater than the values for the NC. HFD supplemented with 20 mg/kg aqueous *E. ribes* extract reduced body weight gain of the rats, ($p < 0.05$). BMI significantly greater in rats fed the HFD than the value for the normal control rats ($p < 0.01$), on the other hand it was reduced in the rats, administered with 20 mg/kg aqueous *E. ribes* extract ($p < 0.05$). However, there was no significant difference in body weight gain and BMI among the NC and ER *per se* treated groups (Table 5).

Effect on Organ Weight and Fat Pad Analysis

No significant changes in weights of heart, kidney, liver and spleen in drug treated albino rats as compared to control and high fat diet fed group. However, drug treatment has variably restricted increase in fat pad weights compared to high fat diet group (Tables 6 & 7).

Table 5. Effect of Aqueous *E. ribes* Extract (20 mg/kg) on Food Intake, Water Intake, and Body Weight Gain and BMI in HFD-induced Obesity in Wistar Rats.

Groups	Food intake (gm/day/rat)	Water intake (ml/day/rat)	Body weight gain (g)	BMI (g/cm2)
NC	10.56 ± 2.27	38.34 ± 4.81	80.13 ± 3.67	4.53 ± 0.04
HFD	15.23 ± 2.67 ns$	41.6 ± 8.87 ns$	131.21 ± 18.9**	5.52 ± 0.20*
HFD + ER	14.02 ± 0.21 ns	38.5 ± 1.98 ns	99.8 ± 4.29#	4.77 ± 0.85#
HFD + ORL	14.60 ± 0.12 ns	37.8 ± 1.91 ns	101.2 ± 9.22#	5.01 ± 0.11#
ER *per s*	*e* 12.89 ± 0.54 ns$	40.8 ± 2.38 ns$	57.8 ± 1.59 ns$	3.99 ± 0.77 ns$

All values were expressed as Mean ± SEM, (n = 6); ** $p < 0.01$, * $p < 0.05$ as compared to the NC group; ## $p < 0.01$, # $p < 0.05$ as compared to HFD group; ns = non significant as compared to the HFD group, ns$ = non significant as compared to the NC group.

Table 6. Effect of Aqueous *E. ribes* Extract (20 mg/kg) on Organ Weight Profile in Albino Rats after 42 days of Treatment Organ Weight (g/100g of Animal Weight).

Groups	Heart	Kidney	Liver	Spleen
NC	0.56 ± 0.27	0.74 ± 0.81	4.13 ± 0.67	0.53 ± 0.04
HFD	0.53 ± 0.67	0.71 ± 0.87	4.21 ± 0.91	0.52 ± 0.20
HFD + ER	0.52 ± 0.21	0.76 ± 0.98	4.84 ± 0.29	0.57 ± 0.85
HFD + ORL	0.50 ± 0.12	0.73 ± 0.91	4.24 ± 0.22	0.51 ± 0.11
ER *per se*	0.59 ± 0.54	0.79 ± 0.38	4.85 ± 0.59	0.54 ± 0.77

All values were expressed as Mean ± SEM, (n = 6).

Table 7. Effect of Aqueous *E. ribes* Extract (20 mg/kg) on Fat Pad Weight Profile in Albino Rats after 42 days of Treatment.

	Fat pad weights (g/100g of animal weight)		
Groups	Mesentric	Perirenal	Uterine
NC	0.246 ± 0.22	0.534 ± 0.82	0.223 ± 0.14
HFD	1.540 ± 0.27	1.171 ± 0.67	0.532 ± 0.50
HFD + ER	0.531 ± 0.24 ##	0.751 ± 0.34#	0.667 ± 0.45
HFD + ORL	0.510 ± 0.62	0.710 ± 0.22	0.651 ± 0.21
ER *per se*	0.359 ± 0.56	0.529 ± 0.14	0.262 ± 0.71

All values were expressed as Mean ± SEM, (n = 6); ## $p < 0.01$, ### $p < 0.001$, # $p < 0.05$ as compared to the NC group; one way ANOVA followed by Dunnet's t-test.

Effect on Serum Lipid Levels

Serum TG levels were significantly higher in HFD fed group compared to that of NC group, HFD + ER group ($p < 0.01$). Levels of serum TC, LDL-C, VLDL-C were similar to tendency of serum triglyceride level ($p < 0.01$). Serum levels of HDL-C were higher in HFD + ER treated group compared to HFD fed and NC group, significant difference was recognized ($p < 0.01$) (Table 8).

