# A mini review on chemistry and biology of *Hamelia Patens* (Rubiaceae)

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## ABSTRACT

*Hamelia patens* Jacq. Commonly known as "redhead," "scarlet," or "firebush." belongs to the Madder family (Rubiaceae), different parts (leaves, stem, flower, root, seeds and even whole plant) of *Hamelia patens* used. It is a perennial bush, and grow in full sun and in shade. It grows to about 6 feet. Neotropical shrub *Hamelia patens* Jacq has been cultivated as an ornamental in the United States, Great Britain, and South Africa. *Hamelia patens* have contained pentacyclic oxindole alkaloids: isopteropodine, rumberine, palmirine, maruquine and alkaloid A, B and C, other chemical constituents are apigenin, ephedrine, flavanones, isomaruquine, narirutins, pteropodine, rosmarinic acid, narirutin, seneciophylline, speciophylline, and tannin. In last few decades several Indian scientists and researchers have studied the pharmacological effects of steam distilled, petroleum ether, chloroform, ethanol & benzene extracts of various parts of *Hamelia* plant on immune system, reproductive system, central nervous system, cardiovascular system, gastric system, urinary system and blood biochemistry.

**Key words:** *Hamelia patens*, alkaloids, Traditional uses

## INTRODUCTION

Plants are one of the most important sources of medicines. Today the large numbers of drugs in uses are derived from plants. The genus *Hamelia patens* (family-Rubiaceae) commonly known as "bayetilla," "trompetilla," "coralillo," or "hierba coral. These two last names are related to the red color of some of its organs.[1-2] The genus *Hamelia* (family Rubiaceae) consists of 16 species of shrubs or small trees native to lowland neotropical areas ranging from southern Florida, the West Indies, and Mexico to Brazil and Argentina.[3] *Hamelia patens* was named a Texas Superstar by Horticulture Department at Texas A&M. University because of its excellent performance during the hot dry Texas summers, it is recommended as a low maintenance native shrub in Florida.[4] and has been promoted as an attractant for hummingbirds and butterflies to gardeners throughout the southern U.S. as a perennial or annual.[5] *Hamelia patens* has been introduced and cultivated for at least 250 years, with six species grown in England in 1839.[6-7] It grows as a tree in the Atlantic tropical lowland of Costa Rica.[8] It is a reliable tropical plant that has found its way into many a landscape because of its proven drought and soil tolerance that basically can grow anywhere. Firebush should be planted in full sun or part shade in well-drained soil beginning in mid-May and on into the summer. Firebush can tolerate many types of soils from heavy clays to high alkaline as long as it has good drainage. *Hamelia patens* does not have pest or disease problems that plague so many of the tropical. To the delight of organic growers, this plant is a prize, because it requires no pesticides, Use a complete fertilizer at planting and then once a month with soluble nitrogen to continue a strong blooming show. The show continues for into the fall when the foliage changes to a striking blood-red color.[9] But it can easily be killed when overwatered.[10] The species can be propagated from seeds, but most commercial ornamentals are produced from cuttings, monthly production may simply be a direct result of seasonal changes in the physical environment.[11] A more complicated level of control would be an adjustment in fruit ripening rate to match changes in fruit removal rates.[12] Firebush flowers throughout the year. It is a hummingbird-pollinated plant.[13] The flowers are also visited by butterflies.[14] *Hamelia patens* in India shows considerable variation in floral morphology, pollination and fruit set in different seasons. *Hamelia patens* at Agra, in order to compare

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these data of cultivated plants with those obtained within its area of natural distribution.[15] *Hamelia patens* has been studied chemically. It is known to contain pentacyclic oxindole alkaloids,[16-17-18] and also contains a new glycoside, 5, 7, 2', 5'-tetrahydroxyflavanone 7-rutiroside, together with narirutin and rosmarinic acids were isolated from the aerial parts of *Hamelia patens*.[19] Firebush contains 17.5 percent crude protein and has an *in vitro* digestibility of 61.6 percent,[20] and has a sugar content of approximately 9%, although significant variation exists both within and among trees.[21] *Hamelia patens* is commonly used as a treatment for wound in central America.[22] The important advantages claimed for therapeutic uses of medicinal plants in various ailments are their safety besides being economical, effective and their easy availability, Because of these advantages the medicinal plants have been widely used by the traditional medical practitioners in their day to day practice. In Mexico it is used for 42 different medicinal purposes, especially to stop bleeding, healing sores, and in menstrual disorders,[23-24] Pimples, malaria, sore[25], skin disease, blisters, eczema, stomachache[26], athlete's foot, relieve pain, skin lesions, rash, insect bites, itching headache, asthma, burn, scurvy, inflammation, rheumatism, nervous shock, post partum pain, uterine and ovarian afflictions, in scant, expel intestinal worms and dysentery.[1-27-28-29-30-31]

## SYNONYMS

*Hamelia erecta* Jacq., *Hamelia coccinea, Hamelia pedicellata* Wernh, *Hamelia latifolia* Reichb. ex DC.

### Common names

Hindi: *Hamelia*

English: Scarlet bush, Fire bush, Hummingbird bush, Polly red head, Texas firecracker, Red head va

Span: Sanalo-todo, Pata de pajaro

Mayan: Ix-canan

## PLANT MORPHOLOGY

This species is bush or small tree, 1.4-3.0 m but sometimes reaches 7 m in height tall, trees produce flowers and fruits throughout the year.[12] the fruits are also eaten by birds which disburse the seeds.[32] Plants may have single or multiple stems. The twigs are orange to purple. Leaves are opposite or grouped in threes or fours, and finely hairy to glabrous. The leaves have petioles 1 to 3.5 cm long and blades that are mostly ovate-elliptic to obovate-elliptic with an acute or acuminate tip. The lateral and especially the mid veins are red or pink. In temperate areas, as the temperatures turn cool in the fall, the foliage turns to a brilliant red, hence the common name. The inflorescence is terminal, a modified dichasium with flowers that are tubular, 12 to 22 mm long, and orange to red in color. The fruit is a berry, spherical to elliptical, 7 to 10 mm long, turning red and then black at maturity. The seeds are orange-brown, 0.6 to 0.9 mm long.[33] firebush has a tap and lateral root system with abundant fine roots. Stem bark is gray and smooth and the inner bark is light green.[34] *Hamelia patens* should be planted in full sun or part shade in well-drained soil beginning in mid-May and on into the summer. It can tolerate many types of soils from heavy clays to high alkaline but mostly prefers loamy or clayey soil, Propagation is by soft wood cuttings in spring or by seed.[10]

## TAXONOMY

Kingdom: Plantea
(Unranked): Angiosperms
(Unranked): Eudicots
(Unranked): Asterids
Order: Gentianales
Family: Rubiaceae
Genus: *Hamelia*
Species: *Hamelia patens*
Botanical name: *Hamelia patens Jacq.*

## CHEMICAL CONSTITUENTS

*Hamelia patens* is rich in active phytochemicals including alkaloids and flavonoids. It contains several of the same oxindole alkaloids as Cat's Claw (Uncaria tomentosa) including pteropodine and isopteropodine; both have been highly studied and even patented as effective immune stimulants. These two chemicals have also recently shown to have a positive modulating effect on brain neurotransmitters (called 5-$HT_2$ receptors) that are targets for drugs used in treating a variety of conditions including depression, anxiety, eating disorders, chronic pain conditions and obesity. Three new oxindole alkaloids have also been discovered in *Hamelia*

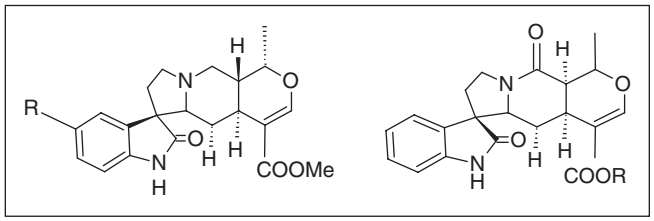


**Figure 1**
*Isopteropodine, R = H*
*Rumberine, R = OH*
*Palmirine, R = OMe*
*Maruquine, R = H*
*Alkaloids A, R = Me*
*Oxindole Alkaloids from Hamelia patens methanol extract of the Leaves Determined by GC-MS.*[1]

*patens* which have never been classified before; they have been named *Hamelia patens* alkaloid A, B and C. Scientists in India discovered that *Hamelia* leaves contain small amounts (00.05%) of ephedrine a stimulant alkaloid that has received some negative press of late. In addition, the aerial parts of the plant have been found to contain rosmarinic acid, a phytochemical that has demonstrated immune modulating and antidepressant activity. The main plant chemicals documented in *Hamelia patens* thus far include: apigenin, ephedrine, flavanones, isomaruquine, isopteropodine, maruquine, narirutin, oxindole alkaloids, palmirine, pteropodine, rosmarinic acid, rumberine, rutin, seneciophylline, speciophylline, tannin and Stigmast-4-ene-3,6-dione.[16-28]

## BIOLOGICAL ACTIVITIES AND CLINICAL RESEARCH

Much of the clinical research on *Hamelia* has validated the traditional uses of the plant. In animal studies (with rats) *Hamelia patens* leaf extracts demonstrated analgesic, diuretic, and hypothermic actions. External use of the leaf in mice showed significant anti-inflammatory activity comparable to that of a prescription anti-inflammatory drug used as a control. Scientists in two different countries have documented antibacterial and antifungal properties against a wide range of fungi and bacteria in several in vitro studies. The plant has also been documented with diuretic effects and was shown to inhibit the growth of tumor and bacteria cells. Spontaneous Activity Stimulation, Cytostatic Activity also reported.[28-36]

### Biological Activities for Compounds of Hamelia patents

Compounds of *Hamelia patents* like Pteropodine, Isopteropodine, Isopteropodine, Pteropodine, Rosmarinic Acid, and Pteropodine have show the significant biological activities.[35]

## MEDICINAL USE OF VARIOUS PARTS OF *HAMELIA PATENS*

A decoction or infusion of the leaves of *Hamelia patens* is generally used internally or externally for bacterial and fungal infections as well as for its anti-inflammatory and pain reducing properties. Typically, if the remedy is taken internally, an infusion is employed; a leaf decoction is prepared for external use. Try planting a beautiful scarlet bush in the garden. While working in the garden on hot days, chew on one of the leaves like the rainforest Indians do; it has remarkable hypothermic and cooling actions which will help keep the body from overheating. The use of this plant in herbal medicine systems has been reported to be safe and non-toxic when taken orally at the traditional remedy dosages. Only one of the animal studies published thus far indicated toxicity, when they injected a methanol extract of *Hamelia patens* leaves into mice at high dosages (1.5 grams per kg of body weight).[28] *Hamelia patens* is also recommended for the treatment of stop the bleeding, wound healing, athlete's foot, relieve pain, skin lesions, rash, insect bites, itching, headache, asthma, burn, scurvy, inflammation, rheumatism, nervous shock, post partum pain, menstrual disorders, uterine, ovarian afflictions, expel intestinal worms, dysentery and also possess different activities like Antibacterial, Antifungal, Analgesic, Anti-inflammatory, Cytostatic, Diuretic, Hypothermic and, Spontaneous Activity.[27-28-35] Leaves and stems are crushed and applied externally to cuts, blisters, rashes, act as a pain reliever for bruises, skin fungus, insect stings, strains, sprains, and other painful or inflamed conditions, and it is a remedy for amenorrhea. To prepare the infusion a handful of leaves is poured in 4l ml of water, and boiled until only half the volume is remaining. The patient must drink the infusion "constantly" in the next days and follow a diet low in fat. This is also administered against flux and dark bleeding after menstruation, or child delivering (Martínez, *et al*., 1995).[36] Scientific research that could sustain *Hamelia patens* purported folk medicinal uses is scant. However, recent experimental evidence indicates that the topical application of an ointment prepared with the ethanol extract of *Hamelia patens* aerial parts increases the breaking strength in wounds induced in rats.[20] Ethno-pharmacological information suggests that *Hamelia patens* may exhibit activity on myometrium contractility Nevertheless, to date there are no studies focused to systematically evaluate this property.[1] It was found that methanol leaf extract of *Hamelia patens* exhibited moderate DPPH radical scavenging activity at concentration of 200 µg/ml.[27] The inhibitory action of *Hamelia patens* methanol extract was studied *in vitro* on uterus, ileum and duodenum the anti-diarrheal activity was evaluated *in vivo* on mice and rats.[35] In EL Salvador, where it is known as *chichipince,* a crude extract of *Hamelia patens* is blended into soap and sold as cleaning agent for wounds.[37]

## CONCLUSION

*Hamelia patens,* the versatile medicinal plants are the unique source of various types of compounds having diverse chemical structure. A very little work has been done on the biological activity and plausible medicinal applications of these compounds and hence extensive investigation is needed to exploit their therapeutic utilities to combat diseases. A drug development programme should be undertaken to develop, modern drugs with the compounds isolated from *Hamelia patens*. Although crude extracts from

various parts of the plants have medicinal applications from time immemorial, modern formulation can be developed after extensive investigation of its bioactivity, mechanism of action, pharmacotherapeutic and toxicity with help of standardization clinical trials. Global scenario is now changing toward the use of nontoxic plant products having traditional medicinal uses; development of modern drugs from the centuries old knowledge on this tree should be emphasized for the control of various diseases. In fact time has come to make good use through modern approaches of drug development a significant amount of research has already been carried out during the past few decades in exploring the chemistry of different parts of the plant, which generate enough encouragement among the scientists in exploring more information about this medicinal plant. An extensive research and development work should be undertaken. *Hamelia patens* and its products for their economics and therapeutic utilitization, therefore this review article might be helpful for scientists and researcher to find new chemicals entities responsible for its claimed traditional activities.

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# Pharmacognostic Standardization of *Cymbopogon citratus* (dc.) stapf leaves

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## ABSTRACT

**Context:** To ensure reproducible quality of herbal products, proper control of starting material is important. The first step towards ensuring quality of starting material is authentication. Thus, in recent years there has been a rapid increase in the standardization of selected medicinal plants of potential therapeutic significance. Despite the modern techniques, identification of plant drugs by pharmacognostic studies is more reliable. **Objective:** The present work has been designed to study the Pharmacognostic parameters of the leaves of *Cymbopogon citratus*. **Materials and methods:** Various standardization parameters like morphological characters, microscopic evaluation, physicochemical evaluations (loss on drying, ash values, extractive values), preliminary phytochemical screening and TLC chromatographic profile of the extract were carried out and the quantitative microscopy were reported. **Results:** 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.

**Key words:** *Cymbopogon citratus*, Pharmacognostic parameters, Preliminary Phytochemical Screening.

## INTRODUCTION

*Cymbopogon citratus,* family Poaceae also called as lemongrass is a widely used herb in tropical countries, especially in Southeast Asia. The essential oil of the plant is used in flavour, fragrancing and aromatherapy, medicinal tea, culinary herb[1] and treatment for skin diseases.[2] It is known as a source of ethno medicines .[3] *C. citratus* is used in different parts of the world in the treatment of digestive disorders, fevers, menstrual disorder, rheumatism and other joint pains .[4] The chemical composition of the essential oil of *C. citratus* varies according to the geographical origin, the compounds as hydrocarbon terpenes, alcohols, ketones, esters and mainly aldehydes have constantly been registered.[5] The essential oil (0.2 to 0.5%, West Indian lemon grass oil) consists of mainly citral.[6] Citral is a mixture of two stereoisomeric monterpene aldehydes, the Trans isomer geranial (40 to 62%) dominates over the cis isomer neral (25 to 38%).[7-8] Flavonoids are also reported to be the phytoconstituents of *Cymbopogon citrates*. It consists of luteolin and and its 6-C and 7-O –glycosides,[9] isoorientin 2'-O-rhamnoside[10] and isolation of the flavonoids quercetin, kaempferol and apiginin[11] from aerial parts. The phenolic compounds elimicin, catecol, chlorogenic acid, caffeic acid and hydroquinone isolated from plant.[12]

Various pharmacological activities of *Cymbopogon citratus,* have been reported such as Anti amoebic,[13] Antibacterial,[14-17] Antidiarrhoeal,[18] Antifungal,[19] Antimalarial,[20] Antiinflammatory[21] and Anti-anxiety.[22] *Cymbopogon citrates* (DC) Stapf (Graminae) (*C. citratus*) are an herb worldwide known as lemongrass. The tea made from its leaves is popularly used as antispasmodic, analgesic, anti-inflammatory, antipyretic, diuretic and sedative.[28] In lemongrass, antioxidant[29], and antinociceptive[30] activities have been studied. A few ethno botanical reports on treatment of fever and headache were investigated.[23]

## MATERIALS AND METHODS

### Plant material

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

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### Preparation of leaf extracts

Leaves of *Cymbopogon citratus (DC.)* stapf were dried in shade and powdered. One hundred grams of powdered leaves were subjected to successive Soxhlet extraction by solvents in increasing order of polarity viz. petroleum ether (60-80°C), chloroform and methanol. Before each extraction the powdered material was dried in hot air-oven below 50°C. Finally, marc was digested with distilled water for 24 hours to obtain the aqueous extract. Each extract was concentrated by distilling off the solvent and then evaporating to dryness on the water-bath. Extracts were weighed and percentage was calculated in terms of the air-dried weight of the plant material.

### Pharmacognostic Evaluation

#### *Organoleptic Evaluation*

Organoleptic features of the plant were evaluated by observing color, odour, taste, size, shape of morphology and special features like texture. A part of quantitative microscopy, stomatal number, stomatal index, was determined by using fresh leaves of plant.

### Microscopic and Histological Techniques

#### *Study of Transverse Sections*

The leaves of *Cymbopogon citratus* were boiled with water until soft. Free hand sections of the leaves were cut transferred on slides cleared by warming with chloral hydrate and mounted in glycerin. The lignified and cellulosic tissues were distinguished using differential staining techniques.[24]

#### *Photomicrography*

Microscopic evaluations of tissues were supplemented with micrographs. Photographs of different magnifications were taken with Nikon Labpot 2 Microscopic Unit. For normal observations bright field was used. For the study of crystals, starch grain and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars.[25]

#### *Powder microscopy*

A few drops of chloral hydrate solution were added to a sample of powered plant material on a slide, was covered with a glass slip and heat gently over a microbunsen. Vigorous boiling was avoided. The slide was examined under the microscope. When the clearing process is completed a drop of glycerol solution was added which will prevent crystallization of the mountain on cooling.

#### *Physicochemical analysis*

Physicochemical analysis i.e., alcohol (90% ethanol) and water soluble extractive values, total ash, acid-insoluble ash, and loss on drying of the powdered drug were determined.[26]

#### *Phytochemical Screening*

The various extracts of *Cymbopogon citratus* were subjected to qualitative chemical examination.[27]

#### *Thin Layer Chromatography Profile*

TLC glass plates (5×15 cm), 0.25 mm thick were prepared using Silica gel G. The plates were activated at 110°C for 30 minutes. The TLC profiles of the extracts were studied using different solvent systems. TLC plates were developed in TLC chamber. Thin layer chromatogram was visualized under 254/366 nm UV light and after keeping in Iodine Chamber.

## RESULTS

### Organoleptic features of the leaves (Figure 1)

Type - Simple leaf
Colour - Upper surface: dark green and lower surface: light green
Odour - lemon like smell
Taste - bitter
Size - 1- 2 meter long and 5- to 10 mm wide.
Shape - leaf blades linear & tapered to both ends, sheath terete
Margin - entire
Surface - flat, very coarse
Venation - parallel

### Microscopic Evaluation

Leaf is dorsiventral with prominent midrib, smooth adaxial surface and rehivate abaxial surface. The lamina is 80-90 µm thick. It consists of prominent adaxial epidermis which has radical oblong thin walled cells. Some of the adaxial epidermal cells are dilated into large circular or radially wedge shaped cells called bulliform cells or motor cells (Figure 2 and 3). The abaxial epidermis is thin and the cells are thick walled, the outer tangential walls form semicircular papillate projection (Figure 3).

The mesophyll tissue is not differentiated into palisade cells and spongy parenchyma. The vascular bundles

**Figure 1:** Leaf of Cymbopogon citratus.

of the lateral veins are equal in size and occur at uniform intervals in a horizontal row. The vascular bundles are surrounded by radiating cylindrical chlorenchyma cells, which represent the palisade mesophyll (Figure 3), in between the vascular bundles, these are vertical band cells; the cells beneath adaxial epidermis is dilated and hyaline, other cells after vertical band are chlorenchymatous.

The vascular bundles are collateral with adaxial group of a few xylem elements and phloem elements. The vascular bundle of the smaller vein is surrounded by a ring of circular dilated cells. Each bundle is capped by small group of fibres both on the upper and lower ends.

The larger vein has two wide, circular metaxylem elements and one or two protoxylem elements. A prominent circular mass phloem is seen in between the metaxylem elements, the entire bundle is unsheathed by thick sclerenchyma cells (Figure 2).

## Midrib (Figure 4 and 5).

The midrib is concavo convex in transactional view. The adaxial side is broadly concave and the abaxial side is convex.

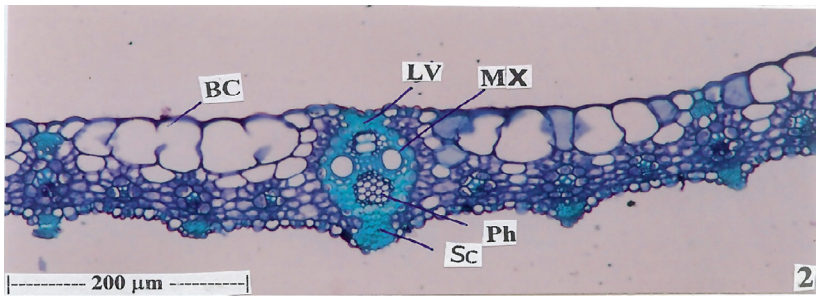


**Figure 2:** Transverse section of leaf through lamina. [BC: Bulliform cells Lv: lateral vein MX: Metaxylem, Ph: phloem, Sc: sclerenchyma.]

