Hair Loss and the Applied Techniques for Identification of Novel Hair Growth Promoters for Hair Re-Growth

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ABSTRACT

Hairs are the characteristic covering of mammalian skin, originated from embryonic ectoderm. In humans, the main function of the hair is an important facet of appearance. Baldness or commonly called Alopecia is defined as the loss of hair from an area of the body. Hair loss affects millions of people, including over 40 % of men over the age of 30 and a significant number of women also. The factors responsible for hair loss are scarring, disease, infection, less blood circulation in the blood capillaries of scalp and sensitivity to the androgen. Although alopecia requires a careful history, close attention to the appearance of the hair loss, and a few simple studies can quickly narrow the potential diagnosis. The alopecia which is caused due to androgen sensitivity is called androgenetic alopecia. Andogenetic alopecia is one of the most common forms of hair loss, usually has a specific pattern of temporal-frontal loss in men and central thinning in women. The importance of hair either hair loss as well as the overgrowth of terminal hair on the body or face, has deleterious effects on self-esteem. Therefore the main focus of this review article is on causes of hair loss and the active ingredients for hair re-growth along with the assessment techniques.

Key words: Alopecia, Hair growth, Natural ingredients, Hair follicle.

INTRODUCTION

Hair is a filamentous biomaterial, which grows approximately 0.3 mm/day or 6 inches per year, while the scalp sheds 100 hairs per day.[1] Hair often refers to two distinct structures: The part beneath the skin, is called the hair bulb. The second part is called hair shaft which is the hard filamentous part that extends above the skin surface.[2] A cross section of the hair shaft may be divided roughly into three zones known as the cuticle which consists of several layers of flat, thin cells laid out overlapping one another as roof shingles. The second innermost layer is called the cortex which contain the keratin bundles in cell structures that remain roughly rod-like. The innermost layer is called the medulla, a disorganized and open area at the fiber's centre.[3] There are different types of the hairs produced by the body. The first and foremost hair which is produced in uterus called lanugo hair. Such type of hair is fine, soft, poorly pigmented and has no central medulla.[4]

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The second one is called Vellus hair, which is non-medullated, fine, and poorly pigmented. The intermediate hair is first observed post-natally as the scalp hair growth subsequent to the initial lanugo hair growth. Intermediate hair is characterized by a relatively rough cuticle, sparse pigmentation and a fragmented or absent medulla.[5] Eyelashes have the largest diameter of all body hair. The specific characteristics of different type of hairs, their growth rate, as well as the estimated numbers of hair follicles, based on the body location are given below in Table-1, 2 and 3 respectively.

HAIR GROWTH AND CYCLE REGULATION

Traditionally, three phases of the growth cycle are recognized: a growth phase (anagen phase I–VI), a regression phase (catagen), and a resting phase (telogen).[6] Two more new phases have been recently identified for hair growth cycle called exogen[7] and kenogen respectively.[8] Under physiological conditions, 85% of the scalp hair is in anagen and approximately 15% is in the telogen phase. The anagen phase of scalp hair follicles typically persists for 2-6 years. The anagen phase of hair follicles of the eyebrows in contrast to scalp hair follicles, are only 70 days, while eyelashes grow for 100-150 days. The duration of body hair follicles are briefly mentioned in table 4.

Table 1: Type of Hairs

Hair type	Characteristics
Lanugo	<30 µm diameter; >2 mm length
Vellus	<30 µm diameter; <2 mm length
Intermediate	>30 µm diameter; <60 µm diameter; >2 mm length
Terminal	>60 µm diameter; >2 mm length

Table 2: Rate of terminal hair growth in adults (Approximately)

Location	Typical hair growth per day (mm)
Chin	0.38
Scalp	0.35
Axilla	0.30
Eyebrow	0.20
Thigh	0.16

DERMAL COMPONENTS OF HAIR FOLLICLE

The dermal portion of the hair follicle can be divided into two compartments, the dermal papilla (DP) and dermal sheath (DS).[9] The DP is located at the base of the hair follicle. The potent inductive ability along with secretory power of the dermal papilla determine the size of the anagen bulb, subsequently the diameter of the hair shaft produced by the hair bulb and the rate of hair growth, respectively.[10]

CAUSES OF HAIR LOSS

There are many factors associated with hair loss (Alopecia), but overall causes for hair loss are attributed to male hormones (Androgens), genetic factors and age. The causing factors for hair loss are listed below.