Effect on Serum Insulin and Leptin Levels

It was observed after 42 days of treatment, HFD + ER group showed a significant ($p < 0.01$) reduction in serum glucose level in relation to the HFD fed group. Serum leptin and insulin and levels in HFD fed group was significantly higher ($p < 0.01$) than that of normal control but significant reduction observed in HFD + ER group ($p < 0.01$). Result of HFD + ORL group was comparable to that of HFD + ER group (Table 9).

Histopathological Studies

Results reveal that drug significantly reduced the fat accumulation in liver, heat and kidney (Figures 4, 5 & 6).

DISCUSSION

Present study was carried out to investigate the anti-obesity effects of aqueous *E. ribes* extract in HFD-induced

Table 9. Effect of Aqueous *E. ribes* Extract (20 mg/kg) on Serum Insulin and Leptin Levels in HFD-induced Obesity in Wistar Rats.

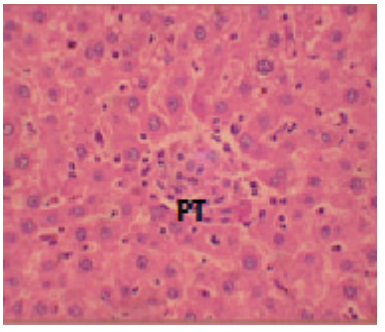
Groups	Insulin (pmol/l)	Leptin (ng/ml)
NC	167 ± 11.05	5.92 ± 1.19
HFD	659.06 ± 3.24	14 ± 09.63
HFD + ER	491.23 ± 1.89 ###	11.69 ± 5.59 #
HFD + ORL	432.37 ± 1.01	10.22 ± 4.56
ER *per se*	159.91 ± 0.67	6.05 ± 1.01

All values were expressed as Mean ± SEM, (n = 6);, ### $p < 0.001$, # $p < 0.05$ as compared to the NC group; one way ANOVA followed by Dunnet's t-test.t

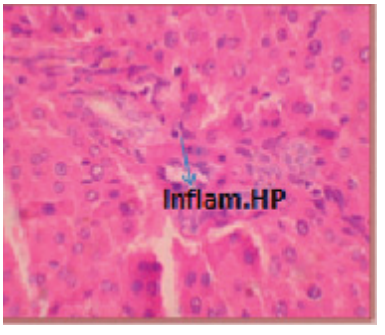
Table 8. Effect of Aqueous *E. ribes* Extract (20 mg/kg) on Serum TC, TG, LDL-C, VLDL-C and HDL-C Levels in HFD-induced Obesity in Wistar Rats.

Groups	TC (mg/dl)	TG (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)	HDL-C (mg/dl)
NC	96.99 ± 1.05	59.92 ± 1.19	49.97 ± 0.31	12.44 ± 0.56	33.56 ± 2.03
HFD	159.06 ± 3.24**	150 ± 09.63**	101.61 ± 5.87**	33.1 ± 1.02**	23.12 ± 0.35*
HFD + ER	139.23 ± 1.89##	132.69 ± 5.59#	78.63 ± 4.08#	27.09 ± 0.21#	34.134 ± 1.24##
HFD + ORL	132.37 ± 1.01##	121.22 ± 4.56##	74.09 ± 0.71##	24.54 ± 0.95##	38.24 ± 0.23##
ER *per se*	89.91 ± 0.67 ns$	56.05 ± 1.01 ns$	42.18 ± 7.01 ns$	10 ± 0.20 ns$	32.66 ± 0.57 ns$

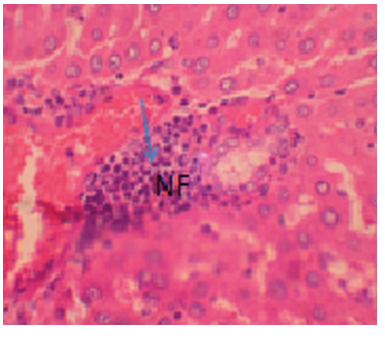
All values were expressed as Mean ± SEM, (n = 6); ** $p < 0.01$, * $p < 0.05$ as compared to the NC group; ## $p < 0.01$, # $p < 0.05$ as compared to HFD group; ns = non significant as compared to the HFD group, ns$ = non significant as compared to the NC group.



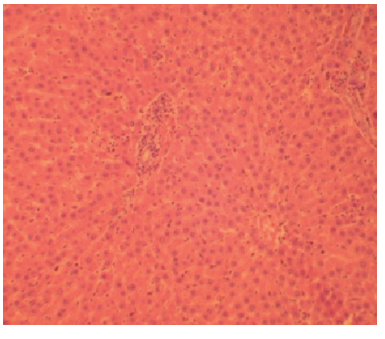
High power photomicrograph of liver from ND* control group showing a normal portal triad (PT) and hepatocytes (H&E × 400).