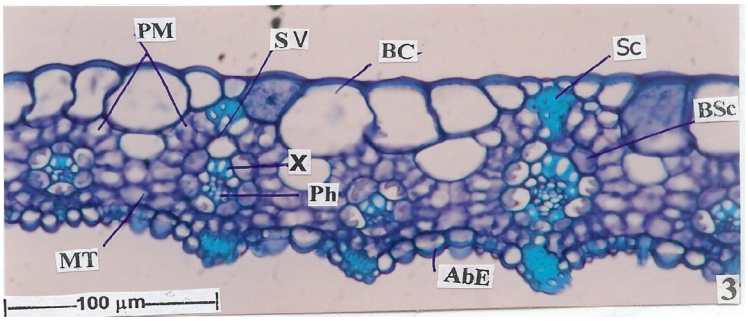


**Figure 3:** Transverse section of leaf through epidermis. [AbE: Abaxial epidermis, BC: Bulliform cells, BSc: Bundle sheath cells MT: Mesophyll tissue, Ph: phloem, PM: palisade mesophyll, Sc: sclerenchyma, Sv: Smaller vascular bundle, X: Xylem]

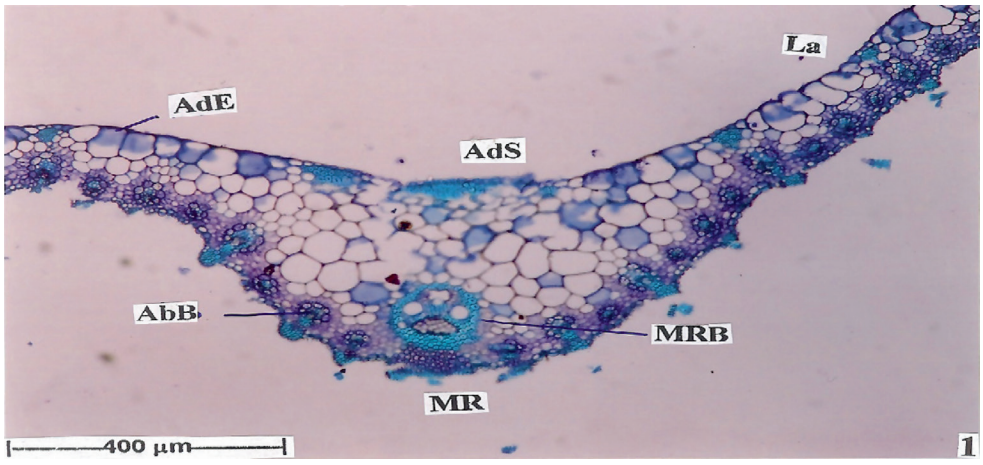


**Figure 4:** Transverse section of leaf through midrib. [Abp: Abaxial bundle, AdE: Adaxial epidermis, AdS: Adaxial slide, La: Lamina, MR: Midrib, MRB: Midrib bundle.]

The midrib is 380 µm in vertical plane and 650 µm in horizontal plane and thus the midrib is cradle shaped in sections. The upper surface of the midrib is smooth while the lower surface has small conical ridges, opposite to the smaller vascular bundle (Plate 5). The epidermal layer in the region of adaxial midrib is thin and less prominent. The abaxial epidermis is thin, but district with spindle shaped cells and thick cuticle.

The ground tissue is parenchymatous, fairly thick walled angular and compact. The vascular system of the midrib consists of a central median larger vascular bundle placed towards the basal part; these are several smaller vascular bundles placed all along to basal abaxial part (Figure 4). The larger median bundle has two wider metaxylem elements and three protoxylem elements, phloem occurs in wide mass in between metaxylem elements. The vascular bundle is surrounded by thick bundle-sheath fibres (Figure 5). The smaller bundles are circular with a small group of xylem and bundle sheath parenchyma cells. A prominent mass of fibres can be seen beneath each smaller vascular bundle.

## Powder microscopy

The leaf pieces were cleared and made transparent by treating then with chloral hydrate. These leaf fragments exhibit several thin and thick, straight parallal lateral veins. In the witercostal regions are seen epidermal cells and stomata. The adaxial epidermis is apostomatic (without stomata). The epidermal cells are vertically rectangular and thin walled (Figure 6).

The abaxial epidermis is stomataferous. The epidermal cells are narrow, rectangular and the anticlinal walls are thick and undiluted (Figure 7 and 8). The stomata occur in regular, parallel longitudinal rows. The stomata are paracytic type with two bracket shaped subsidiary cells lying parallel to the guard cells.

The epidermal tissues were stained with Toluidine blue in order to show the lignification of the cells. The vascular strands of the veins and guard cells stained blue, showing that they have lignified walls. The stomatal number is 700-800/mm$^2$.

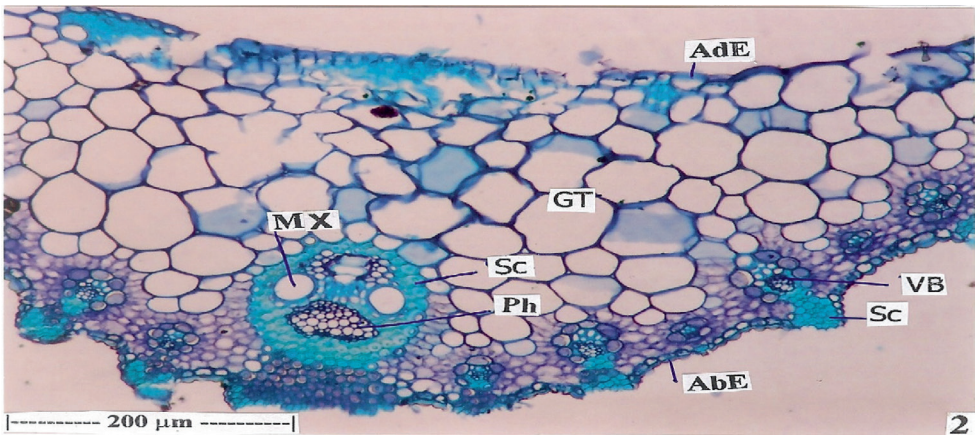


**Figure 5:** Transverse section of leaf through midrib. [AdE: Adaxial epidermis MX: Metaxylem, Sc: Sclerenchyma, GT: Ground tissue, Ph: Phloem, VB: Vascular bundle. AbE: Abaxial epidermis.]

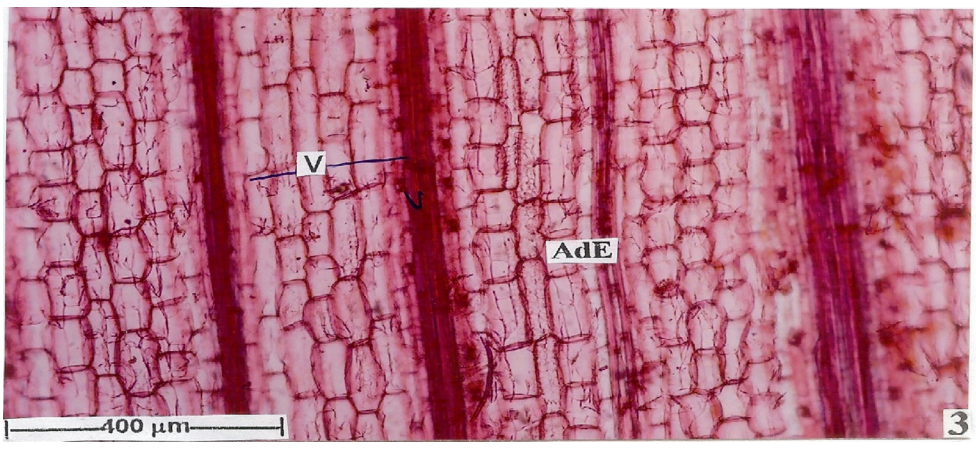


**Figure 6:** Powder Microscopy of leaf shows the adaxial epidermis is apostomatic (without stomata).[ AdE: Adaxial epidermis V: Vessel.]

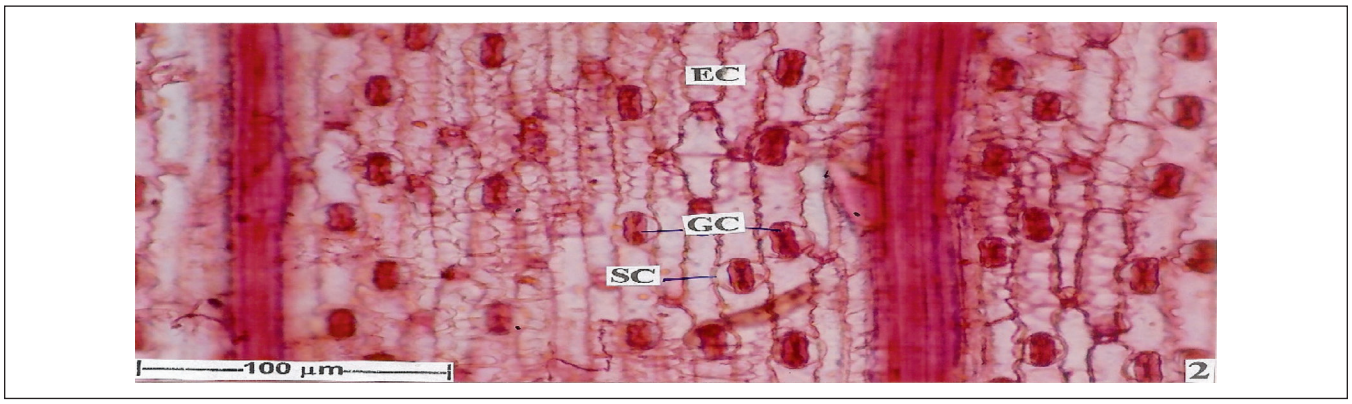


**Figure 7:** Powder Microscopy of leaf shows the epidermal cells are narrow, rectangular and the anticlinal walls are thick and undiluted. [GC: Guard cells, SC: Subsidiary cells, EC: Epidermal cells.]

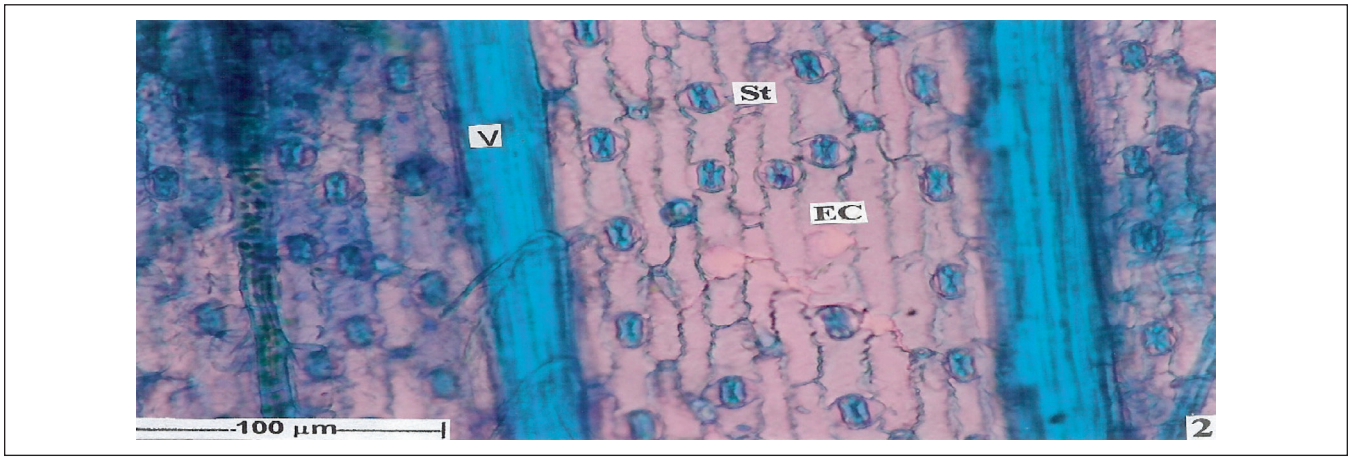


**Figure 8:** Powder Microscopy of leaf shows the epidermal cells vessels and stomata. [St-Stomata, V-Vessels, EC-Epidermal cells]

## Determination of Leaf constants

The surface parameters of leaves of *Cymbopogon citratus* were measured (Table 1).

## Physical Evaluation

The physical parameters of powdered leaves of *Cymbopogon citratus* were evaluated (Table 2).

## Preliminary Phytochemical Screening

The preliminary phytochemical investigation of the petroleum ether (60-80°C), chloroform and methanol and water extracts of *Cymbopogon citratus* leaves shows the presence of carbohydrates, proteins, saponins steroids, flavonoids, phenolic compounds and tannins (Table 3).

## Thin layer chromatography

TLC of the methanolic extract on silica gel G using n-butanol: acetic acid: water (4:1:5) under UV (366 nm) shows one fluorescent zone at Rf value of 0.91 (violet). On exposure to iodine vapour, three spots appeared at Rf values of 0.76, 0.47, 0.21 (all yellow). On spraying with 5% methanolic-sulphuric acid reagent and heating the plate at 105°C for ten minutes a single spot appears at Rf 0.91 (grey).

**Table 1: Surface data of leaves of *Cymbopogon citratus*.**

| Leaf constant | Value per sq mm |
|---|---|
| Stomatal No | 700-800/mm$^2$ |
| Stomatal Index | 29.4 |

**Table 2: Physical parameters of leaves of *Cymbopogon citratus*.**

| Physical Parameter | % (Air dried drug) |
|---|---|
| Loss On Drying | 18.9 |
| Total Ash | 11.2 |
| Acid Insoluble Ash | 4.6 |
| Ethanol Soluble Extractives | 7.8 |
| Water Soluble Extractives | 11.5 |

**Table 3: Phytochemical screening of leaves extracts of *Cymbopogon citratus*.**

| Plant constituent | Extracts | | | |
|---|---|---|---|---|
| | Petroleum Ether Extract | Chloroform Extract | Methanol Extract | Aqueous Extract |
| 1) Alkaloids | – | – | – | – |
| 2) Carbohydrate | – | – | + | + |
| 3) Saponins | – | – | + | + |
| 4) Proteins and Amino acids | – | – | + | – |
| 5) Triterpenoids | – | + | – | – |
| 6) Phenolic compounds and tannins | – | – | + | + |
| 7) Phytosterols | – | + | – | – |
| 8) Flavonoids | – | – | + | – |

## DISCUSSION

As a part of standardization, the macroscopical examination of leaves of *Cymbopogon citratus* 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 fruits of plant can serve as diagnostic parameters. The microscopic evaluation of fruits of *Elaeocarpus sphaericus* and extractive values, ash values and loss on drying of the powdered drug and phytochemical screening of the extract have been carried out which would be of considerable use in the identification of this drug. Percentages of the extractive values, ash value and loss on drying were calculated with reference to the air-dried drug. The percent extractives in different solvents indicate the quantity and nature of constituents in the extracts. The extractive values are also helpful in estimation of specific constituents soluble in particular solvent. Thin layer chromatography (TLC) was examined in short UV (254 nm) and long UV (366 nm) which is particularly valuable for the preliminary separation and determination of plant constituents. This finding is useful to supplement the existing information with regard to identification and standardization of *Cymbopogon citratus* even in the powdered form of the plant drug to distinguish it from drug and adulterant. These studies also suggest that the observed pharmacognostic and physiochemical parameters are of great value in the quality control and formulation development.

## CONCLUSION

The present study may be useful to supplement the information with regard to its standardization and identification and in carrying out further research and its use in traditional system of medicine.

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# HPTLC method Development & Validation for quantification of Markers of Dhatrinisha churna

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## ABSTRACT

**Introduction:** Dhatrinisha churna has been traditionally used in the Ayurvedic system of medicine and by traditional medical practices of India to treat hyperlipidemia. A sensitive, reliable, selective, precise and accurate densitometric High Performance Thin Layer Chromatography (HPTLC) fingerprinting method has been developed for the simultaneous determination and quantification of Curcumin and Ellagic acid in Dhatrinisha churna. **Method:** Quantification of Curcumin and Ellagic acid were done by developed densitometric HPTLC method. Validation of method performs in order to demonstrate its selectivity, accuracy, precision, repeatability and recovery study. **Result:** All calibration curves showed good linear correlation coefficients ($r^2 > 0.997$) within the tested ranges. The chromatogram of Dhatrinisha churna was quantified with respect to Curcumin (1.072% w/w) and Ellagic acid (0.867% w/w). Intra-and inter-day RSDs of retention times and peak areas were less than 1.92%. The recoveries were between 96.60 and 101.40%. **Conclusion:** A method has been developed for the simultaneous quantification of two markers in Dhatrinisha churna. The proposed HPTLC method was found to be simple, précised and accurate and can be used for the quality control of the raw materials as well as formulations.

**Key words:** Dhatrinisha churna; Curcumin; Ellagic acid; HPTLC

## INTRODUCTION

Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of the ayurvedic medicines is the lack of standard quality control profiles. The quality of herbal medicine i.e. the profile of the constituents in the final product has implication in efficacy and safety. Due to the complex nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control parameters. To overcome these problems modern analytical techniques are expected to help in circumventing this problem.[1,2]

From such polyherbal formulations separation, identification and estimation of chemical components is very difficult.[3] Literature survey revealed that the above mentioned marker compound have various pharmacological properties. The rhizome and roots of *Curcuma* species are frequently used in cosmetics and spas for skin nourishment. Pharmacological study reveals its various medical activities such as antioxidant, promotion of blood circulation to remove blood stasis and treatment of cancer. The antioxidants are claimed biological active in protecting the body, the skin collagen and elastic tissue against damaging by reactive oxygen species.[4,5] Gallic acid and ellagic acid are hydrolysable tannins and present in a rich variety of plants like in tea, red wine, fruits, beverages and various medicinal plants. Gallic acid is known to have antiinflammatory, antimutagenic, anticancer and antioxidant activity.[6,7] Ellagic acid has been found to exhibit antimutagenic, antiviral, anticancer, antitumor and antioxidant properties, along with whitening of the skin.[8,9]

The advances in chromatographic techniques made it possible to quantify the chemical constituents in a mixture with comparatively little clean-up using high performance thin layer chromatography (HPTLC).[10] Present study deals with development and validation of methods for quantification of some of the important marker compounds viz. Curcumin and Ellagic acid in Dhatrinisha churna.

Dhatrinisha churna is an Ayurvedic preparation mentioned in the *Chikisthasthan,* Chapter-II, Slock-8 of ayurvedic literature *Susrut Samhita* and Chater-6, Slock-26, 772 of Ayurvedic literature *Charak Samhita*[11] for the treatment of Hyperlipidemia. Dhatrinisha churna has been also used by

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traditional medical practices of India to treat hyperlipidemia. It consists of the mixture of the fine powder of the dried rhizome of *Haridra* (*Curcuma longa* Linn., F.- Zingiberaceae) and dried fruit of *Amalaki* (*Emblica officinalis Gaertn.* Syn. *Phyllanthus emblica* Linn. F. – Euphorbiaceae). Traditionally it is widely used for the treatment of hyperlipidemia and in diabetes. Curcumin[12] and Ellagic acid[13] are the main active markers present in the *Haridra* and *Amalaki* respectively and are mainly responsible for their aid pharmacological action.

In the present investigation, we have developed simple, optimized and validated HPTLC method for the standardization of Dhatrinisha churna. Two chemical markers were selected, one from each medicinal herbs used as raw materials. The method was validated on the basis of its selectivity, linearity, precision, accuracy, limit of detection and limit of quantification according to ICH requirements.

## MATERIALS AND METHODS

### Plant Materials

Individual components of Dhatrinisha churna were procured from a Yucca enterprise, Mumbai, Maharashtra and authenticated by comparison with herbarium specimens. The drugs were cleaned, dried and powdered separately and passed through 40 # sieve. These both powders were mixed well in equal proportion uniformly.

### HPTLC method development

Optimum chromatographic conditions were obtained after running different mobile phase. Many different mobile phase and scanning wavelength were tried for the best separation of peaks. Chromatography was performed at 25 ± 2°C, relative humidity 40%, on 10 cm × 10 cm aluminum foil HPTLC plates coated with 0.2 mm layers of silica gel 60F254 (E. Merck). Solutions (5 µL) were applied to the plates as bands 6 mm wide by use of a CAMAG (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100 µL syringe (Hamilton, Bonaduz, Switzerland). Linear ascending development to approximately 80 mm from the point of application was performed with toluene : ethyl acetate : methanol : formic acid (16: 14: 1: 4 v/v) as mobile phase in a CAMAG 20 cm × 10 cm × 4 cm glass twintrough chamber previously saturated with mobile phase vapor for 25 min. After development, the plate was dried in hot air oven at 105°C for 5 min. Densitometric scanning in absorbance mode at 330 nm was then performed by use of a CAMAG TLC scanner-III linked to Wincats software (V 1.4.3.6336). The slit dimensions were 5.00 mm × 0.45 mm and the scanning speed 20 mm s–1. For calibration, stock solutions of Curcumin and Ellagic acid of different concentration (100-600 ng/band) were applied to the HPTLC plate to prepare a calibration plot and to check for reproducibility. Peak areas were recorded and calibration plots were prepared by plotting average peak area against concentration. Peak area and amount of standards data were treated by linear least-square regression analysis.

### Method Validation

The method was validated according to ICH[14] guideline for linearity, precision, accuracy, selectivity, limit of detection and limit of quantification. Selectivity was checked using an extract of Dhatrinisha churna and a mixture of standards in order to optimize separation and detection. Linearity of the method was performed by analyzing a standard solution of markers by the proposed method in the concentration range 100-600 ng/spot. The accuracy of the proposed method was determined by a recovery study, carried out by adding standard markers in the Dhatrinisha churna. The samples were spiked with three different amounts of standard compounds prior to extraction. The spiked samples were extracted in triplicate and analyzed under the previously established optimal conditions. The obtained average contents of the target compounds were used as the actual values in order to calculate the spike recoveries. Precision was determined by repeatability and interday and intraday reproducibility experiments of the proposed method. A standard solution containing two markers was injected six times; Dhatrinisha churna was also extracted six times to evaluate the repeatability of the extraction process. The mean amounts and standard deviation (SD) value of each constitute were calculated. The Limit of Detection (LOD) and Limit of Quantification (LOQ) of markers compounds were calculated at signal-to-noise ratio of approximate 3:1 and 10:1 respectively.