- Reduction in hair follicle function due to male hormones
- Reduction in metabolic functions of hair follicles and hair bulbs
- Reduction in scalp physiological functions
- Local impairment of the circulation due to tension in the scalp

HAIR GROWTH PROMOTERS

Hair growth promoters are preparations made by adding various pharmaceutical agents or natural ingredients to an alcohol water solution which is applied to the scalp to normalize its function. By increasing the blood circulation in the scalp or inhibiting the activity of 5-α-reductase enzyme activity; the promoters improve hair follicle function which promotes hair growth and prevents hair loss.

Table 3: Estimated number of hair follicles in the skin by body region

Location	Numbers of follicles
Head	1,000,000
Trunk	425,000
Arms	220,000
Legs	370,000
Approximate total	2,000,000

Table 4: Hair cycle duration with body location

S.No.	Location	Hair growth state	Typical Time Duration
1.	Scalp	Anagen	2-6 years
		Catagen	2-3 weeks
		Telogen	3 months
2.	Beard	Anagen	4-14 weeks
		Telogen	10-18 weeks
3.	Arms	Anagen	6-12 weeks
		Telogen	7-13 weeks
4.	Legs	Anagen	19-26 weeks
		Telogen	13-34 weeks

ACTIVE INGREDIENTS FOR HAIR GROWTH PROMOTERS

Among pharmaceutical agents which dilate the capillaries, directly act on the nervous system, such as Swertia extract (Swertinogen), vitamin E and its derivatives, benzoyl nicotinate, capronium chloride and on the other side those compounds which invigorate the circulation by local stimulation such as tincture of chilly (capsicum annumlinne) and tincture of cantharis (Spanish fly) are given below in table-5.

IMPORTANT NATURAL INGREDIENTS THAT POSSIBLY PROMOTE HAIR GROWTH

There are many hair growth stimulants like vitamins (E, C, A, H and B3), antioxidants, amino acids, proteins, fatty acids and polyphenols. An extract of *Asiasari radix* showed the potential for hair growth stimulation with increased protein uptake in a mouse study, and an in vitro study of human follicles revealed the expression of vascular endothelial growth factor (VEGF) in human dermal papillae.[11] Proanthocyanidins is a grape seed extract has been shown to promote hair follicle cells and convert the telogen follicle to an anagen follicle *In-Vitro*.[12] Extract from Ginkgo biloba leaf by an *In-Vitro* study promoted hair growth through effects on proliferation and inhibition of apoptosis of follicular cells.[13] Aloe vera L or *Aloe barbadensis* gel has been used traditionally for the treatment of alopecia, exhibiting improvement.[14] Aloenin is the major ingredient.[15]

Table 5: Natural and Pharmaceutical agents used as hair growth promoters

Action	Pharmaceutical agent
Circulation improvement (Vasodilator)	Swertia extract, Vitamin E and its derivatives, Benzoyl nicotinate, ϒ-orizanol, Cepharanthine & Minoxidil
5 α reductase II inhibitors	Finasteride, Dutasteride or GI198745, Lamin or prezatide copper, folligen or copper chloride, MK-386 or β-dimethyl-4 aza-5-cholestan-3-one, turosteride, essential fatty acid like ϒ-linolenic acid, palmitoleic acid, oleic acid, linoleic acid, evening primrose oil, emu oil etc and curcumin
Dihydrotestosterone receptor blocker	Flutamide, Spironolactone, Cyproterone acetate, Cimetidine and β sitosterol.
Local stimulation	Tincture of chilly (capsaicin) and cantharis, camphor, vanilyl amide nonylate, nicotinic acid benzyl ester etc.
Hair root activating agents	Placenta extract, pantothenic acid and its derivatives, allantoin and quaternium-73
(Nourishing agent) Amino Acids & vitamins	Cystine, cysteine, methionine, serine, leucine and tryptophan
	Vitamins A, B1, B2, B6, E and its derivates, Pantotheinic acid and its derivates and Biotin (Vitamin H)
Anti seborrhea	Sulpur, thioxorone and Vitamin B6