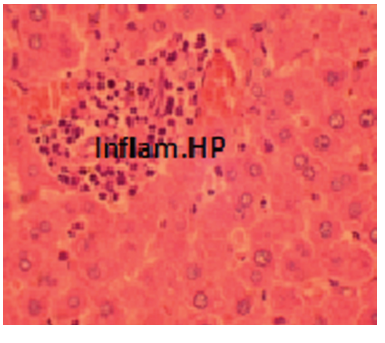
High power photomicrograph of liver from HFD* control group showing swelling of hepatocytes (HP) and narrowing sinusoids (H&E × 400).



High power photomicrograph of liver from *Embelia ribes* treated group showing necrotic focus with collection of inflammatory cells in the portal triad. Darker nuclei show the regeneration of cells resulting in narrowing of sinusoids (H&E × 400).

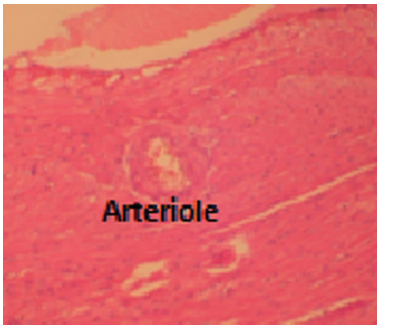


High power photomicrograph of liver from standard group showing no fat deposition (H&E × 400).

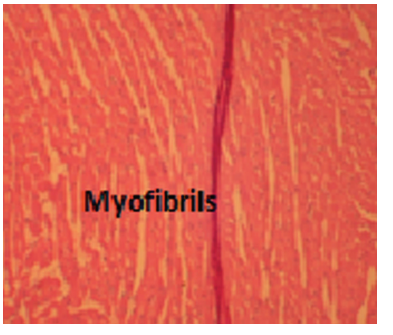


High power photomicrograph of liver from *per se* group showing significant inflammation of hepatocytes and damage to liver (H&E × 400).

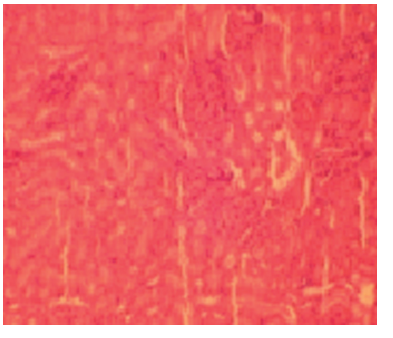
Figure 4. Changes observed in liver of different groups.



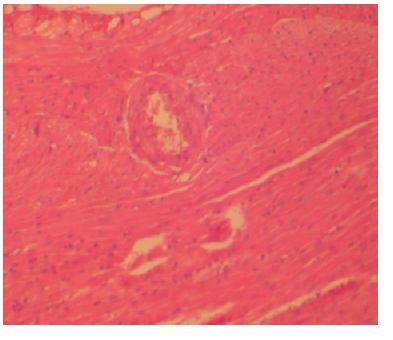
High power photomicrograph of Heart from ND* control group showing normal arteriole (A) and muscles (M-myofibrils) (H&E × 400).



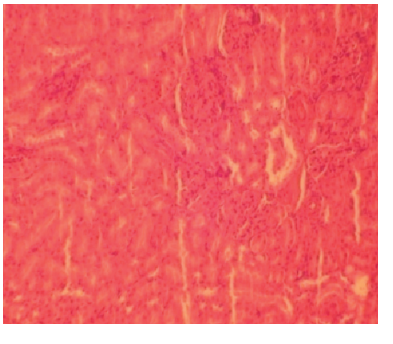
High power photomicrograph of Heart from HFD* control group showing edema (E) and change in colour of heart from pink-red to yellowish pink (H&E × 400).



High power photomicrograph of Heart from *Embelia ribes* treated group without any significant change (H&E × 400).

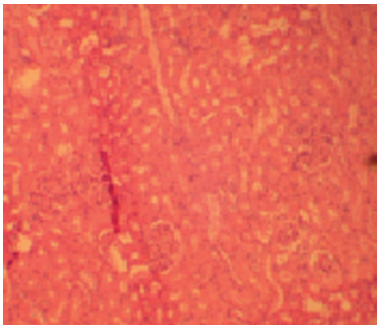


High power photomicrograph of Heart standard group showing normal arteriole (H&E × 400).