## RESULT

### Method development and Quantification

TLC densitometric methods were developed using HPTLC for the quantification of two marker compounds from Dhatrinisha churna. Solvent systems were optimized to achieve best resolution of the marker compounds from the other components of the sample extracts. Several methods tried to seperat out markers from extract such as Choloform: Acetic acid: Methonol (80: 5: 15), Ethyl acetate: Formic acid: Acetic acid (100: 11: 11) etc. Of the various solvent system tried, the one containing toluene: ethyl acetate: methanol: formic acid (16: 14: 1: 4) gave best resolution of curcumin ($R_f$ = 0.74) and ellagic acid ($R_f$ = 0.51) in the presence of other compounds in the sample extract and enabled the quantification of marker compounds. The amount of markers found in Dhatrinisha churna were

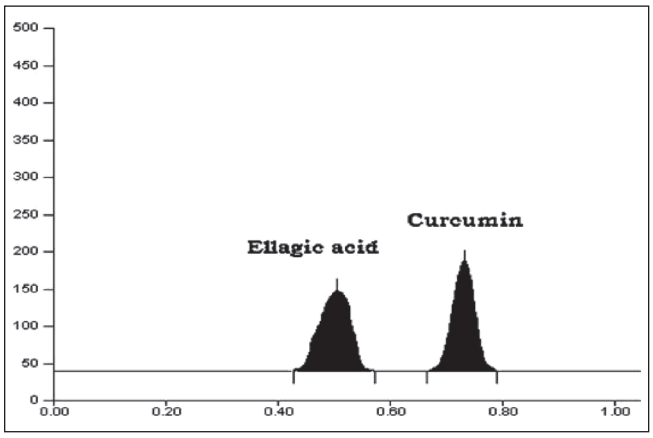


**Figure 1:** HPTLC chromatogram of Curcumin and Ellagic acid.

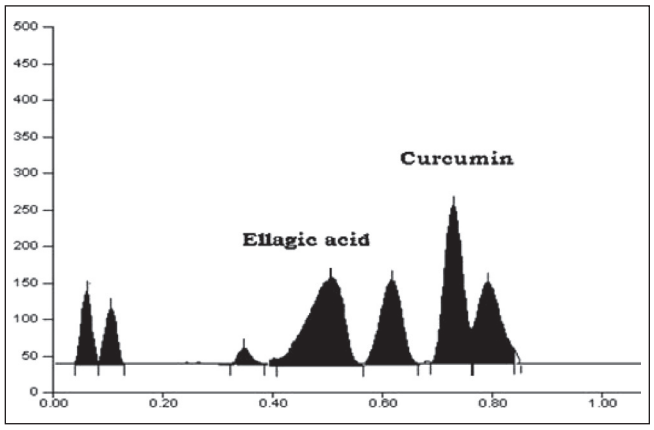


**Figure 2:** HPTLC chromatogram of Dhatrinisha churna.

**Table 1: Regression Parameter, Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ) of the Proposed HPTLC Method for Dhatrinisha churna**

| Markers | Conc. range (ng/spot) | $R_f$[a] | Regression equation | $R^2$ | LOD (ng/spot) | LOQ (ng/spot) |
|---|---|---|---|---|---|---|
| Curcumin | 100-600 | 0.74 ± 0.03 | Y = 47.296 + 0.85X | 0.999 | 24 | 36 |
| Ellagic acid | 100-600 | 0.51 ± 0.04 | y = 21063x + 733.6 | 0.997 | 25 | 72 |

[a]Mean ± SD (n=6)

**Table 2: Repeatability and Recovery Tests for the Two Markers in Dhatrinisha Churna**

| Markers | Contents[a] (mg/g) | Added amount (mg) | Recorded amount[a] (mg) | Recovery rate[a] (%) | RSD (%) |
|---|---|---|---|---|---|
| Curcumin | 10.72 ± 0.04 | 5 | 15.550 ± 2.04 | 96.60 ± 2.04 | 2.37 |
| | | 10 | 20.667 ± 0.71 | 99.50 ± 0.71 | 2.50 |
| | | 15 | 25.930 ± 1.55 | 101.40 ± 1.55 | 2.30 |
| Ellagic acid | 8.67 ± 0.02 | 4 | 12.558 ± 1.08 | 97.20 ± 1.08 | 1.65 |
| | | 8 | 16.774 ± 2.30 | 101.30 ± 2.30 | 1.12 |
| | | 12 | 20.662 ± 0.71 | 99.93 ± 0.71 | 2.12 |

[a] Mean ± SD (n=3)

1.072 ± 0.04% w/w (curcumin) and 0.867 ± 0.02% w/w (Ellagic acid) respectively. The HPTLC chromatogram obtained for Markers and Dhatrinisha churna are shown in Figure 1 & 2 respectively.

## Method Validation

The HPTLC method was validated by defining the selectivity, linearity, accuracy, precision, limits of detection and limit of quantification. For qualitative purposes, the method was evaluated by taking into account the precision in the $R_f$ and selectivity of marker compounds. A high repeatability in the $R_f$ time was obtained for both, standards and extracts even at high concentration. For quantitative purpose linearity, accuracy, precision LOD and LOQ were evaluated. LOD and LOQ values for Curcumin 24 ng/spot and 36 ng/spot & for Ellagic acid 25 ng/spot and 72 ng/spot respectively. Linear correlation was obtained between peak area and concentration of two markers in the range of 100-600 ng/spot. Values of the regression coefficients ($r^2$) of the markers were higher than 0.99,

**Table 3: Precision of the Intra-day and Inter-day HPTLC Measurement for two Markers in Dhatrinisha Churna**

| Compound | Intra-day[b] | | Inter-day[c] | |
|---|---|---|---|---|
| | Contents[a] (% w/w) | RSD (%) | Contents[a] (% w/w) | RSD (%) |
| Curcumin | 1.072 ± 0.04 | 0.92 | 1.072 ± 0.07 | 0.93 |
| Ellagic acid | 0.867 ± 0.02 | 1.92 | 0.867 ± 0.04 | 1.88 |

[a]Mean ± SD (n=6)
[b]Sample were analyzed six times a day
[c]Sample were analyzed once a day over six consecutive days

thus confirming the linearity of the methods. The high recovery values (96.60-101.40%) indicated a satisfactory accuracy. Relative standard deviation of all the parameters was less than 2.5% for the degree of repeatability, indicating the high repeatability of the proposed method. The low coefficient of variation values of intraday and interday precision reveals that the proposed method is precise. The result of validation parameters were shown in Table 1-3.

Therefore, this HPTLC method can be regarded as selective, accurate and precise.

## CONCLUSION

The results indicate that Dhatrinisha churna contains a number of markers that may be responsible for its therapeutic activity. The developed HPTLC method will assist in the standardization of Dhatrinisha churna using biologically active chemical markers. The proposed HPTLC methods for simultaneous estimation of Curcumin and Ellagic acid from Dhatrinisha churna seems to be accurate, precise, reproducible and repeatable. Dhatrinisha churna also contained a number of other constitute, which are currently the subject of further investigation, apart from those standards studied. With the growing demand for herbal drugs and with increased belief in the usage of herbal medicine, this standardization tool will help in maintaining the quality and batch to batch consistency of this important Ayurvedic preparation.

## ACKNOWLEDGMENTS

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# Reversal of memory deficits by ethanolic extract of *Mimusops elengi* Linn. in mice

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## ABSTRACT

**Background:** Management of cognitive disorders like dementia and Alzheimer's disease has been challenging since no potential drug is available with proved efficacy. Some nootropic drugs like piracetam, aniracetam and cholinesterase inhibitors such as Donepezil® have found to exhibit severe toxic effects in elderly. **Objective:** The present study was designed to investigate the reversal of memory deficits by ethanol extract of *Mimusops elengi* Linn. in mice. **Methods:** *M. elengi* [100 and 200 mg/kg] was administered orally for 8 successive days to both young and aged mice. Elevated plus maze and Passive avoidance paradigm were employed to assess short term and long term memory respectively. Light and dark box test, Open field test and Social interaction test were used to assess the possible anxiolytic potentials of *M. Elengi.* To delineate the possible mechanism through which *M. elengi* elicits the anti-amnesic effect, we investigated its influence on central cholinergic activity by estimating the whole brain acetylcholinesterase activity. **Results:** *M. elengi* [100 and 200 mg/kg, p.o.] significantly attenuated amnesic deficits induced by diazepam [1 mg/kg, i.p.], scopolamine [0.4 mg/kg, i.p.] and natural aging. *M. elengi* [100 and 200 mg/kg] decreased transfer latencies and increased step down latencies significantly in the aged mice. It also reversed amnesia induced by diazepam and scopolamine in young mice. *M. elengi* exhibited significant anxiolytic activity in mice. It also decreased whole brain acetyl cholinesterase activity significantly. **Conclusion:** *M. elengi* can be useful in restoring memory in the treatment of various types of dementia.

**Key words:** acetylcholine- anxiety- memory- *Mimusops elengi* - scopolamine.

## INTRODUCTION

Memory is vulnerable to a variety of pathologic processes including neurodegenerative diseases, strokes, tumors, head trauma, hypoxia, cardiac surgery, malnutrition, attention deficit disorder, depression, anxiety, the side effects of medication, and normal ageing[1]. As such, memory impairment is commonly seen by physicians in multiple disciplines including neurology, psychiatry, medicine, and surgery[2]. Memory loss is often the most disabling feature of many disorders, impairing the normal daily activities of the patients and profoundly affecting their families. The key features of these dreaded disorders are memory impairments, deterioration of language, visuospatial, motor, sensory abnormalities, gait disturbance and seizures. There are around 30 million patients suffering from Alzheimer's disease (AD) which is the major cause of dementia, all over the world[3]. In India, AD patients are estimated to be around 3 million[4]. Presently, there are no satisfactory diagnostic procedures and therapeutic regimens available for the management of these cognitive disorders. Despite the severity and high prevalence of these diseases, Allopathic system of medicine is yet to provide a satisfactory remedy. Therefore, neurobiologists all over the world are looking for new directions and alternative strategies for managing cognitive disorders. Cognitive disorders involve disturbance in thinking or memory that represent a marked change from the individual's prior level of functioning[5].

AD is a neurodegenerative disorder affecting major brain areas including the cortex and limbic system, and is characterized by progressive decline in memory with impairment of at least one other cognitive function[6-7]. AD often begins with symptoms like short-term memory loss, and continues with more widespread cognitive and

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emotional dysfunction. So-called late-onset AD (LOAD) occurs after age 65. AD features ongoing deterioration of patients' functioning which results in substantial and long-lasting disability over the approximate 7–10 years from diagnosis to eventual death[8]. Although AD usually shows no symptom on motor or sensory alterations, certain atypical clinical presentations (such as spastic paraparesis) are occasionally found in some patients[9-10].

The most common cause of dementia in the elderly is probably Alzheimer's disease [AD], a chronic, progressive disabling organic brain disorder characterized by disturbance of multiple cortical functions, including memory, judgment, orientation, comprehension, learning capacity and language. Nootropic agents like, Piracetam and Cholinesterase inhibitors like, Donepezil® are commonly used for improving memory, mood and behavior. Anticholinesterases such as Metrifonate[11], Physostigmine, Tacarine, Donepezil[12], Huperzine-A[13-14], Rivastigmine[15], Galanthamine[16] and Eptastigmine[17] have all been shown to reverse amnesia produced by disruption of cholinergic system. Enzyme choline acetyltransferase is involved in the synthesis of acetylcholine and acetylcholinesterase is involved in the degradation of acetylcholine. In the present study,

Plant extracts of *Zingiber officinale*[18], *Nardostachys jatamansi*[19], *Foeniculum vulgare*[20], *Hibiscus sabdariffa*[21], *Ocimum sanctum*[22], and *Desmodium gangeticum*[23], *Piper nigrum*[24], *Glycyrrhiza glabara*[25-26] have been found to posses nootropic effects and they had significantly lowered the whole brain AChE activity thereby elevating acetylcholine levels in the brain.

Piracetam was the first nootropic agent discovered for its antimyoclonic action, effects after stroke and in mild cognitive impairment. Levetiracetam, fosracetam, nefiracetan, pramiracetam, nebracetam and oxiracetam are in various stages of licensing and investigation[27]. However, the resulting adverse effects of these drugs such as diarrhea, insomnia, nausea, bronchitis, loose stools, muscular cramps and other known side effects[28], have made their use limited and it is worthwhile to explore the utility of traditional medicines in the treatment of various cognitive disorders.

*Mimusops elengi* L. [Sapotaceae] is known as bakula in ayurveda[29]. It is a small to large evergreen tree found all over India and is cultivated in gardens as an ornamental tree and is used in the ayurvedic system of medicine for the treatment of various neurological disorders[30-31]. Stem bark of *Mimusops elengi* possesses cardiotonic, stomachic, anthelmentic and astringent properties[32]. The bark powder along with 50 g alum, 5 g sodium chloride, is warmed and used for massaging on teeth in the treatment of pyorrhea by the locals[33]. The fine powder is sniffed to relieve headache, the decoction is used as a general tonic and flower in perfumery[34]. Phytochemical review of the bark of *M. elengi* reveals the presence of taraxerol, taraxerone, ursolic acid, betulinic acid, quercitol, lupeol[35], alkaloid isoretronecyl tiglate and mixture of triterpenoid saponins[36,37]. *M. elengi* is reported to possess anti-ulcer[38] and hypotensive[39] activities. The present study was undertaken to evaluate the effects of ethanol extract of bark of *M. elengi* on scopolamine and ageing induced amnesia in mice.

## MATERIALS AND METHODS

The stem bark of *Mimusops elengi* [ME] was collected from mature trees growing in Gullarghati, Dehradun, Uttaranchal and identified at Department of Pharmacognosy, SBS Institute of Biomedical Sciences and Research, Balawala, Dehradun. A voucher specimen [HKJ/ME-41] has been deposited in the Department. The bark was dried, cleansed and powdered. One kilogram of moderately powdered bark of ME was extracted by refluxing with 90% ethanol in soxhlet extractor for 8-10 h. The extract was evaporated to dryness under reduced pressure and temperature using rotary vacuum evaporator. The yield of dry extract from the crude powder of ME was 12 % w/w. The ethanol extract of ME was suspended in a mixture of Tween 80: Distilled Water in a ratio of 2:8. The suspension was orally administered to animals. The volume of administration was 1 ml/100 g, body weight of mice.

## DRUGS AND CHEMICALS

Scopolamine hydrobromide [Sigma Aldrich, USA] and piracetam [Nootropil®, UCB India Pvt. Ltd., Vapi, Gujarat] were diluted in normal saline and injected intraperitoneally. Phenytoin [Dilantin® suspension, Parke Davis] was administered orally. Volume of administration was 1ml/ 100 g. All the drugs were administered in the morning session i.e. 8 AM- 9 AM on each day.

## ANIMALS

Swiss mice of either sex weighing around 18 g [younger ones, aged 8 weeks] and 25 g [older ones, aged 28 weeks] were used in present study. Animals were procured from disease free animal house of CCS Haryana Agriculture University, Hisar [Haryana, India]. They were acclimatized to the laboratory conditions for 5 days before behavioral studies. The animals had free access to food and water and maintained under 12:12 h light and dark cycles. Institutional Animals Ethics Committee [IAEC] approved the experimental protocol and care of animals was taken as per guidelines of CPCSEA, Dept. of Animal Welfare, Govt. of India.

### Administration of ME

The ethanol extract of *M. elengi* [ME] at different doses [50-2000 mg/kg] was administered orally to mice with the help of a specially designed oral needle connected to a polythene tube. ME was administered at the same time on each day [i.e. 8 AM-9 AM]. During the first four hours after the drug administration, the animals were observed for gross behavioral changes if any for 7 days. The parameters such as hyperactivity, grooming, convulsions, sedation, hypothermia, mortality were observed and doses selected for further studies were 100 mg/kg and 200 mg/kg.

### Locomotor function

Locomotor activity of control and drug-treated animals was measured with the help of a photoactometer (INCO, Ambala, India)[40].

### Elevated plus-maze

Elevated plus-maze served as the exteroceptive behavioral model to evaluate learning and memory in mice. The procedure, technique and end point for testing learning and memory was followed as reported earlier[41,42,43]. The elevated plus maze for mice consisted of two open arms [16 cm × 5 cm] and two covered arms [16 cm × 5 cm × 12 cm] extended from a central platform [5 cm × 5 cm], and the maze was elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an open arm, facing away from the central platform. Transfer latency [TL] was defined as the time taken by the animal to move from the open arm into one of the covered arms with all its four legs. TL was recorded on the first day for each animal. The mouse was allowed to explore the maze for another 2 minutes and then returned to its home cage. Retention of this learned-task was examined 24 h after the first day trial.

### Passive shock avoidance paradigm

Passive avoidance behavior based on negative reinforcement was recorded to examine the long-term memory. The apparatus consisted of a box [27 × 27 × 27 cm] having three walls of wood and one wall of Plexiglas, featuring a grid floor [3 mm stainless steel rods set 8 mm apart], with a wooden platform [10 × 7 × 1.7 cm] in the center of the grid floor. The box was illuminated with a 15 W bulb during the experimental period. Electric shock [20V AC] was delivered to the grid floor. Training was carried out in two similar sessions. Each mouse was gently placed on the wooden platform set in the center of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, shocks were delivered for 15 sec and the step-down latency [SDL] was recorded. SDL was defined as the time taken by the mouse to step down from wooden platform to grid floor with its entire paw on the grid floor. Animals showing SDL in the range [2-15 sec] during the first test were used for the second session and the retention test. The second-session was carried out 90 min after the first test. When the animals stepped down before 60 sec, electric shocks were delivered for 15 sec. During the second test, animals were removed from shock free zone if they did not step down for a period of 60 sec. Retention was tested after 24 h in a similar manner, except that the electric shocks were not applied to the grid floor. Each mouse was again placed on the platform, and the SDL was recorded, with an upper cut-off time of 300 sec[44-45].

## COLLECTION OF BRAIN SAMPLES

The animals were sacrificed by cervical decapitation under light anesthesia on the 8th day, 90 mins after administration of the last dose of ME. Immediately after decapitation whole brain was carefully removed from the skull. For preparation of brain homogenate, the fresh whole brain was weighed and transferred to a glass homogenizer and homogenized in an ice bath after adding 10 volumes of 0.9% w/v sodium chloride solution. The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant liquid was used for estimation of brain acetylcholinesterase activity.

### Estimation of brain acetyl cholinesterase [AChE] activity

The time frame of cholinesterase activity estimation was similar to behavioral tests i.e. 8 AM- 11 AM on each day. On the 9th day the animals were euthanized by cervical dislocation carefully to avoid any injuries to the tissue. The whole brain AChE activity was measured using the Ellman method[46]. The end point was the formation of yellow color due to the reaction of thiocholine with dithiobisnitrobenzoate ions. The rate of formation of thiocholine from acetylcholine iodide in the presence of tissue cholinesterase was measured using a spectrophotometer. The sample was first treated with 5, 5'-dithionitrobenzoic acid [DTNB] and the optical density [OD] of the yellow colour compound formed during the reaction at 412 nm every minute for a period of three minutes was measured. Protein estimation was done using Folin's method. AChE activity was calculated using the following formula:

$$R = \frac{\delta \text{ O.D.} \times \text{Volume of Assay [3 ml]}}{E \times \text{mg of protein}}$$

Where R= rate of enzyme activity in 'n' mole of acetylcholine iodide hydrolyzed/min/mg protein; δ O.D. = Change in absorbance/min; E = Extinction coefficient = 13600/M/cm.

### Light and dark box test

The apparatus consisted of a rectangular box (45 × 27 × 27cm), partitioned into two compartments connected by

a 7.5 × 7.5 cm opening in the wall between compartments. An animal was placed in the center of the light compartment and was observed for 5 min for the time spent in open (white/light) compartment[47]. Percent time spent I the light compartment was determined as follows: % = 100 × number of seconds spent in compartment/300 total seconds (5 min observation time).

### Open field test

The open field test, which provides simultaneous measures of locomotion, exploration and anxiety, was used for this study. The open field is a 400 × 400 × 300 mm arena with thin black block stripes pained across the floor, dividing it into 16 quadratic blocks. Mouse was placed in the center of arena and an observer quantified the spontaneous ambulatory locomotion of each mouse for 5 min. During this period, the number of squares crossed and number of rearing were measured[48].

### Social interaction test

The social interaction arena was an open topped box (22 × 15 × 12 cm). Mice were isolated for 1 h before the test. After introduction to the test arena, the mice were observed for cumulative time spent in genital investigation, sniffing, a partner, following, grooming, kicking, biting, wrestling, climbing over and under, neck licking and boxing[49].

#### *Statistical Analysis*

All the results were expressed as mean ± Standard error. The data was analyzed using ANOVA followed by Tukey-kramer test.

## RESULTS

### Acute toxicity study

No mortality was observed following oral administration of ME even with the highest dose [2000 mg/kg]. ME had no toxic effect on the normal behavior of the mice. However doses more than 1500 mg/kg profuse watery stools.

### Effect on locomotor activity

In the present study, ME (100 and 200 mg/kg) did not show any significant change in the locomotor function of animals (score 219 ± 1.8 and 212 ± 13) as compared to control group (score 215.4 ± 11) when tested using a photoactometer.

### Effect on transfer latency (TL) using elevated plus maze

Aged mice showed higher transfer latency (TL) values on first day and on second day (after 24 hr) as compared to young mice, indicating impairment in learning and memory (i.e. ageing-induced amnesia). Piracetam (200 mg/kg, ip) pretreatment for 8 days decreased transfer latency on 8$^{th}$ day and after 24 hr, i.e. on 9$^{th}$ day as compared to distilled water treated group, indicating improvement in both learning and memory (Fig 1). Scopolamine (0.4 mg/kg) and diazepam (1 mg/kg) increased TL significantly ($P < 0.01$) in young mice on first and second day as compared to control, indicating impairment of memory (Fig 2).

ME (100 mg/kg, po) decreased the TL on 8$^{th}$ day and 9$^{th}$ day in young and aged mice ($P < 0.05$) when compared to control groups. Higher dose of ME (200 mg/kg, po) more significantly enhanced the learning and memory of aged animals rather than the young mice as reflected by marked decrease in TL on 8$^{th}$ day and 9$^{th}$ day when subjected to elevated plus maze tests (Fig 1). The higher dose of ME pretreatment for 8 days successively protected young mice ($P < 0.001$) against scopolamine, diazepam and ageing induced amnesia (Fig 2).

### Effect on SDL using Passive avoidance paradigm

ME [100 and 200 mg/kg, p.o.] profoundly increased step down latency [SDL] significantly as compared to control group on the second day indicating improvement in memory of young mice (Fig 3). Scopolamine hydrobromide [0.4 mg/kg, i.p.] decreased SDL on second day after training, indicated impairment of memory. ME [200 mg/kg, p.o.] administered orally for 8 days significantly [$P < 0.001$] reversed amnesia induced by both scopolamine and natural aging (Fig. 4).

### Effect on whole brain acetylcholinesterase activity

The whole brain AChE activity with phenytoin [12 mg/kg, p.o.] demonstrated significant rise in AChE activity as compared to control and piracetam [200 mg/kg, p.o.]. ME [100 and 200 mg/kg, p.o.] significantly [$P < 0.001$] lowered AChE activity [Fig. 5].

### Effect on Light and dark box test

Diazepam (0.5 mg/kg) significantly increased the time spent in light compartment ($P < 0.001$) compared to normal group (table 1). Significant increase in the time spent in the light compartment $P < 0.05$ was seen with administration of ME [100 mg/kg] and ME [200 mg/kg] as compared to normal.

### Effect on Open field test

ME 200 mg/kg showed good anxiolytic activity as compared with normal mice. There was marked decrease in locomotion activity in animals treated with ME [100 mg/kg] and ME [200 mg/kg] as the number of squares crossed in the perimeter was decreased between the ME [200 mg/kg] treated groups and differed significantly from the control groups (table 2). The frequency of rearing also decreased significantly.