NATURAL PRODUCTS FOR HAIR CARE AND TREATMENT

Cysteine is a major amino acid; enhanced hair growth in a mouse screening study was used to evaluate the hair-growth-promoting effects of plant extracts. The extracts were painted on the backs of mice for 30-45 days and protein synthesis was measured using the cysteine uptake assay, using cultured murine vibrissae follicles.[11] Bergamot and boxthorn applied topically increased the cutaneous activity of superoxide dismutase, collagen, and decreased malondialdehyde with an observable increase hair growth.[16] The Chinese herb extract "Dabao" was applied topically and resulted in modest hair growth as compared to the placebo (42% compared to 37%).[17] The main ingredient of Ginseng is gensenoside-Rb (1) or G-Rb (1) shows hair growth activity while other extracts are ineffective.[18] The leaves and flowers of *Hibiscus rosa-sinensis* for its potential to stimulate hair growth. Topical preparations were applied to the backs of albino rats and to cell cultures of hair follicles from albino rat neonates. From the study it was evident that, compared to the flower, the leaf extract was more potent as hair growth promoter.[19] *Hydrangea macrophylla* extract promotes hair growth through the suppression of TGF-beta, which delays the catagen cycle. The mechanism may be accounted by the fact that TGF-beta is activated by caspase in the lower portion of the follicle and the outer layer of the outer root sheath. This mouse study suggests that TGF beta suppression could be used to treat alopecia.[20] *Illicium anisatum* has been shown to increase blood flow in a mouse model. In an in vitro study of mouse vibrissae follicles, a water-soluble extract of *Illicium anisatum* leaves, fruits, and roots (shikimic acid and glycosides, and polysaccharides) produced better growth than controls. Similar acetone extracts inhibited the growth of hair follicles. Shikimic acid induced insulin growth factor-1, keratinocyte growth factor, and VEGF in the hair follicle. The results of this study suggest that *Illicium anisatum* water extract could be a useful additive to hair growth products.[21] In a mouse study, the topical application of an extract of *Sophora flavescens* dried root induced increases in growth factors such as insulin-like growth factor-1 (IGF-1) and keratinocyte growth factor (KGF) in dermal papillae cells and inhibited type II, 5-α-reductase activity. This result suggests that sorphora has potential as a natural hair growth promoter.[22] A number of natural fatty acids include gamma-linolenic, linoleic, palmitic, elaidic, oleic, and stearic acids primarily having the 5-alpha-reductase inhibition activity. In a mouse study, an acetone extract of *Boehmeria nipononiveа* exhibited 5-alpha-reductase inhibition and a hair growth effect.[23] In a double-blind clinical trial, six out of ten patients with androgenetic alopecia have been shown improvement when treated with Saw palmetto, a liposterolic extract of *Serenoa repens*, and beta-sitosterol.[24]

METHODS FOR EVALUATING HAIR GROWTH PROMOTERS

Methods for the evaluation of Hair growth promoters can be broadly classified in to three categories.

Tissue culture

In-Vivo study

On human volunteers

Tissue culture based screening

Jahoda and Oliver were the first two scientists who initiate to grow the dermal papillary cells for the evaluation of hair growth promoters.[25] The quantification of the cells proliferation done by MTT tetrazolium salt assay.[26] The most common primary and established cell lines used for the hair growth studies are dermal papillary and HaCat cell line.[28]

In-Vivo study

Various species of animals like mice, rats, sheep and monkeys are widely used for the hair studies. The mice model is most widely reported for hair growth promotion studies and the most common parameters are qualitative hair growth study (Hair growth initiation and completion time) as well as quantitative hair growth study by measuring hair length.