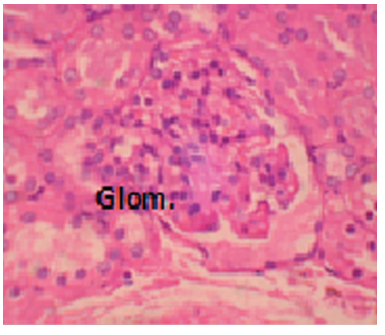


High power photomicrograph of Heart from *per se* group without any significant change with normal arteriole (H&E × 400).

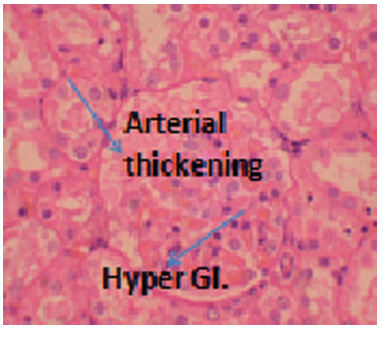
Figure 5. Changes observed in heart of different groups.



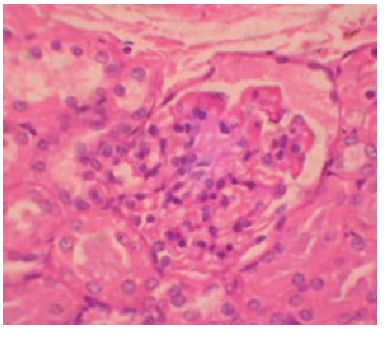
Low power photomicrograph of Kidney from ND* control group showing normal glomerulus (H&E × 100).



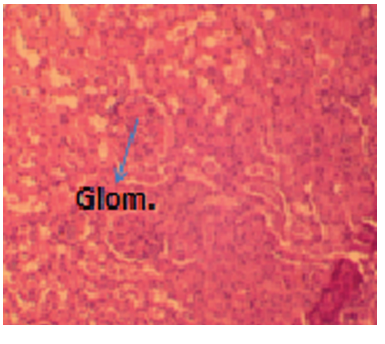
High power photomicrograph of Kidney from HFD* control group showing normal glomerulus (G) (H&E × 400).



High power photomicrograph of Kidney from *Embelia ribes* treated group showing hypercellular glomerulli (HG) and inflammatory cells and arterial thickening (H&E × 400).



High power photomicrograph of Kidney from standard group showing normal glomerulus (G) (H&E × 400).



Low power photomicrograph of Kidney from *per se* group showing normal glomerulli (G) (H&E × 100).

Figure 6. Changes observed in kidney of different groups

obesity in Wistar rats. Obesity, which affects up to 30% of the adult population in developed countries. Obesity is associated with serious mortalities including high incidence of type 2 diabetes, hyperlipidemia, hypercholesterolemia, cardiovascular disease and many more. HFD is thought to be one of the main factors for development of obesity. We selected a rat model of diet induced obesity based on numerous experimental studies indicated that consumption of HFD leads to increased body weight and fat mass, induce alterations in carbohydrate and lipid metabolism, lead to insulin resistance, increase production and release of leptin in laboratory animals and humans.[10,11,12,13]

The present study results showed that compared to that of NC, Serum TG, TC, LDL-C, VLDL-C levels were significantly elevated in HFD fed rats (HFD group) decreased in HFD + ER treated group more than that of HFD fed group; indicating potential lipid lowering activity of the test drug. This study also showed that body weight gain and BMI in HFD group were increased more than of NC group, significantly decreased in HFD + ER group.

Among various fat deposits, visceral fat mass correlated to insulin resistance in animal model [14]. Individuals with visceral by obesity have impaired insulin action[15], and prevents insulin resistance by removing visceral fat mass.[16] From adipose tissue, two groups of inflammatory proteins are produced (i) IL-6 and TNF-α [inflammatory mediators], (ii) leptin, adiponectin and resistin [adipocytokines].[17] Human obesity is characterized by variable degrees of hyperleptinemia and leptin resistance.[18] The result of the present study indicates insulin sensitivity improved in rat fed with the drug. The drug induced melioration of insulin resistance which is supported by down regulation of leptin. Thus, visceral fat accumulation and improved insulin resistance in rat model with HFD induced obesity may possibly be through down regulation of leptin, TNF-α, SREBP1γ, and PPARγ2 gene expression. Uygun et al reported that leptin might contribute to hepatic steatosis by promoting insulin resistance and also by altering insulin signaling in hepatocytes, which consequently promote increased intracellular fatty acid.[19] Hence, drug treatment to rats with HFD prevents increase of these levels due to decrease of the body fat content. Conclusively, observed reduction in body weight gain, BMI, serum lipids, glucose, insulin, leptin levels suggests that aqueous extract of *E. ribes* possess significant antiobesity potential.

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