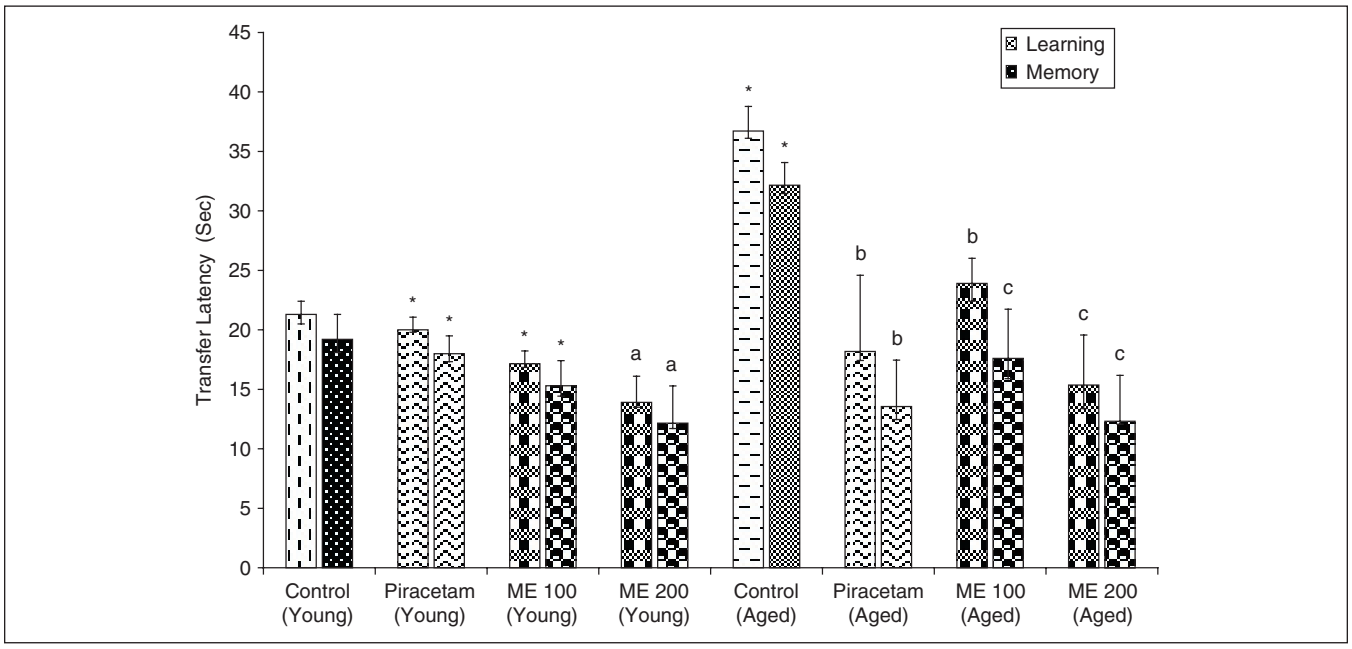


**Figure 1:** Effect of *M. elengi* (ME, 100 and 200 mg/kg, p.o.) on transfer latency of young and aged mice using elevated plus maze.
*Values are mean ± S.E.M. (n=6).*
**indicates P< 0.01 as compared to control group of young mice.*
*[a]indicates P< 0.001 as compared to control group of young mice.*
*[b]indicates P< 0.01 as compared to control group of aged mice.*
*[c]indicates P< 0.001 as compared to control group of aged mice.*
*(One way ANOVA followed by Tukey-kramer multiple comparison tests)*

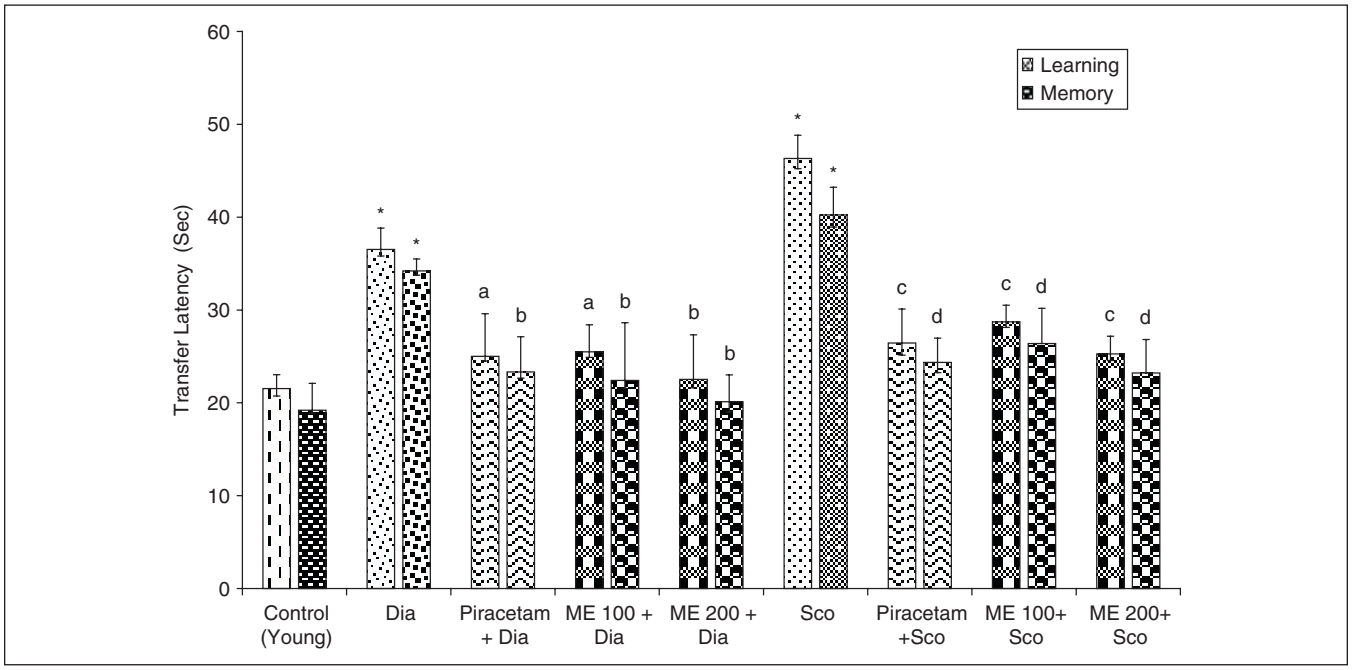


**Figure 2:** Effect of *M. elengi* (ME, 100 and 200 mg/kg, p.o.) on diazepam (Dia, 1 mg/kg, i.p.) and scopolamine (Sco, 0.4 mg/kg, i.p.) induced amnesia in young mice using elevated plus maze.
*Values are mean ± S.E.M. (n=6).*
**indicates P< 0.01 as compared to control group of young mice.*
*[a]indicates P< 0.01 as compared to diazepam (Dia) group alone.*
*[b]indicates P< 0.001 as compared to diazepam (Dia) group alone.*
*[c]indicates P< 0.01 as compared to scopolamine (Sco) group alone.*
*[d]indicates P< 0.001 as compared to scopolamine (Sco) group alone*
*(One way ANOVA followed by Tukey-kramer multiple comparison tests)*

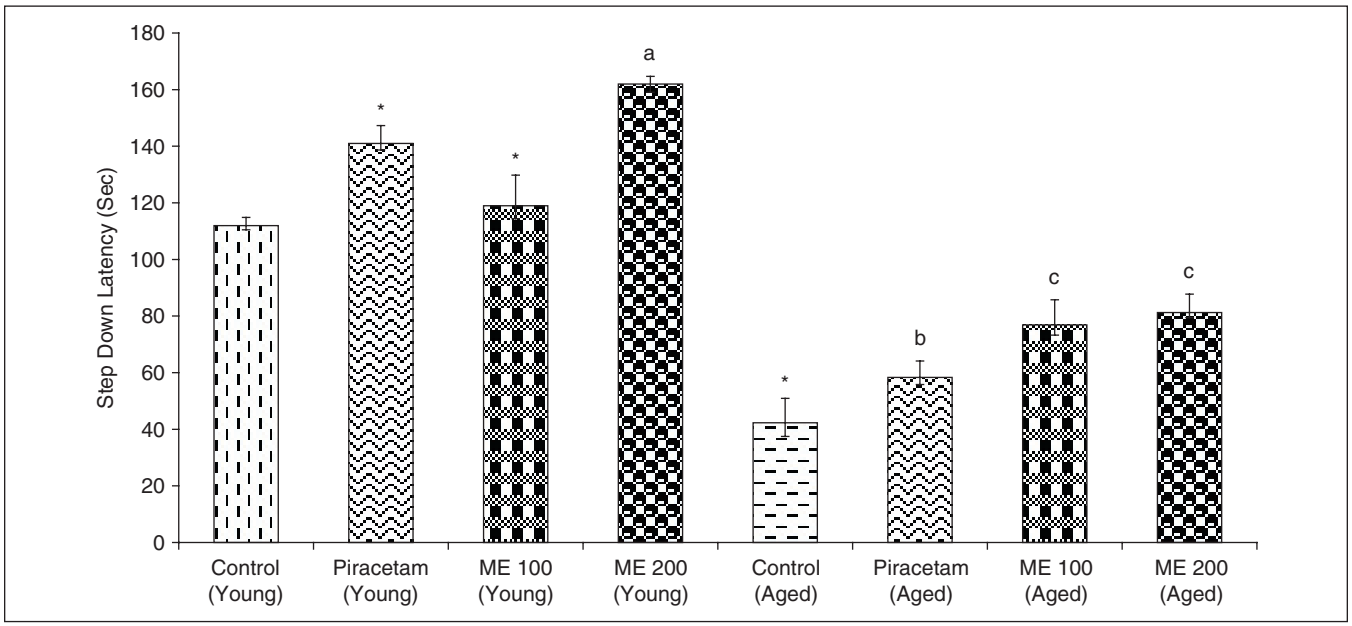


**Figure 3:** Effect of *M. elengi* (ME, 100 and 200 mg/kg, p.o.) on step down latency of young and aged mice using passive avoidance apparatus.
*Values are mean ± S.E.M. (n=6).*
**indicates P< 0.01 as compared to control group of young mice.*
*[a]indicates P< 0.001 as compared to control group of young mice.*
*[b]indicates P< 0.01 as compared to control group of aged mice.*
*[c]indicates P< 0.001 as compared to control group of aged mice.*
*(One way ANOVA followed by Tukey-kramer multiple comparison tests)*

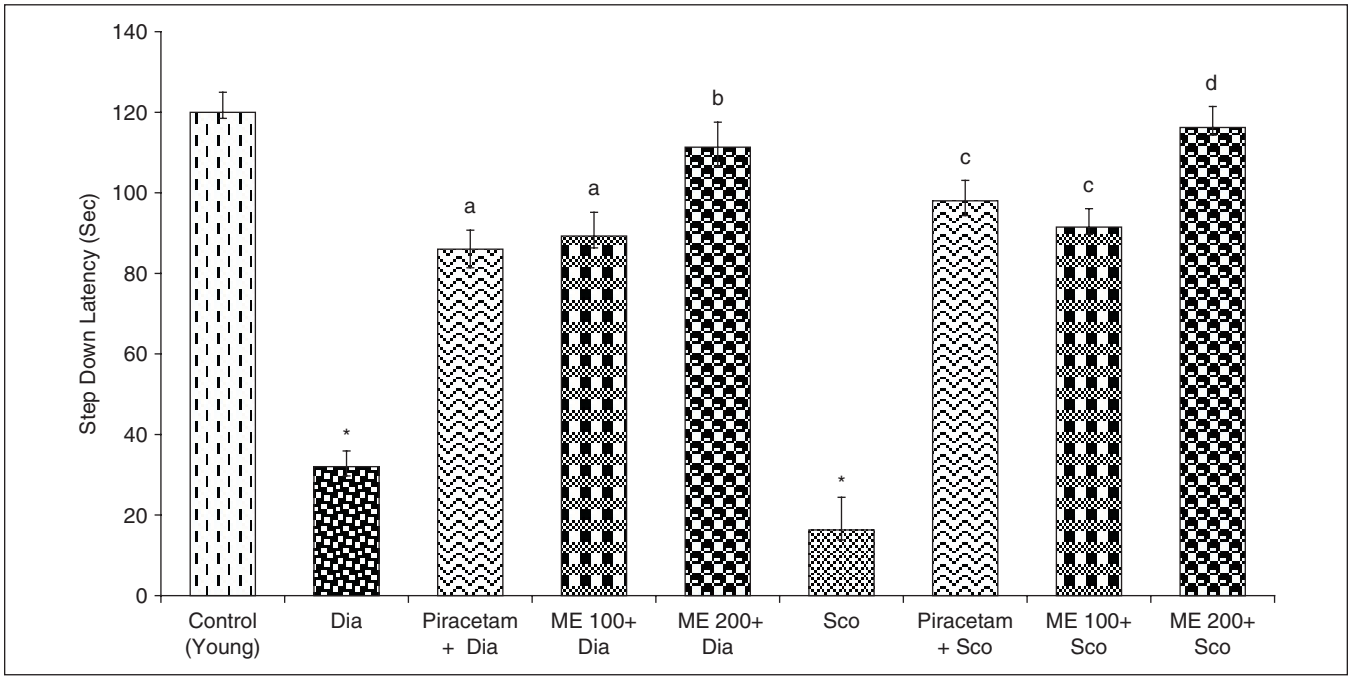


**Figure 4:** Effect of *M. elengi* (ME, 100 and 200 mg/kg, p.o.) on diazepam (Dia, 1 mg/kg, i.p.) and scopolamine (Sco, 0.4 mg/kg, i.p.) induced amnesia in young mice using passive avoidance apparatus.
*Values are mean ± S.E.M. (n=6).*
**indicates P< 0.01 as compared to control group of young mice.*
*[a]indicates P< 0.01 as compared to diazepam (Dia) group alone.*
*[b]indicates P< 0.001 as compared to diazepam (Dia) group alone.*
*[c]indicates P< 0.01 as compared to scopolamine (Sco) group alone.*
*[d]indicates P< 0.001 as compared to scopolamine (Sco) group alone*
*(One way ANOVA followed by Tukey-kramer multiple comparison tests)*

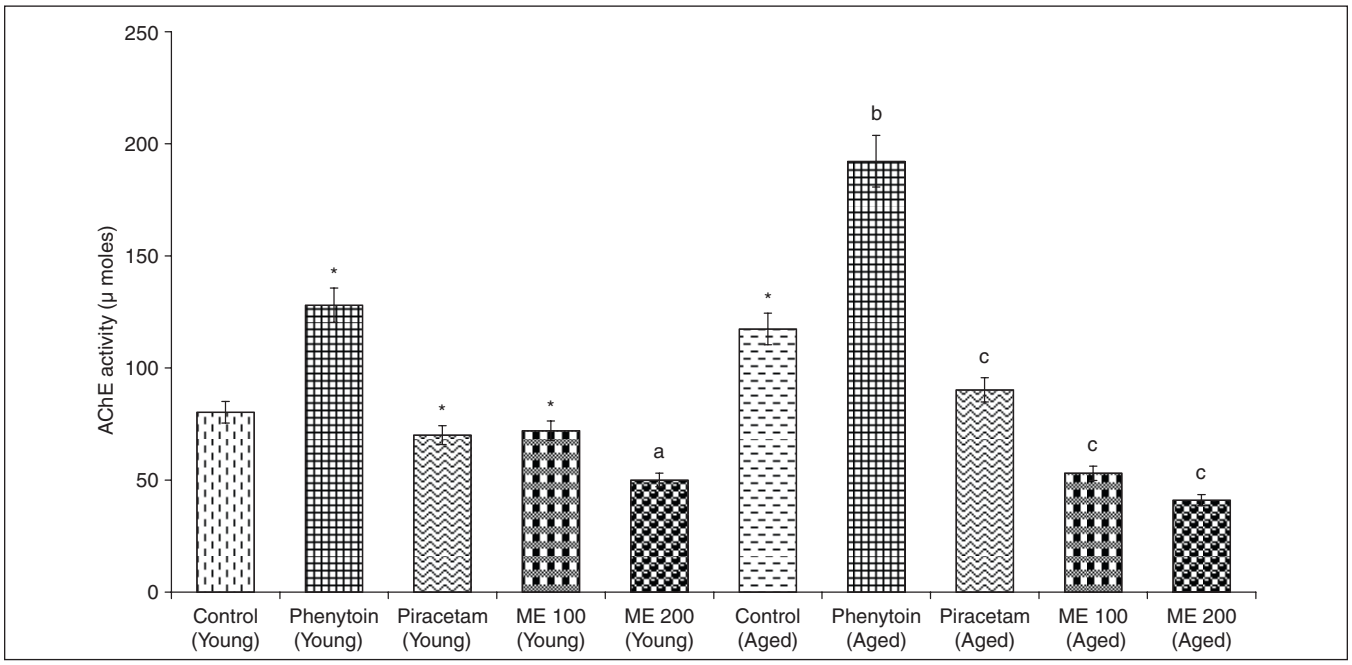


**Figure 5:** Effect of *M. elengi* (ME, 100 and 200 mg/kg, p.o.) on brain cholinesterase (AChE) activity of young and aged mice using Ellman's colorimetric method. Phenytoin (12 mg/kg, p.o.) was used as negative control.
*Values are mean ± S.E.M. (n=6).*
**indicates P< 0.01 as compared to control group of young mice.*
*[a]indicates P< 0.001 as compared to control group of young mice.*
*[b]indicates P< 0.01 as compared to control group of aged mice.*
*[c]indicates P< 0.001 as compared to control group of aged mice.*
*(One way ANOVA followed by Tukey-kramer multiple comparison tests)*

**Table 1: Effect of *M. elengi* (ME, 100 and 200 mg/kg, p.o.) on time spent by mice behavior in open field test**

| Treatment | Dose (p.o.) mg/kg | Number of squares crossed | Rearing |
|---|---|---|---|
| Normal | 1 | 139 ± 5.59 | 23.6 ± 1.20 |
| Diazepam | 0.5 | 63.5 ± 4.17[a] | 10.21 ± 1.19[a] |
| ME | 100 | 66.07 ± 4.14 | 12.90 ± 0.80 |
| ME | 200 | 51.44 ± 9.60 | 9.11 ± 0.69[a] |

N=6 in each group. Values are expressed as Mean ± SE. *P* values [a] <0.001, [b]<0.05 as compared to normal treated group. Statistical test employed was ANOVA followed by Tukey-Kramer multiple comparison test.

**Table 2: Effect of *M. elengi* (ME, 100 and 200 mg/kg, p.o.) on time spent by mice behavior in social interaction test**

| Treatment | Dose (p.o.) mg/kg | Time spent (sec) in social interaction |
|---|---|---|
| Normal | 1 | 39.9 ± 2.9 |
| Diazepam | 0.5 | 68.13 ± 1.13[a] |
| ME | 100 | 43.54 ± 4.11 |
| ME | 200 | 61.86 ± 1.14[b] |

N=6 in each group. Values are expressed as Mean ± SE. *P* values [a] <0.001, [b]<0.01 as compared to normal treated group. Statistical test employed was ANOVA followed by Tukey-Kramer multiple comparison test.

### Effect on Social interaction test

Diazepam significantly increased the time spent in social interaction among mice as compared to the time spent in social interaction among mice as compared to its effect in the control group (table 3). ME [100 mg/kg] and ME [100 mg/kg] significantly increased the time spent in the social interaction as compared to control group, indicating anti anxiety effect in mice.

**Table 3: Effect of *M. elengi* (ME, 100 and 200 mg/kg, p.o.) on time spent by mice behavior in light and dark test**

| Treatment | Dose (p.o.) mg/kg | Time spent (sec) in social interaction |
|---|---|---|
| Normal | 1 | 63.16 ± 3.46 |
| Diazepam | 0.5 | 128.36 ± 5.20[a] |
| ME | 100 | 92.49 ± 11.36[b] |
| ME | 200 | 87.19 ± 9.43[b] |

N=6 in each group. Values are expressed as Mean ± SE. *P* values [a] <0.001, [b]<0.01 as compared to normal treated group. Statistical test employed was ANOVA followed by Tukey-Kramer multiple comparison test.

## DISCUSSION

Alzheimer's disease is a neurodegenerative disorder associated with a decline in cognitive abilities and severe behavioral abnormalities such as irritability, aphasia, apraxia, agnosia and restlessness[50]. Alzheimer patients frequently have non-cognitive symptoms, such as depression, apathy and psychosis, which impair their day-to-day activities[51-52].

Enhancement in the life-span of human beings in developed and developing countries has resulted in proportionate increase in the number of patients suffering from senile dementia. Alzheimer's disease (AD) is said to be the leading cause of dementia in elderly individuals. AD individuals exhibit deterioration in mental functions rendering them incapacitated to perform normal daily activities. However, evidence shows that AD can also afflict young individuals as early as 40 years of age[53]. Neuritic plaques (consisting of a core of β- amyloid aggregates covered by dead neurons, microglia and apolipoprotein E) and neurofibrillary tangles are the major pathological hallmarks of an Alzheimer brain[54]. Cholinergic drugs such as Donepezil® improve learning, memory and attention. The non-cognitive aspects of dementia however are linked to serotonin and dopamine rather than acetylcholine because these neurotransmitter systems most probably influence mood, emotional balance and psychosis[55-56].

The symptoms of dementia are oxidative damage, impaired neurotransmission and degeneration of neuronal circuits in the affected brain areas[57]. Oxidative damage accompanies Alzheimer's disease [AD], and cholinesterase inhibitors are recommended for use in mild-to moderate Alzheimer's disease. In exteroceptive behavioral models, the stimulus lies outside the body whereas; it lies within the body in case of interoceptive behavioral models. Passive avoidance behavior is a classic paradigm to assess memory with strong aversive component, based on negative reinforcement and is used in the present study to examine long-term memory[58]. Interoceptive behavioral models such as diazepam, scopolamine and natural aging induced amnesia are widely cited as models simulating human dementia in general and Alzheimer's disease in particular[59].

Nootropics are a class of psychotropic agents with selective facilitatory effect on integrative functions of the central nervous system, particularly on intellectual performance, learning capability and memory[60-61]. Piracetam, the first representation of a class of nootropic agents, has been shown to improve memory deficits in geriatric individuals. Repeated injections of piracetam had improved learning abilities and memory capacities of laboratory animals[62].

Acetylcholine is considered the most important neurotransmitter involved in the regulation of cognitive functions. Cognitive dysfunction has been shown to be associated with reduced cholinergic transmission and the facilitation of central cholinergic transmission with improved memory[63-64]. Selective loss of cholinergic neurons and decrease in cholinacetyltransferase activity was reported to be a characteristic feature of senile dementia of the Alzheimer's type[65-66]. There are extensive evidences linking the central cholinergic system to memory[67-69].

Anticholinesterases such as Metrifonate[70-71], Physostigmine, Tacarine, Donepezil, Huperzine-A[72], Rivastigmine[73], Galantamine[74] and Eptastigmine[75] have all been shown to reverse amnesia produced by disruption of cholinergic system. Enzyme choline acetyltransferase is involved in the synthesis of acetylcholine and acetylcholinesterase is involved in the degradation of acetylcholine. In the present study, *M. elengi,* significantly lowered the whole brain AChE activity thereby elevating acetylcholine levels in the brain.

Both piracetam and *M. elengi* ME meet major criteria for nootropic activity, namely improvement of memory in absence of cognitive deficit[76]. Cognitive deterioration occurring in patients with probably AD is associated with progressive loss of cholinergic neurons and consequent decline in levels of acetylcholine [Ach] in brain[77]. Cholinergic deficits occur in the brain of patients with AD and vascular dementia[78-79]. Phenytoin is known to reduce hippocampal ACh concentration and causes cognitive impairment[80-83],some medicinal plants and phytochemicals have been found useful for amnesic conditions[84]. In the present study, the aqueous extract of *M. elengi* significantly inhibited the AChE activity in the whole brain homogenate of mice, indicating its potential in the attenuation of learning and memory deficits especially in aged mice.

Until recently, little attention has been paid to anxiety symptoms in dementia. However, anxiety is common in this population, and associated with poor outcome and quality of life[85]. Anxiety is more common in individuals with dementia than in individuals without dementia[86]. In several studies between awareness of cognitive deficits and anxiety in dementia raises interesting possibilities. First, being awa`re of one's cognitive decline may generate anxiety[87]. *M. elengi* exhibited profound anti-anxiety activity in mice when tested on Light and dark box test, Open field test and Social interaction test models.

Considering the lack and need of drugs with proven effectiveness in improving learning and memory, the specific memory improving effects of *M. elengi* reported here is of enormous interest and deserves further investigations using more experimental paradigms for further confirmation of memory improving potential of *M. elengi* in the treatment of various cognitive disorders.

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# Hepatoprotective and Free Radical Scavenging Activities of Extracts and a Major Compound Isolated from the Leaves of *Cineraria abyssinica* Sch. Bip. exA. Rich.

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## ABSTRACT

In Ethiopian traditional medicine the aqueous decoction of the leaves of *Cineraria abyssinica* Sch. Bip. exA. Rich (Asteraceae) is used for the treatment of various ailments including liver diseases, however, to date, there appears to have been no scientific report on the phytochemistry and claimed hepatoprotective activity of the plant. The main purpose of this study was, therefore, to carry out hepatoprotective and antioxidant activities of the leaf extracts of *C. abyssinica*. Hepatoprotective activities of the aqueous and 80% methanolic extracts as well as the methanol fraction of the leaves of *C. abyssinica* were investigated against carbon tetrachloride-induced liver damage in rats. Intraperitoneal administration of 2 ml/kg of $CCl_4$ (50% in liquid paraffin) significantly ($p < 0.001$) raised the plasma levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the toxin group compared with the values in the control group. Pretreatment of rats with 200 mg/kg of the aqueous, 80% methanol extracts and the methanol fraction reduced the toxin-induced rise in plasma ALP (65%, 75.4%, 85%), ALT (46.1%, 42.3%, 75%), and AST (58%, 98%, 79%), respectively. The standard drug, silymarin (100 mg/kg) reduced serum ALP (88%), ALT (92%), and AST (87.3%). Bioactivity-guided fractionation of the methanol fraction resulted in the isolation of the flavonol glycoside rutin, whose structure was assigned on the basis of spectroscopic methods. The results of biochemical analysis were further verified by histopatholgical examination of the liver, which showed improved architecture, absence of necrosis and a decrease in inflammation, compared with the findings in the toxin group of animals. Both the extracts and rutin showed potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities. Acute toxicity studies showed that the total extracts of the plant are nontoxic up to a dose of 3 g/kg. The present study revealed for the first time the presence of a hepatoprotective and antioxidant phytochemical in the leaves of *C. abyssinica* that scientifically validates the traditional use of the plant and its potential for the treatment of liver disorders.

**Key words:** *Cineraria abyssinica,* Asteraceae, hepatoprotective, rutin, free radical scavenging

## INTRODUCTION

Liver disease is one of the major causes of morbidity and mortality in public, affecting humans of all ages throughout the world. Despite the great stride in allopathic medicine, modern drugs available for liver diseases have so many limitations. They are limited in number, they do not provide a complete cure and they are unaffordable to most people in the developing countries. This situation stresses the importance of worldwide public–private partnerships to enhance the research enterprise, bring new agents to market in a more cost-effective fashion, and provide effective therapies to suffering patients at costs that are within their reach.[1-3]

Traditional medicines continue to provide front-line pharmacotherapy for many millions of people worldwide. In the absence of safe and reliable antihepatotoxic modern drugs, several medicinal plants have been used worldwide in various traditional herbal recipes for the prevention and

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treatment of liver disease. In recent years there has been a growing focus to follow systematic research methodology and to scientifically evaluate the basis for traditional herbal medicines which are claimed to possess hepatoprotective activity.[1,4]

*Cineraria abyssinica* Sch. Bip. exA. Rich (Asteraceae) commonly known by its vernacular name '*Etsemefirh*', is an erect or scrambling, annual or perennial herb that can grow up to 20-100 cm high. It has repeatedly branched stem, with alternate, simple to lyrately pinnatified petiolate leaves and radiate capitula with yellow florets. It extends from Ethiopia into Yemen and Saudi Arabia.[5] Based on the information provided by the traditional community from Harar, eastern part of Ethiopia, the aqueous decoction of the leaves of *C. abyssinica* is employed for the treatment of various ailments such as hypertension, cancer, diabetes, diarrhea, kidney and liver diseases. However, despite its wider use in traditional medicine, there are no prior reports on the phytochemistry and pharmacological effects of this plant. The present research was therefore, undertaken to examine the possible hepatoprotective action of the plant using *in vivo* $CCl_4$-induced hepatotoxicity test in rats and to examine its *in vitro* DPPH free radical scavenging effect.

## MATERIALS AND METHODS

### Plant material

The leaves of *C. abyssinica* were collected from and around the town of Harar in the Harari People Region, 525 km East of Addis Ababa, Ethiopia in September 2008. The plant was authenticated by Ato Melaku Wondafrash of the National Herbarium, Addis Ababa University, where a voucher specimen has been deposited (Collection number, B 01).

### Animals

Wistar albino male rats (200-250 g) and mice (25-30 g) obtained from the Ethiopian Health and Nutrition Research Institute (EHNRI) animal house were used for the experiments. The animals were housed under standard laboratory conditions and were fed commercial rat feed and tap water *ad libitum*. The animals were fasted overnight with free access to water and acclimatized for one week in the new environment before experiments were carried out. All animal experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline[6] and approved by the Institutional Review Board of the School of Pharmacy, Addis Ababa university.

### Chemicals and instruments

All the chemicals and reagents used for the experiments were analytical grade. Ultraviolet (UV) spectra were run on a Shimadzu UV-1800 spectrophotometer. Infra red (IR) spectra were taken on a Shimadzu IR Prestige-21 spectrophotometer in KBr pellets. $^1$H NMR and $^{13}$C NMR spectra were recorded in DMSO-$d_6$ using a Bruker A-400 spectrometer with TMS as an internal standard. Electro spray mass spectra were obtained with LCQ Deca XP, ESI, negative mode spectrometer.

### Preparation of crude extracts

A hydroalcoholic extract of *C. abyssinica* was prepared by macerating 300 g of the powdered shade-dried leaves with 80% methanol (3x, each for 72 h) with occasional shaking. The combined filtrates were then dried in a rotary vacuum evaporator at a temperature not exceeding 40°C. Aqueous extract was prepared by boiling the plant material for 30 min followed by cooling, filtering and lyophilizing of the extract.

### Preparation of solvent fractions and isolation of a compound

The air-dried powdered leaves of *C. abyssinica* (300 g) were successively extracted in a Soxhlet apparatus using solvents of increasing polarity, starting from chloroform then acetone and methanol. The solvents were removed using a rotary vacuum evaporator at a temperature not exceeding 40°C. The most active methanol fraction was subjected to silica gel preparative thin layer chromatography (PTLC) using butanol: acetic acid: water (4:1:5, upper phase) as a mobile phase. The yellowish powder obtained was further purified by LH-20 column chromatography using methanol as solvent and the purity of the eluate was checked by analytical TLC.

### Identification of the isolated compound

The isolated compound was identified as rutin by comparison of its spectral data ($^1$H and $^{13}$C-NMR) with those reported in the literature.[7,8] Furthermore, comparison of the ESI-mass spectra of the isolated compound was found to be superimposable on those of standard rutin.

### Acute toxicity tests

Acute toxicity studies were carried out on the aqueous and 80% methanolic leaf extracts of *C. abyssinica* according to Daisya *et al.*[9] Normal healthy male mice fasted for 12 h were randomly divided into drug-treated 'test' groups and vehicle-treated 'control' group, of 6 mice per group. Each of the extracts (0.5, 2.0 and 3.0 g) suspended in 1% carboxyl methyl cellulose (CMC) was separately administered orally to the mice in each of the test groups. The mice in the control group were treated with vehicle alone (1% CMC). Two h after treatment, the mice in both the test and control groups were given free access to food and water, and behavioral changes were observed over a period of 24 h. Mortality, if any, caused by the extract within this period of time was also observed.

### *In vivo* hepatoprotective activity studies

The model described by Narayan *et al.*[10] was employed. The rats were divided into seven groups consisting of five animals each. Animals in group A served as normal and they were given only vehicle (0.7% CMC suspension 1 ml/kg b.w.) orally for 6 days. Animals in group B served as toxin control and they were administered with $CCl_4$ (50% solution of $CCl_4$ in liquid paraffin, 2 ml/kg b.w., i.p.) on the 4$^{th}$ day and with vehicle on rest of the days. Animals in groups C-E were treated orally with 200 mg/kg of the aqueous extract, the 80% methanolic extract and the methanol fraction, respectively, suspended in 0.7% CMC for 6 days and $CCl_4$ (2 ml/kg b.w.) on the 4$^{th}$ day i.p, while the animals of groups F and G received rutin and silymarin (100 mg/kg b.w. suspended in 0.7% CMC), respectively, for 6 days orally and $CCl_4$ (2 ml/kg b.w.) on the 4$^{th}$ day i.p. On the 7$^{th}$ day, the animals were anesthetized by ether and blood was collected in heparinized tubes from the retro orbital plexus of each animal and serum was separated by centrifugation at 365 rpm for 15 min and analyzed for various biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP).

### Histopathological study

Immediately after collection of blood from each animal, they were sacrificed by cervical dislocation and the liver was separated, washed with normal saline and stored in 10% formalin. Small pieces of the liver fixed in 10% formalin, were processed for embedding in paraffin. Sections of 5-6 µm were cut and stained with hematoxylin and eosin (H & E) and examined under the microscope for histopatholgical changes. Images were captured using Olympus DP12 CCD camera at original magnification of 10x (Olympus DP12 Microsystems Digital Imaging Olympus, Japan).

### 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity test

The method of Sokmen *et al.*[11] was used in this experiment. Firstly, 5 ml of 0.004% DPPH in methanol was mixed with 50 µl of various concentrations (1000, 500, 250, 125, 50, µg/ml) of the crude extracts, fractions, the isolated compound or ascorbic acid (a reference compound) separately. Following 30 min of incubation at room temperature in the dark, the absorbance of the mixture in the samples was measured using a spectrophotometer (Unico$^{TM}$2100) at 517 nm against methanol as blank. Percentage radical scavenging activity of the samples was evaluated by comparing with a control (5 ml DPPH solution and 50 µl methanol). Each sample was measured in triplicate and averaged. The percentage radical scavenging activity (RSA) was calculated using the following formula:

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

where, $A_0$ is absorbance of the control, and $A1$ is absorbance of samples after 30 min. Free radical scavenging activities of the crude extracts, fractions, rutin and ascorbic acid were expressed as $IC_{50}$. The $IC_{50}$ value was defined as concentration (in µg/ml) of sample that inhibits 50% of the formation of DPPH radical.

### Data and statistical analysis

Data are expressed as mean ± SD (standard deviation). Statistical evaluation of data was performed by using one-way analysis of variance (ANOVA) and Turkey post test. Values were considered to differ significantly for $P < 0.01$ to $P < 0.001$. The % protection of the test material was calculated by the following formula:

$$\% \text{ Protection} = \left(\frac{(a-b)}{(a-c)}\right) \times 100$$

where, a is the mean value of the marker produced by hepatotoxin; b is the mean value of the marker produced by toxin plus test material; and c is the mean value produced by the vehicle control.

## RESULTS AND DISCUSSION

### Acute toxicity

In the present study, a preliminary toxicity study was designed to demonstrate the appropriate safe dose range that could be used for subsequent experiments rather than to provide complete toxicity data on the extract. Up to a dose of 3 g/kg both the aqueous and 80% methanolic leaf extracts of *C. abyssinica* were found to be nontoxic. No mortality was observed in the extract-treated mice and also the extracts did not produce significant changes in behaviors such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions and coma.

### *In vivo* hepatoprotective activity studies

In bioassay-directed searching for hepatoprotective agents from natural sources, employing the closely relevant model system to human liver toxicosis could be an effective way to identify therapeutically applicable agents.[12] $CCl_4$ is a well-known hepatotoxic chemical commonly used as a chemical inducer of experimental liver injury.[13] Since the changes associated with $CCl_4$-induced liver damage in animal model system are closely related to hepatotoxicity in acute viral hepatitis in human[14], $CCl_4$-mediated hepatotoxicity was chosen as the experimental model in this study. The main causes of acute liver injury by $CCl_4$ are free radicals of its metabolites. $CCl_4$ is reductively bioactivated by cytochrome P450 2E1 into a trichloromethyl radical ($^{\bullet}CCl_3$), which is subsequently converted into trichloromethyl peroxyl radical ($^{\bullet}OOCCl_3$) in the presence of oxygen. These highly unstable reactive free radical metabolites may cause cellular

damage by initiating lipid peroxidation and covalently binding to macromolecules. They form covalent bonds with unsaturated fatty acids, or take a hydrogen atom from the unsaturated fatty acids of membrane lipids, resulting in the production of chloroform and lipid radicals. The lipid radicals react with molecular oxygen, which initiates peroxidative decomposition of phospholipids in the endoplasmic reticulum. The peroxidation process results in the release of soluble products that may affect cell membrane. Cell membrane integrity is broken and enzymes such as ALT, AST and ALP in cell plasma leak out.[15-17]

Normally, the concentration of transferase in hepatocyte is about 1000-5000 times higher than in serum. When liver is injured by $CCl_4$, membrane permeability of liver parenchyma cell intensified, the activities of ALP, ALT and AST in serum increased sharply as a consequence and serum aminotransferase activities have long been considered as sensitive indicators of hepatic injury in both experimental and clinical setup.[18]

Table 1 shows that $CCl_4$ significantly ($P<0.001$) increased serum ALP, ALT and AST activities compared with the normal control group. This substantial rise is an indicative of cellular leakage and loss of functional integrity of hepatocytes. However, pretreatment of the rats with the 200 mg/kg of crude extracts, methanol fraction or 100 mg/kg of rutin as well as the standard drug, silymarin inhibited these alterations significantly ($P<0.01$ to $P<0.001$).

The aqueous extract reduced serum ALP (65.0%), ALT (46.1%) and AST (58.0%) while, the 80% methanol extract reduced serum ALP (75.4%), ALT (42.3%), and AST (98.0%). The activity of the methanol fraction in preventing $CC1_4$-induced elevation of serum transaminase activities (ALP (85.0%), ALT (75.0%), and AST (79.0%)) was better than those of the aqueous and 80% methanol crude extracts.

Bioassy-giuded fractionation of the active methanol fraction of the plant over PTLC followed by sephadex LH-20 purification led to the isolation of the flavonol glycoside rutin. To the best of our knowledge, this is the first report on the isolation of rutin from *C. abyssinica*. Rutin has been extensively studied and is known to exhibit multiple pharmacological activities including antiviral[19], antitumor[20], antiallergic[21], anti-inflammatory[22], antihypertensive[23], antidiabetic[24], gastroprotective[25], anticonvulsant[26], nephroprotective[27] and antioxidant[28]. In the present study, rutin showed pronounced hepatoprotective activity that was comparable to that of silymarine. Treatment of rats with 100 mg/kg of rutin ameliorated $CCl_4$-induced hepatocellular damage as evidenced by the significant reduction in serum ALP (85.0%), ALT (79.3%) and AST (87.0%) levels. The hepatoprotective activity of rutin was also previously reported. Rutin isolated from *Artemisia scoparia* Thunb (Asteraceae), a traditional plant used for treatment of liver diseases in Pakistan, has been reported to possess hepatoprotective activity against paracetamol- and $CCl_4$-induced hepatocellular damage in mice.[29] In another study, administration of rutin to rats pretreated with ethanol has been shown to decrease the levels of liver marker enzymes, lipid peroxidation and significantly elevated the activities of liver superoxide dismutase, catalase, glutathione, glutathione peroxidase, vitamins C and E when compared to untreated ethanol supplemented rats.[30] Furthermore, Radwan *et al.*[31] reported that rutin prevents radiation induced hepatotoxicity.

Biochemical effects of the crude extracts, fractions and the compound isolated from the leaves of *C. abyssinica* were supported by the results of histopathological examination, as evidenced by a decrease in the incidence and severity of histopathological hepatic lesions (Figure 1). The histopathological results depicted in Figure 1 demonstate that liver section of normal control (0.7% CMC treated) rats showing normal hepatic cells with well preserved cytoplasm, prominent nuclei and well brought out central

**Table 1: Effect of the crude extracts, methanol fraction, and rutin isolated from the leaves of *Cineraria abyssinica* in comparison with that of silymarin on activities of serum enzymes in rats injected with 50% $CCl_4$ in paraffin (2 ml/kg) i.p.**

| Group | Dose | Serum marker | | |
|---|---|---|---|---|
| | | ALP (IU/l) | ALT(IU/l) | AST (IU/l) |
| Control (vehicle) | 0.7% CMC | 239.6 ± 31.817[a] | 104.2 ± 2.950[a] | 262.8 ± 57.495[a] |
| Toxin | 0.7% CMC + $CCl_4$ (2ml/kg) | 624.4 ± 209.220 | 338.6 ± 89.804 | 584.6 ± 39.665 |
| Aqueous extract | 200 mg/kg + $CCl_4$ (2 ml/kg) | 375.2 ± 102.380[b] (65.0) | 230.6 ± 30.188 (46.1) | 398.8 ± 90.663[b] (58.0) |
| 80% Methanol extract | 200 mg/kg + $CCl_4$ (2 ml/kg) | 334.2 ± 59.757[b] (75.4) | 239.4 ± 26.435 (42.3) | 270.2 ± 65.515[a] (98.0) |
| Methanol fraction | 200 mg/kg + $CCl_4$ (2 ml/kg) | 299.0 ± 64.935[a] (85.0) | 163.6 ± 63.650[b] (75.0) | 331.2 ± 24.056[b] (79.0) |
| Rutin | 100 mg/kg + $CCl_4$ (2 ml/kg) | 298.6 ± 81.362[a] (85.0) | 152.8 ± 108.540[a] (79.3) | 305.2 ± 60.644[b] (87.0) |
| Silymarin | 100 mg/kg + $CCl_4$ (2 ml/kg) | 286.8 ± 29.781[a] (88.0) | 123.2 ± 14.990[a] (92.0) | 303.8 ± 199.150[b] (87.3) |

[a]significantly different ($P<0.001$) as compared to toxin group and [b]significantly different ($P<0.01$) as compared to toxin group; n = 5, numbers in parenthesis shows % protection.

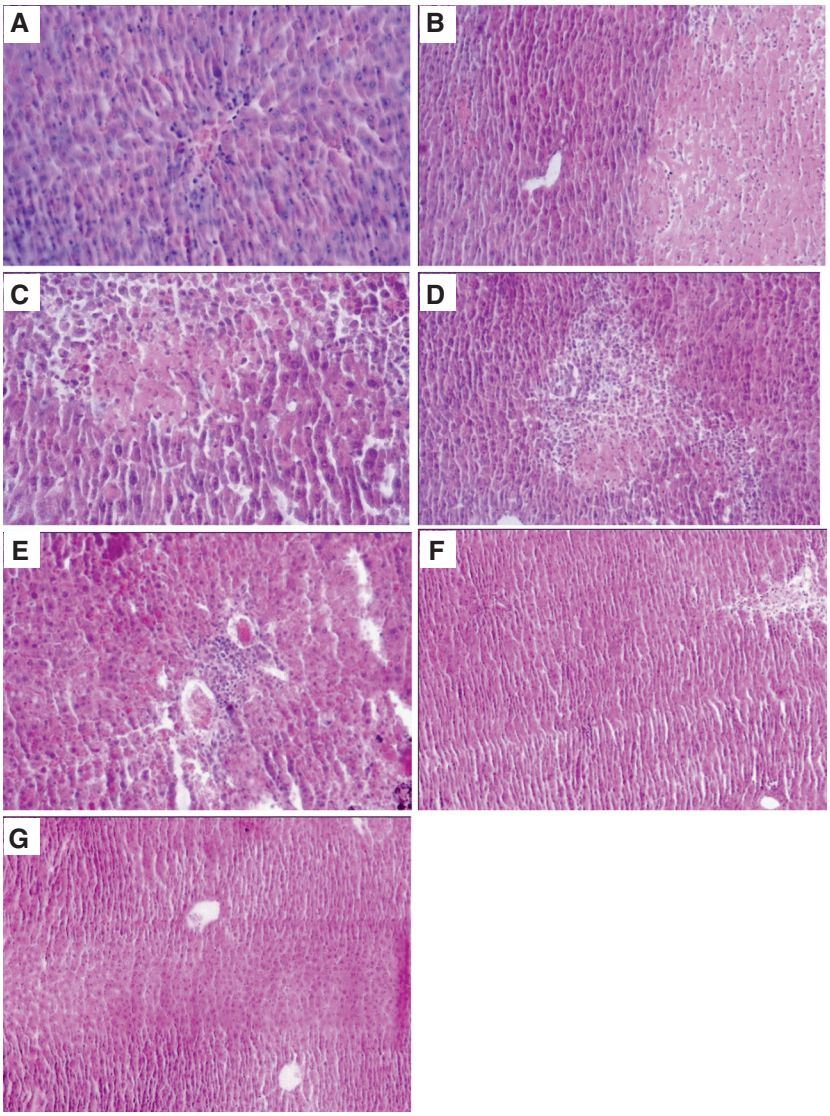


**Figure 1:** Microphotographs of hematoxylin and eosin (H & E) (X10) stained histological section of liver (A) vehicle or normal control: rats treated with 0.7% CMC; (B) $CCl_4$ control; (C) rats treated with 200 mg/kg of the aqueous leaf extract of *Cineraria abyssinica* and $CCl_4$ (2 ml/kg i.p.); (D) rats treated with 200 mg/kg of the 80% methanol leaf extract of *Cineraria abyssinica* and $CCl_4$ (2 ml/kg i.p.); (E) rats treated with 200 mg/kg of the methanol fraction of the leaves of *Cineraria abyssinica* and $CCl_4$ (2 ml/kg i.p.); (F) rats treated with 100 mg/kg of rutin isolated from *Cineraria abyssinica* and $CCl_4$ (2 ml/kg i.p.); (G) rats treated with 100 mg/kg of silymarin and $CCl_4$ (2 ml/kg i.p.).

vein (Figure 1A). Compared with the normal group, liver tissue in rats treated with 0.7% CMC plus $CCl_4$ (toxin group) revealed extensive liver injuries, characterized by severe hepatocellular degeneration and necrosis (Figure 1B). However, the histopathological hepatic lesions induced by administration of $CC1_4$ were remarkably ameliorated by liver of rats treated with the crude extracts, fractions, rutin or silymarin with different degree. Pretreatment of rats with 200 mg/kg of the aqueous extract showed protection to $CCl_4$-induced liver damage as shown by a reduction in liver necrosis (Figure 1C). The small necrosis seen in the aqueous treated group supports the relatively higher transaminase level, compared with the other treated groups observed in the serum markers analysis. Whilst the liver of rats administered with the hydroalcoholic extract showed minor necrosis and focal inflammation (Figure 1D), the liver of those given the methanol fraction had minor focal inflammation without necrosis (Figure 1E). Normal liver histology was observed in rats treated with rutin and silymarin (Figures 1F and 1G). Thus the histological changes associated with the hepatoprotective activity of the crude extracts, methanol fraction, rutin and silymarin strongly support the results of the serum enzymes estimation.

## *In vitro* radical scavenging activity studies

Reactive oxygen and nitrogen species (ROS and RNS) contribute to the pathogenesis of various acute and chronic

**Table 2: DPPH scavenging activity $IC_{50}$ values of the crude extracts, solvent fractions, and rutin isolated from the leaves of *Cineraria abyssinica* in comparison with ascorbic acid**

| Test substance | $IC_{50}$ (µg/ml) |
|---|---|
| Aqueous extract | 6.27 |
| 80% Methanol extract | 5.78 |
| Methanol fraction | 6.82 |
| Acetone fraction | 8.53 |
| Chloroform fraction | 12.41 |
| Rutin | 3.53 |
| Ascorbic acid | 3.57 |

liver diseases, such as acetaminophen overdose, haemochromatosis, alcoholic liver injury, toxin exposure and viral hepatitis.[32-35] As mentioned earlier, the main cause of hepatic cell death of $CCl_4$ is focused on their metabolic activation into highly reactive radicals, depletion of endogenous antioxidants, and covalent binding of the reactive metabolites to cellular proteins.[16,17,36] As a consequence, antioxidants have been proposed as an adjunct therapy for various liver diseases.[37] So in the present study in order to delineate the possible hepatoprotective mechanism of the plant, DPPH free radical scavenging activity was carried out.

In the current study, the crude extracts, fractions, rutin and ascorbic acid were shown to serve as an antioxidant agents or hydrogen donors that can scavenge free radical. As shown in Table 2, rutin showed the highest activity ($IC_{50}$ = 3.53 µg/ml). Therefore, the hepatoprotective activity of the plant crude extracts, methanol fraction and rutin may be partly attributed to their free radical scavenging activities.

## CONCLUSION

The results of the present investigation provide strong evidence that the aqueous and 80% methanol crude extracts as well as the methanol fraction of *C. abyssinica* significantly inhibit acute liver toxicity induced by $CCl_4$ in rats, as shown by a reduction of serum liver enzyme activities and the preservation of liver histopathology. The hepatoprotective action of the plant may be attributed to the presence of rutin which showed potent *in vitro* radical scavenging activity. The results of the study also provide scientific evidence for the traditional use of the plant in the treatment of liver diseases.

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# Evaluation of Anti-inflammatory Effect of Ashwagandha: A Preliminary Study *in vitro*

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## ABSTRACT

**Introduction:** Ashwagandha (*Withania somnifera*) is an important medicinal plant in Indian traditional system of medicine and traditionally has been used for several medicinal purposes in India. The present study was conducted to evaluate the anti-inflammatory effect of hydroalcoholic extract of ashwagandha against denaturation of protein *in vitro*. **Methods:** The test extract at different concentrations was incubated with egg albumin in controlled experimental conditions and subjected to determination of absorbance and viscosity to assess the anti-inflammatory property. Diclofenac sodium was used as the reference drug. **Results:** The present results exhibited a concentration dependent inhibition of protein (albumin) denaturation by the ashwagandha extract. The effect of diclofenac sodium was found to be less when compared with the test extract. **Conclusion:** Form the present findings it can be concluded that ashwagandha possessed marked anti-inflammatory effect against denaturation of protein *in vitro*. The effect was plausibly due to the alkaloid and withanolide contents of ashwagandha.

**Key words:** *Withania somnifera*, anti-inflammatory, protein denaturation, viscosity.

## INTRODUCTION

Inflammation is a bodily response to injury, infection or destruction characterised by heat, redness, pain, swelling and disturbed physiological functions. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. It is the body response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair. It is triggered by the release of chemical mediators from injured tissue and migrating cells.[1] The commonly used drug for management of inflammatory conditions are non-steroidal anti-inflammatory drugs (NSAIDs), which have several adverse effects especially gastric irritation leading to formation of gastric ulcers.[2,3] Natural products have contributed significantly towards the development of modern medicine. Recently traditional medicine worldwide is being re-evaluated by extensive research on different plant species and their active therapeutic principles. The rich wealth of plant kingdom can represent a novel source of newer compounds with significant anti-inflammatory activities. The major merits of herbal medicine seem to be their perceived efficacy, low incidence of serious adverse effects and low cost.

Ashwagandha, also known as Indian ginseng and winter cherry, consists of dried roots of *Withania somnifera* (L.) Dunal. (Family: Solanaceae). It is a perennial plant indigenous to India, grown and cultivated throughout subtropical India. It has been recognized as an important herb in the Ayurveda, the traditional system of Indian medicine for more than 3000 years. Traditionally it has been used for several important medicinal purposes in the Indian subcontinent. Recently there has been renewed interest on ashwagandha for its effectiveness in several disease conditions, adaptogenic, immunomodulator and other health benefits.[4,5] Previous researchers have reported several pharmacological properties of ashwagandha on animals and humans.[6-10] The present study was conducted to evaluate the anti-inflammatory effect of ashwagandha extract against the denaturation of protein *in vitro*.

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## MATERIALS AND METHODS

### Plant material

The dried roots of Ashwagandha (*Withania somnifera* (L.) Dunal. family: Solanaceae) were procured in the month of July, 2011 from Kangalicharan & Sons., Kolkata, West Bengal, India and identified at the Botanical Survey of India, Howrah, West Bengal, India. Just after procurement, the roots were ground mechanically into a coarse powder and kept into an air-tight container for use in the study.

### Drugs and chemicals

Diclofenac sodium was obtained from Organic Chemical Industries Pvt. Ltd., Kolkata 70001, West Bengal, India. Double distilled water from all-glass still was used throughout the study.

### Preparation of extract

The powdered plant material (50 g) was extracted with 50% aqueous ethanol (400 ml) by boiling under reflux for 30 minutes. The extract was filtered and evaporated to dryness to yield the dry extract (HAWS, yield: 37.44%). The dry extract was kept in a refrigerator until use. Different concentrations of HAWS for anti-inflammatory assay were prepared freshly from the dry extract by dissolving in double-distilled water immediately prior to use.

### Evaluation of anti-inflammatory effect *in vitro*

The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of HAWS so that final concentrations become 31.25, 62.5, 125, 250, 500, 1000 µg/ml. Similar volume of double-distilled water served as control. Then the mixtures were incubated at 37 ± 2ºC in a BOD incubator (Labline Technologies) for 15 mins and then heated at 70ºC for 5 mins. After cooling, their absorbance was measured at 660 nm (SHIMADZU, UV 1800) by using vehicle as blank and their viscosity was determined by using Ostwald viscometer. Diclofenac sodium at the final concentration of (78.125, 156.25, 312.5, 625, 1250, 2500 µg/ml) was used as reference drug and treated similarly for determination of absorbance and viscosity. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition} = 100 \times \left[\frac{V_t}{V_c} - 1\right]$$

Where, $V_t$ = absorbance of test sample, $V_c$ = absorbance of control.

The extract/drug concentration for 50% inhibition ($IC_{50}$) was determined by plotting percentage inhibition with respect to control against treatment concentration.

## RESULTS AND DISCUSSION

There are certain problems associated with animal use in experimental pharmacological research such as ethical issues and the lack of rationale for their use when other suitable methods are available or could be investigated. Hence, in the present study the protein denaturation bioassay was selected for *in vitro* assessment of anti-inflammatory property of hydroalcoholic extract of ashwagandha (HAWS). Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto-antigens in certain arthritic diseases may be due to denaturation of proteins *in vivo*.[11,12] Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development.

In the present investigation, the anti-inflammatory effect of HAWS was evaluated against the denaturation of egg albumin *in vitro*. The results are summarized in Table 1. The present findings exhibited a concentration dependent inhibition of protein (albumin) denaturation by HAWS throughout the concentration range of 31.25 to 1000 µg/ml. Diclofenac sodium (at the concentration range of 78 to 2500 µg/ml) was used as reference drug which also exhibited concentration dependent inhibition of protein denaturation (Table 2); however, the effect of diclofenac sodium was found to be less as compared with HAWS. This was further confirmed by comparing their $IC_{50}$ values (Table 3).

The increments in absorbances of test sample with respect to control indicate stabilization of protein i.e. inhibition of protein (albumin) denaturation by HAWS and reference

**Table 1: Effect of HAWS against protein denaturation**

| Concentration (µg/ml) | % Inhibition | Viscosity (cp) |
|---|---|---|
| Control | - | 1.45 |
| 31.25 | 20 | 0.79 |
| 62.5 | 40 | 0.83 |
| 125 | 300 | 0.83 |
| 250 | 500 | 0.90 |
| 500 | 600 | 0.94 |
| 1000 | 1100 | 1.03 |

**Table 2: Effect of diclofenac sodium against protein denaturation**

| Concentration (µg/ml) | % Inhibition | Viscosity (cp) |
|---|---|---|
| Control | - | 1.45 |
| 78.125 | 12.5 | 0.80 |
| 156.25 | 12.5 | 0.86 |
| 312.5 | 25 | 1.0 |
| 625 | 50 | 1.13 |
| 1250 | 212.5 | 1.15 |
| 2500 | 812.5 | 1.26 |

**Table 3: $IC_{50}$ values of HAWS and diclofenac sodium against protein denaturation**

| Treatments | $IC_{50}$ values (µg/ml) |
|---|---|
| HAWS | 65 |
| Diclofenac sodium | 625 |

drug diclofenac sodium.[13] This anti-denaturation effect was further supported by the change in viscosities. It has been reported that the viscosities of protein solutions increase on denaturation.[14] In the present study, the relatively high viscosity of control dispersion substantiated this fact. Presence of HAWS prevented this, implying inhibition of protein denaturation. Here, viscosities decreased with respect to control where no test extract/drug was added. However, the viscosities were found to decrease with concomitant decrease in concentration of test extract and reference drug as well. Although, the viscosities of the test samples (extract/drug), of all concentrations were always less than that of control. The observed decrease in viscosities may be due to decrease in concentration of test extract/drug, or other uncertain physicochemical factors. Nevertheless, the viscosity data indicated inhibition of protein (albumin) denaturation. The effect of concentration of test agent on viscosity behaviour of denatured protein dispersion requires further studies.

The major constituents of ashwagandha are several alkaloids and steroidal lactones commonly called withanolides which are responsible for its wide ranging biological effects.[15,16] In the present study, anti-inflammatory effect of ashwagandha can be attributed to its alkaloid and withanolide contents. The effect may be due to synergistic effect rather than single constituent.

It has been reported that one of the features of several non-steroidal anti-inflammatory drugs is their ability to stabilize (prevent denaturation) heat treated albumin at the physiological pH (pH: 6.2 – 6.5).[17] Therefore, form the results of the present preliminary study it can be concluded that ashwagandga possessed marked anti-inflammatory effect against the denaturation of protein *in vitro*. Previous workers have reported anti-inflammatory activity of ashwagandha in experimental animal models.[18] The present findings corroborated this property of ashwagandha *in vitro*. Further definitive studies are necessary to ascertain the mechanisms and constituents behind its anti-inflammatory actions both *in vivo* and *in vitro*.

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# Phytochemical Evaluation and Antioxidant Study of *Jatropha curcas* Seeds

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## ABSTRACT

*Jatropha curcas* L. is a soft-wooded shrub found both in wild and cultivated conditions across the country. The seeds of *J. curcas* have been used as a purgative, antihelminthic, abortifacient as well as for treating ascites, gout, paralysis, and skin diseases. Present study includes physicochemical, phytochemical, antioxidant and HPTLC analysis of *J. curcas* seeds. Ethanolic, aqueous and hydroalcoholic extracts from *J. curcas* were screened for antioxidant evaluation using DPPH free radical scavenging activity. Protocatechuic and Gallic acid, two potential antioxidants present in this species, has been studied through HPTLC which may be utilized for the proper standardization of the drug.

In *J. curcas,* $IC_{50}$ of ethanolic, aqueous and hydroalcoholic extract were found to be 46 ± 3.46 µg/ml, 36.66 ± 0.57 µg/ml and 32.66 ± 1 µg/ml respectively. Ascorbic acid is used as standard which showed $IC_{50}$ of 2.66 ± 0.11 µg/ml. HPTLC studies showed the presence of protocatechuic acid and gallic acid at $R_f$ 0.69 and 0.32 with $r^2$ of 0.998 and 0.994 respectively with mobile phase toluene: ethyl acetate : formic acid (5:5:0.5).

*J. curcas* seed extract showed significant in vitro antioxidant activity, 50% hydroalcoholic extract showed the most potent activity. Quantification of protocatechuic and Gallic acid in 50% hydroalcoholic extract of *J. curcas* has been performed and was found to be 0.146% and 0.092% respectively.

**Key words:** Antioxidant, DPPH, Gallic acid, *J. curcas,* Protocatechuic acid

## INTRODUCTION

*Jatropha curcas* L. is a drought-resistant shrub belonging to the Family Euphorbiaceae, wildly found all across the country and is also cultivated in Central and South America, south-east Asia and Africa[1]. Some of the ethnomedical uses of the extracts of *J. curcas* leaves and roots include a remedy for cancer, as an abortifacient, antiseptic, diuretic, purgative and haemostatic[2]. The seeds of *J. curcas* have been used as a purgative, antihelminthic, abortifacient as well as for treating ascites, gout, paralysis, and skin diseases. The seed oil has been used as an ingredient in the treatment of rheumatic conditions, itch and parasitic skin diseases, fever, jaundice and gonorrhoea, as a diuretic agent, and a mouth-wash. The seed is known to contain purgative oil, curcin, a phytotoxin known to cause dehydration, cardiovascular collapse as a result of haemorrhagic gastro-enteritis, and central nervous system depression[5]. Several cases of *J. curcas* nut poisoning in humans after accidental consumption of the seeds have been reported with symptoms of giddiness, vomiting and diarrhoea and in the extreme condition even death has been recorded[6].

It is also used traditionally for the treatment of sciatica, dropsy, paralysis, piles, snake bites, rheumatism, dysentery, diarrhoea and certain skin diseases[7-14]. The plant is known famous for its bio-diesel[15]. The levels of essential amino acids except lysine in *J. curcas* meal protein are higher than those of the FAO reference protein for a growing child of 2-5 years[16].

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## METHODS AND MATERIALS

### Plant material

The plant specimen i.e. seeds of *Jatropha curcas* L. were collected from Banthara, Lucknow, India in 2010. The plant was authenticated by Dr. AKS Rawat, NBRI. A voucher specimen (262541) has been submitted in institute's herbarium.

### Extraction of plant material

The fresh seeds were collected, thoroughly washed with water to remove all debris. The seeds were shade dried. The dried seeds were powdered by using electric grinder at 100 mesh size. Extraction was performed by soxhlation process in two steps. Firstly the powdered seeds are defatted under soxhlet assembly using 250ml of 98% petroleum ether for 6 hours. This is followed by 9 hours soxhlation of defatted seeds powder by using 250 ml of ethanol, aqueous and 50% hydroalcoholic solvent separately. The final extracts obtained were passed through Whatman No. 1 filter paper. The filtrates obtained were concentrated under vacuum in a rotary evaporator at 40°C and stored at 4°C for further use. The crude extracts were obtained by dissolving a known amount of dry extract in 98% ethanol to obtain a stock solution of 1000 µg/ml. The stock solutions of all three extracts were serially diluted with methanol to obtain lower dilutions (10, 15, 20, 25, 30, 35, 40, 45, 50 and 60 µg/ml).

### Physicochemical and Phytochemical Studies

Physicochemical and Phytochemical studies were performed according to the methods[17,18,19], such as extractive values, total ash, acid insoluble ash, total sugar, starch, tannin, and phenols on shade-dried powdered material.

### *In vitro* antioxidant Activity

Antioxidant activity of the plant extracts and standard was assessed on the basis of the radical scavenging effect of the stable DPPH free radical.[20] The diluted working solutions of the test extracts were prepared in methanol. Ascorbic acid was used as the standard in solutions ranging from 1 to 10 µg/ml. 0.002% DPPH solution in methanol was prepared. Then 2 ml of this solution was mixed with 2 ml of sample solution and the standard solution to be tested separately. These solution mixtures were kept in the dark for 20 min and optical density was measured at 517 nm using a Shimadzu spectrophotometer against methanol. The blank used was 2 ml of methanol with 2 ml of DPPH solution (0.002%).

The optical density was recorded and percent of inhibition was calculated according to the methods[21] using the formula given below:

% of inhibition of DPPH activity = (A–B/A) × 100, where, A is optical density of the blank and B is optical density of the sample.

### HPTLC Studies

Reagents used were from Merk (Germany) and standard Gallic and protocatechuic acid was procured from Sigma-Aldrich (Steinheim). Air dried (45-55°C) powdered seeds of *J. curcas* (2.0g) in triplicate were extracted separately with 3 × 10 ml 50% hydroalcoholic solvent. Extracts were concentrated under vacuum and redissolved in methanol, filtered and finally made up to100 ml with methanol prior to HPTLC analysis.

### Chromatographic Conditions

Chromatography was performed on Merk HPTLC precoated silica gel $60GF_{254}$ (20X20 cm) plates. Methanolic solutions of samples and standard compounds gallic and protocatechuic acid of known concentrations were applied to the layers as 6 mm-wide bands positioned 15 mm from the bottom and 15 mm from side of the plate, using Camag Linomat V automated TLC applicator with nitrogen flow providing a delivery speed of 150 nl/s from application syringe. These conditions were kept constant throughout the analysis of samples.

### Detection and Quantification of Gallic and Protocatechuic acid

Following sample application, layers were developed in a Camag twin trough glass chamber that had been pre-saturated with mobile phase of toluene : ethyl acetate : formic acid (5:5:0.5) till proper separation of bands up to 8 cm height. After development, layers were dried with an air dryer and gallic and protocatechuic acid was simultaneously quantified using Camag TLC scanner model 3 equipped with Camag Wincats IV software. Following scan conditions were applied: slit width 5 mm x 0.45 mm; wavelength 310 nm for gallic acid and 280 nm for protocatechuic acid and absorption-reflection mode. In order to prepare calibration curves, stock solution of gallic and protocatechuic acid (1 mg/ml) was prepared and various volumes of these solutions were analyzed through HPTLC, calibration curves of peak area Vs. concentration were prepared for quantification.

## RESULTS AND DISCUSSION

### Phytochemical screening

Hydroalcoholic extract of *J. curcas* seeds showed positive test for flavonoids, proteins, carbohydrates, glycosides, phenolic compounds and saponins.

### Physicochemical Studies

Parameters such as Extractive values (Water, alcohol and petroleum ether soluble), total ash and acid insoluble ash values, total Sugar, total starch, total tannins and total phenolics were determined. Results are shown in Fig 1.

### Antioxidant activity

Invitro antioxidant study of *J. curcas* seeds was performed using three different seed extracts viz. ethanolic extract, aqueous extract and hydroalcoholic extract. Ascorbic acid is used as standard. In this study hydroalcoholic extract was found to be most potent among all three extracts which showed the least $IC_{50}$ value of 32.66 ± 1 µg/ml, whereas

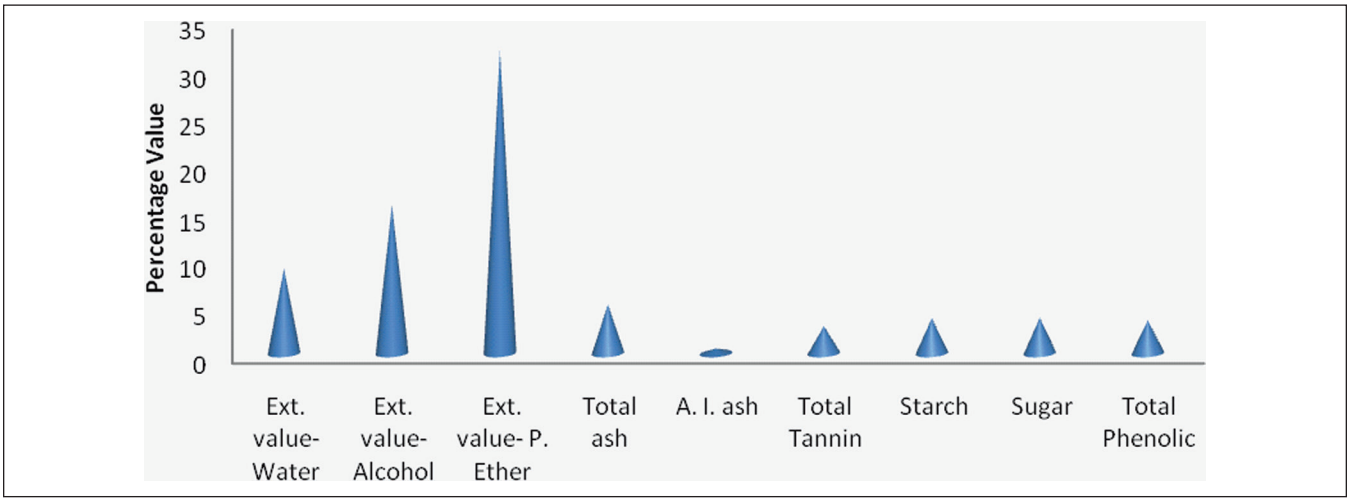


**Figure 1:** Physico-chemical values of *J. curcas* seed

**Table 1: Antioxidant Activity**

| S. No. | Samples | $IC_{50}$(µg/ml) |
|---|---|---|
| 1 | Ascorbic acid | 2.66 ± 0.11 |
| 2 | *J. curcas* ethanolic extract | 46 ± 3.46 |
| 3 | *J. curcas* aqueous extract | 36.66 ± 0.57 |
| 4 | *J. curcas* hydroalcoholic | 32.66 ± 1 |

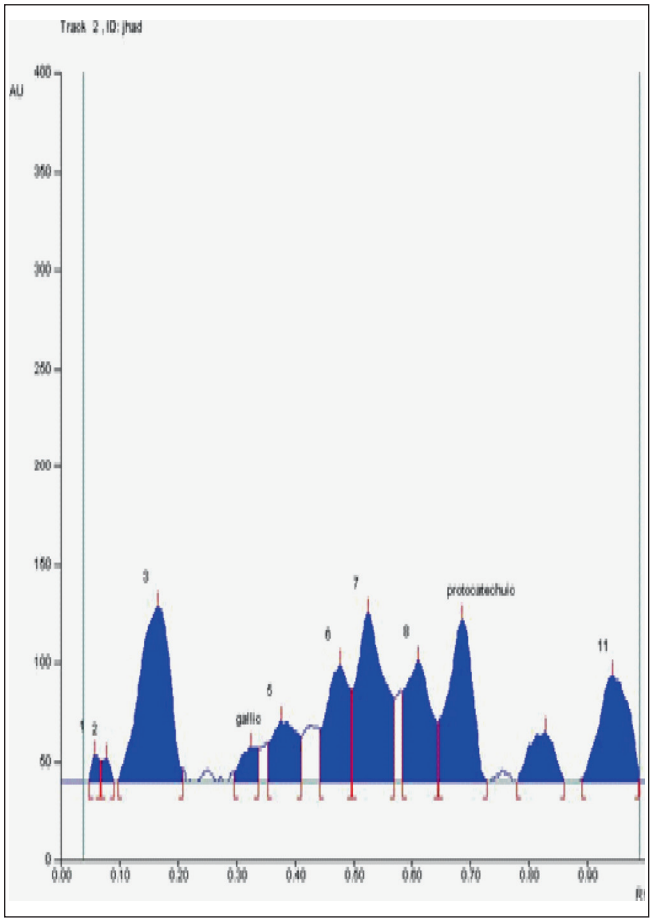


**Figure 2:** Densitogram obtained from extract, showing gallic and protocatechuic acid

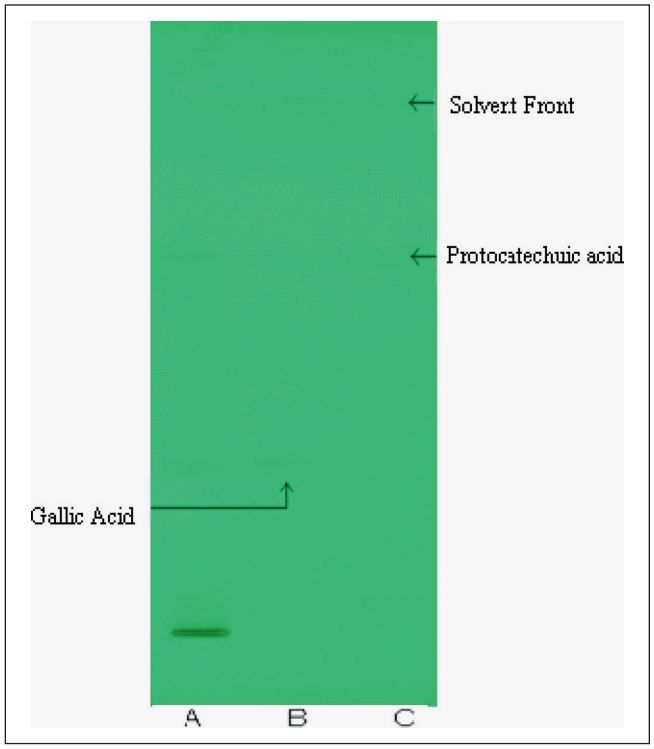


**Figure 3:** Banding pattern of *J. curcas* hydroalcoholic Extract, Gallic and Protocatechuic acid
*A. J. curcas hydroalcoholic extract*
*B. Gallic acid; C. Protocatechuic acid*

aqueous and ethanolic extracts showed $IC_{50}$ value of 36.66 ± 0.57 µg/ml and 46 ± 3.46 µg/ml respectively. Standard ascorbic acid showed $IC_{50}$ value of 2.66 ± 0.11 µg/ml. Results are shown in Table 1.

## Results of HPTLC

The HPTLC of hydro-alcoholic extract of *J. curcas* seeds was performed with different phenolic marker compounds

at six point calibration curve in which gallic acid and protocatechuic acid were observed and quantified. A Densitogram and Banding pattern obtained from extract shows gallic and protocatechuic acid, Fig 2 & 4.

Gallic acid was estimated up to 0.092% in hydroalcoholic extract, $r^2 = 0.994$, $y = 70.572+11.934*x$, $R_f = 0.32$, Fig 3. In same extract protocatechuic acid was quantified up to 0.146%, $r^2 = 0.998$, calibration equation: $y = 701.4 + 9.283 * x$. $R_f = 0.69$, Fig 5.

## CONCLUSION

*J. curcas* seed extract showed significant in vitro antioxidant activity, hydroalcoholic extract showed the most potent activity that could protect against oxidant and free radical injuries, in addition to having their medicinal properties. Thus, the effective source of *J. curcas* seeds could be employed in all medicinal preparations to combat myriad diseases associated with oxidative stress and related disorders. Presence of phenolic compounds in all three extracts suggests that the antioxidant activity may be due to the polyphenolic content. The presence of protocatechuic and gallic acid quantified in this species may be utilized for the proper standardization of the drug.

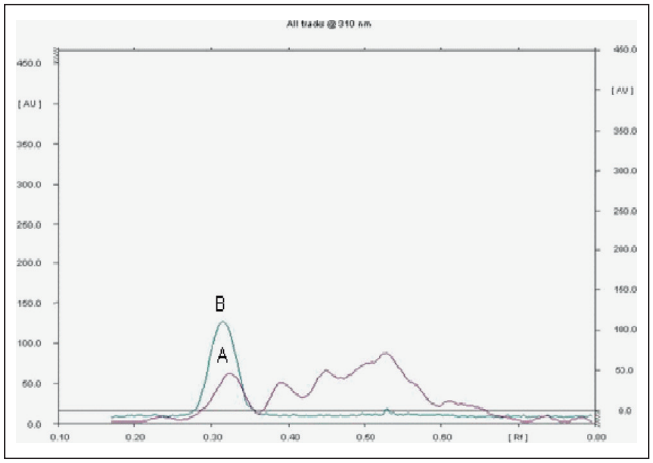


**Figure 4:** TLC chromatogram of extract and standard (Gallic acid)
*A. J. curcas hydroalcoholic extract* *B. Gallic acid*

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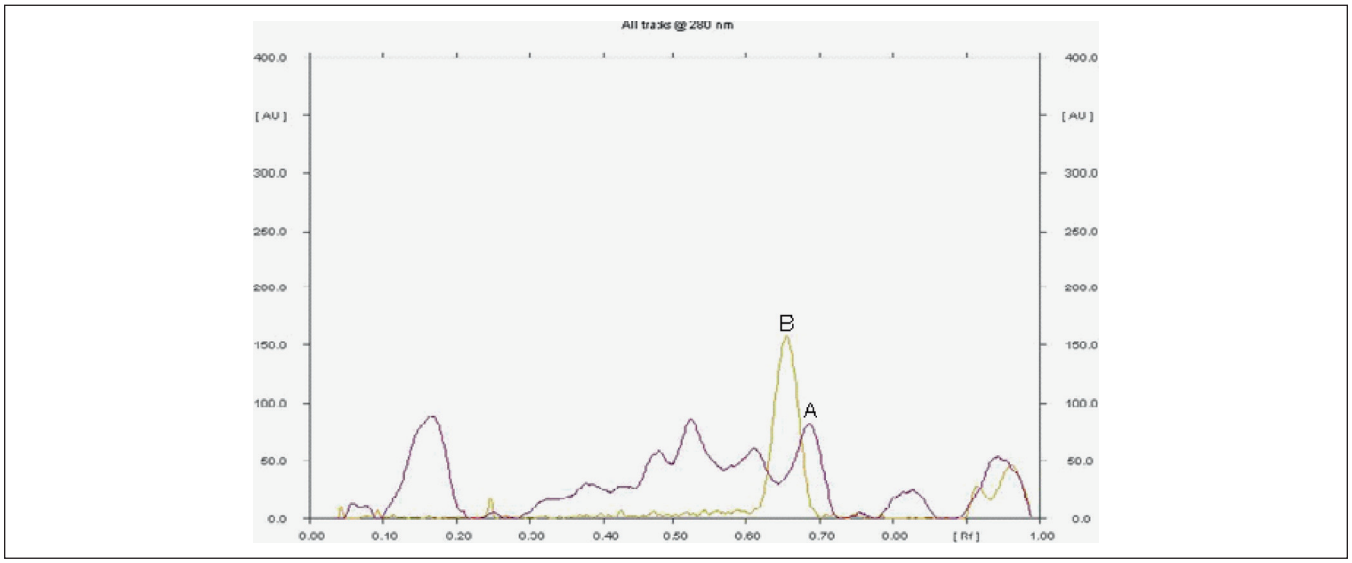


**Figure 5:** TLC chromatogram of extract and standard (Protocatechuic acid)
*A. J. curcas hydroalcoholic extract*
*B. Protocatechuic acid*

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# Antimicrobial and Antifungal Activities of Ethanol and Aqueous Crude Extracts of *Hymenocardia acida* Stem Against Selected Dental Caries Pathogens

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## ABSTRACT

The ethanol and aqueous crude extracts of *Hymenocardia acida* stem showed inhibitory activity against all the tested microorganisms, *Staphylococcus aureus, Staphylococcus auricularis, Streptococcus pyogenes, Streptococcus mutans, Candida albicans, Aspergilus flavus, Microsporium gypseum* and *Bacillus subtilis.* The largest zone of inhibition was obtained with the ethanolic extract (100 mg/ml) against *S. mutans* (16.73 ± 0.07 mm) and the least zone of inhibition was obtained with the aqueous extract (100 mg/ml) against *S. pyogenes* (5.83 ± 0.03 mm). Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were also determined against the selected microorganisms showing zones of inhibition ≥ 6.25 mm for MIC and ≥ 12.25 mm for MBC. Phytochemical screening indicates the availability of secondary metabolites in the plant. This study reveals that the stem of *H.* acida possess antifungal and antibacterial activities respectively and can be used as a potential source of antimicrobial ingredients to cure dental carries pathogens.

**Key words:** Bactericidal, inhibition, secondary metabolites.

## INTRODUCTION

Reported[1] that some of the chewing sticks being used are obtained from the following plants: *Garcinia manni, Musalaria accuminita, Terminalia glaucescens, Anogeissus leiocarpus, Pseudocedrela kotschyi, Zanthoxyllum gilleti* and *Azadirachta indica.*

Investigation further revealed that some of these chewing sticks possess anti-microbial activity against oral microbial flora such as *Staphylococcus aureus* and *S. auricularis*[1], *Candida albicans, Aspergillus flavus, Microsporium gypseum* and *Trichophyton metagrophytes*[2].

Confirmed[3] that chewing sticks have potential of preventing oral ailments. A majority of plants tested in his study revealed that chewing sticks are capable of inhibiting gram-positive and negative bacteria such as *Bacillus subtilis, Porphyromonus gingivalis* and *Fusobacterium nucleatum*. Chewing sticks with antimicrobial activity could become a potential source of new drugs for oral diseases. Oral microorganisms are known for their pathogenesis in tooth decay, gingivitis, periodontitis, and their ability to cause teeth loss[4].

Utilization of non-timber forest products (NTFP) are widely used as chewing sticks for dental cleaning in tropical Africa, they impact varying taste and sensation such as a tingling peppery taste, a bitter taste and numbness[5,6]. Posited that chewing sticks, in addition to providing mechanical stimulation of the gums, also destroy microbes; these advantages of the chewing sticks over the conventional toothpaste and brush has been attributed to the strong teeth of Africans[7]. *Hymenocardia acida* belong to the family Euphorbiaceae is a small savannah tree or shrub about 9 m high. Branchlets become rusty brown as the bark peels. The bole is short, often flattened and usually crooked. The branches form a fairly heavy, somewhat rounded crown. Leaves thin, leathery, elliptic-oblong up to 8.75 cm long and 3.75 cm broad, apex obtuse to rounded, base obtuse; petiole slender, up to 1.8 cm long.

## OBJECTIVES OF THE STUDY

Chewing sticks with antimicrobial activity could become a potential source of new drugs for oral diseases, hence

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this study is aimed at testing the phytochemistry, antimicrobial activities, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) levels of the aqueous and alcoholic extracts of stem of Hymenocardia acida used as chewing sticks collected from Edo state, Nigeria.

## MATERIALS AND METHODS

### Collection and identification of plant material

The stems of *Hymenocardia acida* plant were collected from Edo North Senatorial District of Edo State. The plants were identified by Dr J.F Bamidele of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State.

### Preparation and extraction of plant material

The fresh stems *Hymenocardia acida* were cut from the plants, rinsed in water and spread on trays and dried under the sun. The plant materials were then transferred to the oven set at 45$^0$C for 20-30 minutes before being reduced to fine powder with the aid of a mechanical grinder. The powdered plant materials were then collected and stored in a tightly covered glass jars and kept for further studies.

For ethanol extraction, 100 g of the powdered stem and root materials were soaked in 600 mls of ethanol. The resultant solution was filtered using Whatman filter paper No 1 after 48hours under room temperature (25°C). For aqueous extraction, 100 g of the powdered stems and root materials were boiled in 600 mls of water for 24 hours after which the resultant solutions were filtered using Whatman filter paper No 1.

The two extracts were concentrated through evaporation process using a water bath set at 100$^0$C. The extracts were then stored in a refrigerator until required for use.

### Preparation of stock solution of extracts

Fresh stock (known concentration) solution of each extract was prepared for each experiment. To prepare a required concentration of the extract, a specific weighed amount of the concentrated extract was dissolved completely in an appropriate volume of distilled water. To prepare 100 mg/ml concentration of extract, 1 gm of either of the extract was dissolved in 10 ml of distilled water in a sample bottle, corked and shaken vigorously to obtain a homogenous solution.

### Phytochemical screening

The phytochemical tests were carried out on the aqueous and ethanolic extracts using standard procedures as described by[8,9].

### Source of microorganisms

Pure stock cultures of *Staphylococcus aureus, Staphylococcus auricularis, Streptococcus pyogenes, Streptococcus mutans, Candida albicans, Aspergilus flavus, Microsporium gypseum* and *Bacillus subtilis* isolated from patients with dental diseases were obtained from the "Department of Medical Microbiology", "Department of Dentistry University of Benin", and "University of Benin Teaching Hospital (UBTH)". These pure isolates were used and maintained in slants of Nutrient Agar (NA) and Potato Dextrose Agar (PDA) at 4$^o$C until when needed for further studies.

### Microbial inoculums preparation for susceptibility testing

The inocula of the bacterial isolates were prepared by growing each pure isolate in nutrient broth at 37$^o$C for 24 hrs. The fungal isolates were grown in Potato dextrose broth at 28 ± 2$^o$C for 48 hrs.

After incubation, 1 ml of the diluted cultures of the microbial isolates in normal saline using a Pasteur pipette was inoculated unto the solidified nutrient agar at 40$^o$C for bacteria and Potato dextrose agar for fungi.

### Antimicrobial assay

Antimicrobial activity was evaluated by noting the zone of inhibition against the test organisms[10]. Two colonies of a 24-hour plate culture of each organism were transferred aseptically into 10 ml sterile normal saline in a test tube and mixed thoroughly for uniform distribution. A sterile cotton swab was then used to spread the resulting suspension uniformly on the surface of oven-dried Nutrient agar and Potato dextrose agar plates for bacteria and fungi, respectively. Three (3) adequately spaced wells of diameter 4 mm per plate were made on the culture agar surface respectively using a sterile metal cup-borer. 0.2 ml of each extract and control were put in each hole under aseptic condition, kept at room temperature for 1 hour to allow the agents to diffuse into the agar medium and incubated accordingly. Conventional antibiotics were used as positive controls for bacteria and fungi respectively; distilled water was used as the negative control. The plates were then incubated at 37$^o$C for 24 hours for the bacterial strains and at 28$^o$C for 72 hours for fungal isolates. The zones of inhibition were measured and recorded after incubation. Zones of inhibition around the wells indicated antimicrobial activity of the extracts against the test organisms. The diameters of these zones were measured diagonally in millimeter with a ruler and the mean value for each organism from the triplicate cultured plates was recorded. Using the agar-well diffusion technique, an already made gram positive and gram negative (Asodisks Atlas Diagnostics, Enugu, Nigeria) standard antibiotic sensitivity disc bought from a laboratory chemical equipment store in Benin city was used

as positive control for bacteria while Ketoconanzone was used as positive control for fungi. Distilled water was used as negative control for all the test organisms.

### Determination of minimum inhibitory concentrations (MICs) of the extracts

The lowest concentration of the extracts that will inhibit the growth of test organisms is the Minimum Inhibitory Concentration (MIC). The initial concentration of the plant extract (100 mg/ml) was diluted using double fold serial dilution by transferring 5 ml of the sterile plant extract (stock solution) into 5 ml of sterile Normal saline to obtain 50 mg/ml concentration[11]. Different concentrations were prepared from the crude extract by doubling dilution in distilled water. The different concentrations were 50, 25, 12.5, 6.25 and 3.125 mg/ml respectively. Each dilution was introduced into nutrient agar plates and potato dextrose agar plates already seeded with the respective test organism. All test plates were incubated at 37°C for 24 hrs for bacteria and 28°C ± 2°C for 72 hrs for fungi. The minimum inhibitory concentration (MIC) of the extracts for each test organism was regarded as the agar plate with the lowest concentrations without growth[10].

### Minimum bactericidal concentration (MBC)

The Minimum Bactericidal Concentration (MBC) of the plant extracts were determined by the method described by[12,13]. Samples were taken from plates with no visible growth in the MIC assay and subcultured on freshly prepared nutrient agar plates and Potato dextrose agar plates and later incubated at 37°C for 48 hours and 28 ± 2°C for 72 hours for bacteria and fungi respectively. The MBC was taken as the concentration of the extract that did not show any growth on a new set of agar plates.

### Determination of the antibiotic susceptibility of bacteria isolates

The disc diffusion method[14] was used for the determination of microbial sensitivity. The antibiotic discs employed were: septrin, chloranphenicol, sparfloxcarcin, ciprofloxacin, amoxicillin, augmenting, gentamicin, pefoxacin, ofloxacin, streptomycin, zinnacef and recophin. The zones of inhibition were measured and interpretation was in accordance with manufacturer's instructions.

## RESULTS AND DISCUSSION

In Table I the results of the phytochemical analysis of aqueous and ethanolic stem extracts of *H. acida* revealed the presence of some secondary metabolites such as alkaloids, flavonoids, cardiac glycosides, anthraquinones, phlobotannins, tannins, saponins and carbohydrates. Phlobotannins and terpenoids were absent in ethanol extracts of *H. acida*. Table II

**Table 1: Phytochemical screening of the aqueous and ethanol extracts of *Hymenocardia acida* stem used as chewing sticks**

| *H. acida* (stem) | Test plant extracts | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Chemical components | | | | | | | | | |
| | Alkaloids | Flavonoids | Anthraquinones | Saponins | Tanins | Cardiac Glycosides | Steroids | Terpenoids | Phlobatanins | Carbohydrates |
| Aq | + | + | + | + | + | + | + | + | + | + |
| Et | + | + | + | + | + | + | + | – | – | + |

KEY: + = Present, – = Absent, Aq = Aqueous, Et = Ethanol

**Table 2: Zone of inhibition of Aqueous and Ethanol extracts (100 mg/ml) of *Hymenocardia acida* against selected oral pathogens**

| *H. acida* (stem) | Test Organisms | | | | | | |
|---|---|---|---|---|---|---|---|
| | *S. aureus* | *S. auricularis* | *M. gypseum* | *S. pyogenes* | *S. mutans* | *B. subtillis* | *A. flavus* | *C. albicans* |
| Aq | 6.83 ± 0.03 | 6.20 ± 0.58 | 7.20 ± 0.06 | 5.83 ± 0.03 | 6.70 ± 0.06 | 8.13 ± 0.07 | 8.70 ± 0.06 | 5.90 ± 0.06 |
| Et | 15.60 ± 0.06 | 15.80 ± 0.06 | 10.30 ± 0.58 | 13.90 ± 0.06 | 16.73 ± 0.07 | 16.30 ± 0.06 | 8.90 ± 0.06 | 13.90 ± 0.06 |

NB: Means ± S.E.M; n=3, Means ± S.E.M within a row are significantly different, P< 0.01.

**Table 3: Zone of inhibition in mm of various concentrations of the ethanol extract of *Hymenocardia acida* on test organisms**

| Test organisms | Concentration of extract (mg/ml) | | | | | | Sterile distilled water |
|---|---|---|---|---|---|---|---|
| | 3.125 | 6.5 | 12.5 | 25 | 50 | 100 | |
| *S. aureus* | – | – | 5.20 ± 0.06[a] | 8.50 ± 0.06[b] | 9.03 ± 0.09[c] | 14.40 ± 0.06[d] | – |
| *S. auricularis* | – | – | 2.40 ± 0.06[a] | 4.77 ± 0.09[b] | 6.67 ± 0.09[c] | 11.30 ± 0.06[d] | – |
| *S. pyogenes* | – | – | – | 6.03 ± 0.15[a] | 7.43 ± 0.09[b] | 13.33 ± 0.12[c] | – |
| *S. mutans* | – | – | – | 6.53 ± 0.09[a] | 8.13 ± 0.09[b] | 14.90 ± 0.12[c] | – |
| *M. gypseum* | – | – | – | 3.43 ± 0.12[a] | 6.03 ± 0.20[b] | 10.40 ± 0.12[c] | – |
| *B. subtilis* | – | – | – | 2.73 ± 0.07[a] | 5.47 ± 0.09[b] | 8.77 ± 0.67[c] | – |
| *A. flavus* | – | – | – | – | – | 12.70 ± 0.60[a] | – |
| *C. albicans* | – | – | – | – | 3.53 ± 0.09[a] | 5.03 ± 0.15[b] | – |

NB: Means ± S.E.M; n=3, Means ± S.E.M within a row are significantly different, P < 0.01.
– = No inhibition.

**Table 4: Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal**

| *H. acida* (stem) (mg/ml) | Test Bacteria | | | | |
|---|---|---|---|---|---|
| | *S. aureus* | *S. auricularis* | *S. pyogenes* | *S. mutans* | *B. subtillis* |
| MIC | 12.50 | 12.50 | 25.00 | 25.00 | 12.50 |
| MBC | 25.00 | 25.00 | 50.00 | 25.00 | 25.00 |

Concentrations (MBCs) in mg/ml of the ethanol extracts of *Hymenocardia acida* plant against the test bacteria
Means ± S.E.M; n=3, Means ± S.E.M within a row are significantly different, P< 0.01.
– = No inhibition.

shows the antimicrobial properties of the ethanol extract of the *H. acida* on the test icroorganisms. The entire test organisms were sensitive to the ethanol extracts at a concentration of 100 mg/ml. Plant extracts were more susceptible to *A. flavus* (fungus) followed by *B. subtilis* (gram +ve rod bacteria), *S. mutans* (gram +ve), *S. auricularis* (gram +ve), *S. aureus* (gram +ve), *C. albicans* (fungus), *S. pyogenes* (gram +ve) and *M. gypseum* (fungus) respectively. Table III revealed that the antimicrobial activity of the aqueous extract of the *H. acida* plant extracts were significantly different from one to another on each organism. The ethanol extract has the highest antibacterial and antifungal activity against all the tested oral microorganisms with inhibition diameters of 17.20 ± 0.06 mm and 5.83 ± 0.03mm respectively at 100 mg/ml. Table IV presents the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) values of the ethanol extracts. The ethanol extract of *H. acida* showed minimum inhibitory concentration (MIC) of 12.5 mg/ml against *B. subtilis* and *S. auricularis* while for the fungi 6.25 mg/ml concentration was sensitive to *M. gypseum* and it is fungicidal (MFC) at 12.5 mg/ml to *M. gypseum* (Table V) and shows minimum bactericidal concentration (MBC) of 25 mg/ml against *B. subtilis, S. mutans* and *S. auricularis* (Table IV).

Table VI shows the activity of the commercial antibiotics (standard sensitivity disc) on the test bacteria. It revealed a sensitivity zone of inhibition diameter varying from 4.0 mm – 28.3 mm against the bacterial isolates used. Table VII revealed that ketoconanzone (commercial fungi antibiotic) was active against all the test fungi. It had the highest activity against *M. gypseum* with inhibition diameter of 26 mm, followed by *C. albicans 24 mm* and *A. flavus* 17 mm.

**Table 5: Minimum Inhibitory Concentrations (MICs) and Minimum fungicidal Concentrations (MFCs) in mg/ml of the ethanol extracts of *Hymenocardia acida* plant against the test fungi**

| | Test fungi | | |
|---|---|---|---|
| *H. acida* (stem) (mg/ml) | *M. gypseum* | *A. flavus* | *C. albicans* |
| MIC | 6.25 | 100.00 | 50.00 |
| MFC | 12.50 | 100.00 | 50.00 |

NB: Values are means ± S.E.M (n=3);
Values within a row with different alphabet are significantly different, P < 0.01;.

The presence of bioactive compounds (qualitative phytochemicals) has been known to show medicinal activity as well as exhibit and regulate some physiological activity[15,16]. Saponins have been reported to be an antifungal agent, while tannins prevent the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable to them[17] and tannins have been traditionally used on inflamed surfaces of mouth and treatment of catarrh[18,19]. Also reported that tannins have antioxidant properties.

It was observed that susceptibility increased with increased concentration of the extracts and that ethanol extracts exhibited more activity, potency and consistency than the

**Table 6: Sensitivity zone of inhibition of commercial antibiotics (standard sensitivity disc) on the test bacteria**

| Test isolates | Zone of inhibition (in mm) for commercial antibiotics | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CN | APX | R | CPX | E | SXT | PEF | OFX | S | AM |
| *S. Aureus* | 28.3 | 7.4 | – | 24.5 | 9.7 | – | 21.6 | 27.0 | – | – |
| *S. auricularicus* | 27.0 | 4.6 | – | 20.9 | 7.7 | – | 17.8 | 28.1 | – | 10.5 |
| *S. pyogenes* | 19.7 | 6.8 | – | 20.1 | 5.2 | – | 14.6 | 24.5 | – | – |
| *S. mutans* | 20.6 | 4.0 | – | 18.7 | 8.8 | – | 14.5 | 20.9 | – | – |
| *B. subtilis* | 24.8 | 5.5 | – | 19.0 | 8.6 | – | 11.8 | 19.9 | – | 7.7 |

PEF = Pefloxacin (10 µg/ml), – = No inhibition
CN = Gentamicin (20 µg/ml APX = Ampiclox (30 µg/ml)
OFX = Ofloxacin (10 µg/ml) AM = Amoxacillin (30 µg/ml)
R = Rocephin (25 µg/ml) CPX = Ciprofloxacin (10 µg/ml)
S = Streptomycin (30 µg/ml) SXT = Septrin (30 µg/ml)
E = Erythromycin (10 µg/ml) APX = Ampiclox (10 µg/ml)

**Table 7: Sensitivity zone of inhibition of commercial fungi antibiotics (ketoconanzone) on the test fungi**

| Test fungi | Ketoconanzone (200 mg/ml) |
|---|---|
| *Aspergillus flavus* | 17 mm |
| *Candida albicans* | 24 mm |
| *Microsporium gypseum* | 26 mm |

aqueous extract. These results support earlier studies which observed that plant extracts in organic solvent provided more consistent antimicrobial activity compared with those extracted in water[20,21].

It was also observed that the extracts were active when compared with the negative control (sterile distilled water) against all the test organisms (Table III). The control recorded no visible activity. The positive control (standard sensitivity disc) used on the test bacteria revealed that gentamycin, perfloxacin, ampiclox, ofloxacin, ciprofloxacin, and erythromycin had inhibitory effects on all the test bacteria (Table VI). Amoxacillin has activity against *S. auricularis* and *B. subtilis* with inhibitory diameter of 10.5, 11.9 and 7.7 mm respectively. *S. aureus* was more sensitive to the commercial antibiotics with zone of inhibition of 28.3 mm for gentamycin, 27.0 mm for ofloxacin, 24.5 mm for ciprofloxacin and 21.6 mm for perfloxacin. Rocephin, streptomycin and erythromycin showed no inhibition zone on any of the tested organisms.

## CONCLUSION

Diet plays a major role in preventing dental caries, the practice of dental hygiene is also important. Even when people would prefer to use toothbrushes, they do not have access to toothpaste due to high cost or remoteness. Therefore, continued access to popular and effective sources of chewing sticks with anti-bacterial and anti-fungal properties is important as a primary health care measure. The results from these studies provide evidence for the ethnomedicinal use of the tested plant as chewing sticks.

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# *In vitro* Anthelmintic Activity of Stem Extracts of *Piper betle* Linn Against Pheritima Posthuma

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## ABSTRACT

In this study the anthelmintic activity of ethanolic and aqueous extracts of stems of *Piper betle* Linn was performed. Indian adult earthworms were used for the assessment of anthelmintic activity. Albendazole (40 mg/ml) was used as standard and normal saline water was used as vehicle respectively. Observations were made for the time taken to paralysis and death. In ethanolic extract [P (min) = 1.15, D = 2.16], the activity was found to be more effective as compared to the standard drug Albendazole [P (min) = 2.34, D (min) = 5.68] and aqueous extract [P (min) = 4.38, D (min) = 7.16]

The mode of action of Albendazole is to cause paralysis of worms and to expel them in the feaces. Albendazole causes degenerative alterations in the intestinal cells of the worm. Degenerative changes in organs like endoplasmic reticulum, the mitochondria results in decreased production of adenosine triphosphate (ATP), which is the energy required for the survival of the helminthes. Due to diminished energy production, the parasite is immobilized and eventually dies. The ethanolic extract of stems of *Piper betle* Linn not only demonstrated anthelmintic property but they also caused death of the worms when compared with marketed standard preparation i.e. Albendazole (40mg/ml) and different concentrations of aqueous extract. It is concluded that stems of *Piper betle* Linn is potent anthelmintic. Since this is the preliminary work, separation of chemical constituents which are responsible for the activity may be done in the future.

**Key words:** Anthelmintic activity, *Piper betle* Linn, Stems extracts, Pheritima posthuma

## INTRODUCTION

According to the World Health Organization two billion people are suffering from parasitic worm infections. Parasitic worms also infect living organisms and crops, affecting food production with a resultant economic impact. The morbidity due to parasitic diseases has been increasing in our population, including lymphatic filariasis (a cause of elephantiasis), onchocerciasis, and schistosomiasis.[1,2] Anthelmintic substances having considerable toxicity to human beings are present in foods derived from livestock, posing a serious threat to human health. Therefore there is very much need of drug discovery programmes. There is thus a small repertoire of chemotherapeutic agents available for treatment (Table1). In some respects, this situation has been exacerbated by the remarkable success of ivermectin over the last twenty years, which has decreased motivation for anthelmintic drug discovery programmes. This prompts concern, as anthelmintic resistance has been widely reported in livestock and it may also only be a matter of time before this phenomenon occurs in parasites of humans.[3] Anthelmintics are drugs that either kill or expel infesting helminthes (worms.) Helminthiasis is a disease in which a part of the body is infected with worms such as pinworm, roundworm or tapeworm. Typically, the worms reside in the gastrointestinal tract but may also burrow into the liver and other organs[4].

Broad spectrum anthelmintics are effective against parasitic flat worms and nematodes (e.g. Piperazine citrate, Diethylcarbamazepine citrate, Benzimidazole, Albendazole, and Thiobendazole). However, the majority of drugs are more limited in their action e.g. Praziquantel, (a drug used in the treatment of schistosomiasis and thought to act by disrupting calcium homeostasis)[5], has no activity against nematodes.

The Betle *(Piper betle* Linn), commonly known as paan is a vine belonging to the Piperaceae family. *Piperaceae* family

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belonging to superorder *Nymphaeiflloraea*, order *Piperales* and genus *Piper*. Genus piper comprises about 10 genera, 2000 species. The genus *Piper* (*Piperaceae*) is largely distributed in tropical and subtropical regions of the world.[6] Betle leaves have been chewed along with the nut areca since very ancient times. It can also be used as carminative, stomachic, anthelmintic, tonic, and aphrodisiac. Leaves of the plant *Piper betle* Linn are chewed by people at frequent intervals by patients suffering from hookworms, immature worms are thrown out with the frequent expectoration.[7-9] *Piper betel* has light yellow aromatic essential oil, with sharp burning taste. Leaf possesses activities like antidiabetic, antiulcer, antiplatelet aggregation, antifertility, cardiotonic, antitumour, antimutagenic, respiratory depressant.[10-15]

This became the basis of selection of this plant and particularly the stems. This experiment present research work aims to throw light upon the pharmacognostic, phytochemical and pharmacological account of the stems of *Piper betle* Linn.

### Traditional uses

The leaves of *Piper betle* are pungent , bitter, sweetish, acrid, heating, carminative, stomachic, anthelmintic, tonic, aphrodisiac, laxative useful in vata, kapha foul smell in the mouth, ozoena, bronchitis, elephantiasis of leg, improves appetite should not be taken in eye diseases, leprosy, poisoning, thirst, alcoholism, asthma, loss of consciousness.(Ayurveda)

The leaf has a sharp taste and good smell, improves taste and appetite, tonic to the brain, heart, liver, strengthens the teeth, lessens thirst, clear the throat vulnerary and styptic (unani).

### Chemical constituents

Leaves of *Piper betle* Linn contain protein 3.1%, carbohydrate 6.9%, minerals 2.3%, and tannins 2%. It contains calcium, phosphorus, iron, iodine and potassium is also present. Vitamin B, vitamin C and vitamin A. Leaf contains bitter compounds that are about 0.7 to 2.6%. It also contains an aromatic compound and stable oils like phenol and terpene. Besides this it contains eugenol, chavibetol and hydroxychavicol.

The active ingredients of betle oil, which is obtained from the leaves, are primarily a class of allylbenzene compounds. Though particular emphasis has been placed on chavibetol (betle-phenol; 3-hydroxy-4-methoxyallylbenzene), chavicol (p-allyl-phenol; 4-allyl-phenol).[15,16]

**Table 1: Key drugs registered for the treatment of parasitic worms in humans**

| Schistosomiasis (blood fluke) | Intestinal round worms |
|---|---|
| Antimonials | Piperazine |
| Metrifonate | Benzimidazoles |
| Oxamnaquine | Morantel |
| Praziquantel | Pyrantel |
| | Levamisole |
| Cestodiasis (tape worm) | Avermectins and milbemycins |
| Niclosamide | Closantel (and halogenated salicylamides) |
| Benzimidazoles | Emodepside |
| Praziquantel | |
| Fasciolasis (liver fluke) | Filariasis (tissue round worms) |
| Praziquantel | Diethylcarbmazine |
| Closantel | Suramin |
| (and halogenated salicylamides) | Ivermectin |

## MATERIALS AND METHODS:

### Procurement and authentication of the crude drug

The stems of *Piper betle* Linn were collected in the month of October from Baramati region, Maharashtra, India. Since the plant consist of aromatic principles; they were then

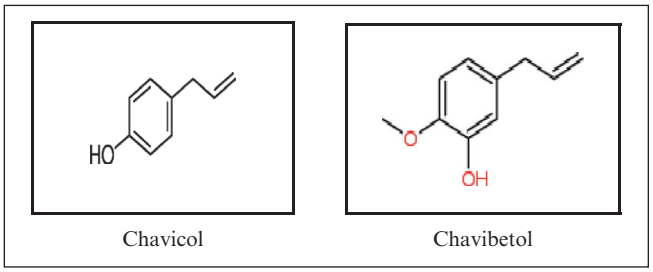


**Figure 1:** Anthelmintic activity of ethanolic extract of Piper betel stems.
*Results are expressed as Mean ± (n=6) from 6 observations.*
**Vehicle worms remain alive up to 24 hrs observations.*

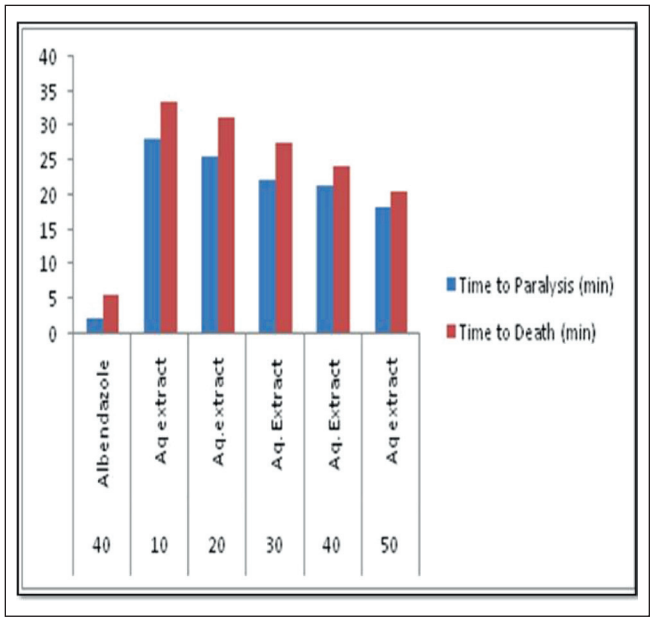


**Figure 2:** Anthelmintic activity of aqueous extract of Piper betel stems.
*Results are expressed as Mean ± (n=6) from 6 observations.*
**Vehicle worms remain alive up to 24 hrs observations.*

shade dried. Herbarium of the plant was prepared and it was authenticated by Agharkar Research Institute, Pune (SIOP/ authenticated/ 2010-11/3951).

### Storage of crude drug material

The stems were powdered finely in the conventional mixer and stored in airtight self locking polybags. Since the *Piper betle* Linn consist of volatile oil and other active constituents.

### Worms Collection

Indian adult earthworms i.e. *Pheritima posthuma* were used to study anthelmintic activity. The earthworms were collected from the water logged area of soil, Pune, washed with normal saline to remove all fecal matter. The earthworms of 5-8 cm in length and 0.2-0.3 cm in width were used for all experimental protocol.

### Preparation of stem extracts

The stems of *Piper betle* Linn were shade dried. They were then milled using commercial laboratory blender. The powder of the stems of *Piper betle* Linn was defatted with petroleum ether. (40-50 degree) The ethanolic and aqueous extracts were successfully obtained from the defatted material by using soxhlet extraction method. All the extracts obtained were concentrated to dryness in vacuum at 40 $^{0C}$ and stored at 4$^{0C}$ in the refrigerator until further used. The extracts were subjected to phytochemical and pharmacological evaluation.

### Phytochemical screening

Freshly prepared extracts were subjected to phytochemical screening tests for various constituents like proteins, fats, carbohydrates, alkaloids, steroids, and flavanoids by standard reagents.[17]

### Experimental Animals

Indian adult earthworms (*Pheritima posthuma*) were collected from moist soil and washed with normal saline to remove all fecal matter was used for the anthelmintic study. The earthworms of 3-5 cm in length and 0.1 - 0.2 cm in width were used for all the experimental protocol due to their anatomical and physiological resemblance with the intestinal Roundworm parasites of human beings.

### Drugs

Standard drug: Albendazole (40mg/ml) (Bendex Suspension, Protec division of Cipla Ltd.)

Chemicals:-Ethanol A.R (Thomas baker chemicals Pvt Ltd), Distilled water, Dimethyl sulfoxide (Thomas baker chemicals Pvt Ltd), Vehicle used: Saline water.

### Assessment of Anthelmintic activity

The assay was performed on adult Eritrean earthworm *Pheritima posthuma*, due to its anatomical and physiological resemblance with the intestinal round worm parasite of human being.[9] All animals were divided into six group containing six earthworms in each. Ethanolic extract of *Piper betle* Linn was dissolved in minimum amount of dimethyl sulfoxide while aqueous extract in water. All the earthworms were washed in a normal saline solution and then earthworms from each group were released into 10 ml of respective formulations as follows: Vehicle (5%DMSO in normal saline), Albendazole (40mg/ml), Ethanolic extract (10-50mg/ml) and aqueous extract(10 -50mg/ml).[18]

Observations were made for the time taken to paralysis (was said to occur when the worm did not revive even in normal saline solution) and death (was concluded when the worms lost their motility followed with fading away of their body colors).[19,20]

## RESULTS

Preliminary phytochemical analysis showed the presence of flavanoids, tannins and steroids like phytoconstituents in the ethanolic extracts and aqueous extract respectively. (Table 2)[21,22] Ethanol and aqueous extracts were used to evaluate anthelmintic activity has shown dose dependant activity. The Mean ± S.D. values (statistical analysis) were calculated for each extracts. The result of anthelmintic activity on earthworm *Pheritima posthuma* was given in Table 3 and Table 4, reveals that, the different concentration used for both aqueous and alcoholic extracts has shown paralysis and death of earthworms and it was compared in the same concentration with albendazole as reference drug. Indian adult earthworms were used for the assessment of anthelmintic activity as experimental animals. Albendazole (40 mg/ml) was used as standard and normal saline water was used as vehicle respectively. Observations were made for the time taken to paralysis and death.[23] Paralysis was said to occur when the worm did not revive even in normal saline solution and death was concluded when the worms lost their motility even when vigorously shaken or dipped

**Table 2: Preliminary Phytochemical Investigations of Stem Extracts**

| Piper betle Stem Extract | Carbohydrates | Flavonoids | Proteins | Saponins | Alkaloids | Steroids | Tannins |
|---|---|---|---|---|---|---|---|
| Ethanolic extract | -ve | +ve | -ve | -ve | -ve | -ve | +ve |
| Aqueous Extract | -ve | -ve | -ve | -ve | -ve | +ve | -ve |

**Table 3: Assessment of Anthelmintic activity (Ethanolic extract)**

| Sr. No. | Treatment | Dose(mg/ml) | Time to Paralysis (min) | Time to Death (min) |
|---|---|---|---|---|
| 1 | Albendazole | 40 | 2.34 ± 0.12 | 5.68 ± 0.13 |
| 2 | Ethanolic extract | 10 | 3.53 ± 0.23* | 7.21 ± 0.34* |
| 3 | Ethanolic extract | 20 | 2.56 ± 0.22* | 5.43 ± 0.23* |
| 4 | Ethanolic extract | 30 | 2.2 ± 0.11* | 4.14 ± 0.22* |
| 5 | Ethanolic extract | 40 | 1.45 ± 0.24** | 3.01 ± 0.21** |
| 6 | Ethanolic extract | 50 | 1.15 ± 0.25** | 2.15 ± 0.24** |

Values are Mean ± S.D analyzed by Dunnett's test *= p<0.01, **= p<0.05

**Table 4: Assessment of Anthelmintic activity (Aqueous extract)**

| Sr. No. | Treatment | Dose(mg/ml) | Time to Paralysis (min) | Time to Death (min) |
|---|---|---|---|---|
| 1 | Albendazole | 40 | 2.34 ± 0.54 | 5.68 ± 0.34 |
| | Aq. Extract | 10 | 8.24 ± 0.51 | 12.56 ± 0.44 |
| 2 | Aq. Extract | 20 | 7.19 ± 0.32 | 10.55 ± 0.36 |
| 3 | Aq. Extract | 30 | 6.23 ± 0.23 | 8.44 ± 0.50 |
| 4 | Aq. Extract | 40 | 5.58 ± 0.54* | 8.12 ± 0.39* |
| 6 | Aq. Extract | 50 | 4.38 ± 0.42* | 7.16 ± 0.31* |

Values are Mean ± S.D analyzed by Dunnett's test *= p<0.01, **= p<0.05

in warm water (50°C), followed with fading away of their body colors.[24,25] In ethanolic extract [P (min) = 1.15, D (min) = 2.16], the activity was found to be more effective as compared to the standard drug Albendazole [P(min) = 2.34,D(min) = 5.68] and aqueous extract [P(min) = 4.38,D(min) = 7.16]

## DISCUSSION

Earthworms have an anatomical resemblance with the intestinal worms like tapeworms, roundworms, pinworms etc. They also show physiological similarity in mechanism of action.[26] The present study reveals that stems of *Piper betle* Linn showed mark and potent anthelmintic activity. Alcoholic extract had shown promising result as anthelmintic activity and water extracts has also shown activity up to lesser extent.

Observations were made for the time taken to paralysis and death of individual worms in standard drug that is Albendazole. It causes degenerative alterations in the tegument and intestinal cells of the worm by binding to the colchicine-sensitive site of tubulin, thus inhibiting its polymerization or assembly into microtubules. The loss of the cytoplasmic microtubules leads to impaired uptake of glucose by the larval and adult stages of the susceptible parasites, and depletes their glycogen stores. Degenerative changes in the endoplasmic reticulum, the mitochondria of the germinal layer, and the subsequent release of lysosomes result in decreased production of adenosine triphosphate (ATP), which is the energy required for the survival of the helminthes.[27,28,29] Due to diminished energy production, the parasites are immobilized and eventually die at different concentrations of ethanolic and aqueous extracts of *Piper betle* Linn.

The data revealed that the ethanolic extract showed remarkable anthelmintic activity as compared to the standard drug and aqueous extract. The function of the anthelmintic drugs like Albendazole is to cause paralysis of worms and to expel them in the faces. The ethanolic extract of stems of *Piper betle* Linn not only demonstrated anthelmintic property but they also caused death of the worms when compared with marketed standard preparation i.e. Albendazole (40mg/ml) and different concentrations of aqueous extract. Phytochemical analysis of extracts of *Piper betle* Linn revealed the presence of tannins and flavanoids in ethanolic extract. Tannins were shown to produce anthelmintic activities chemically tannins are polyphenolic compounds.[30] Some synthetic phenolic anthelmintic e.g. niclosamide, oxyclozanide, bithionol etc., are reported to interfere with energy generation in helminthes parasites by uncoupling oxidative phosphorylation[31]. Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite[32,33]

## CONCLUSION

It is concluded based on the findings of the present study that the stems of *Piper betle* Linn are potent anthelmintic. Phytochemical screening states the presence of tannins and flavanoids in stem extracts; therefore anthelmintic activity may be due to tannins or flavanoids. However, dose and the form in which they can be used, require standardization. Further research efforts are required for

the depilation of chemical constituent and study of the individual constituents which all are responsible for this activity.

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