On human volunteers

The final evaluation of hair growth promoters need to be completed on human volunteers. During testing on humans; there are many problems which have to solved, for example Individual differences, variation between different locations, the difficulty of controlling the people undergoing the tests and the poor understanding of daily and seasonal fluctuations. However in human volunteers study, the quantitative data can be obtained more easily but the costs are high and subject management requires a lot of efforts. The list of hair growth evaluation techniques are given below in Table-6.

CONCLUSION

In summary a number of reasons for hair loss and wide range of natural and synthetic chemical entities are available in the markets, so called hair growth promoting oils or tonic for hair re-growth. An ideal molecule for hair growth has yet to be developed. The most commonly used hair growth promoters like Minoxidil is one of them showing number of side effects include burning or irritation of the eye; itching; redness or irritation at the treated area; unwanted hair growth elsewhere on the body, and headache along with reversible hair loss, upon discontinuation of medication and its effectiveness has largely been demonstrated in younger men (18 to 41 years of age); while Finasteride or Propecia shows a no. of different type of side effects include impotence, abnormal ejaculation, decreased ejaculatory volume, abnormal sexual function, gynecomastia, erectile dysfunction, ejaculation disorder and testicular pain. Therefore now the hair biologist are trying to come up with some new novle molecules which should be free from these side effects and can be widely used in all types of hair loss or alopecia including AGA.

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Table 6: Methods for evaluating hair growth promoters

1) Evaluation using cells or tissue culture	Culturing of Dermal papillary cells and measurement of the cell proliferation by MTT assay. Culturing of HaCat cell line and measurement of the cell proliferation by MTT assay. Culturing of Normal human epidermal keratinocytes and measurement of the cell proliferation by MTT assay. Immunoflourescence assay for Ki-67 as a (Proliferation marker). *In-Vitro* Hair culture.
2) Evaluation using Animals	**Mice** Measure hair length Measure hair initiation and completion time **Rabbits** Measure hair length and weight Record of start growth Vascular Permeability test **Hamsters** Measure size of sebaceous gland (*In-Vivo*) Measure inhibition of 5 α reductase (*In-Vitro*) Measure inhibition of DHT receptor (*In-Vitro*) **Monkeys** Measure hair growth at front of head in red faced monkey. Photographic evaluation method, observe hair growth situation. Measure inhibition of 5 α reductase (hair root).
3) Evaluation using humans	Hair wash test Measure blood flow Trichogram method Unit area trichogram Phototrichogram (PTG) Hair pull test Hair Weighing

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Phytochemistry and Pharmacological Properties of *Etlingera elatior*: A Review

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ABSTRACT

Etlingera elatior are large ginger plants growing in clumps. Rhizomes are stout, strongly aromatic and found just below ground level. Crushed leaves emit a pleasant sour fragrance which is distinctive of the species. Leaves are entirely green with young leaves sometimes flushed pink. Inflorescences, borne on erect stalks protruding from the ground, are large and attractive with showy bracts. Native to Malaysia and Indonesia, *E. elatior* is widely cultivated in Southeast Asia. The species is used as food, condiment, medicine, and ornament. The current knowledge on the phytochemistry of leaves, inflorescences, and rhizomes of *E. elatior* is reviewed. Some insights on the pharmacological properties of the species are discussed. They include antioxidant, antibacterial, antifungal, tyrosinase inhibition, cytotoxic, and hepatoprotective activities.

Key words: *Etlingera elatior*, phytochemistry, pharmacological properties, leaves, rhizomes, inflorescences

INTRODUCTION

Gingers are perennial herbs belonging to the family Zingiberaceae. They produce aromatic rhizomes that are subterranean or above ground.[1] Each rhizome can produce leafy shoots. Inflorescences are terminal, borne either on leafy shoots or on erect shoots near the base of the plant. Ginger rhizomes are consumed as spice or condiment and are used in traditional medicine. In recent years, gingers are gaining popularity as ornamental plants as their inflorescences and foliage are colourful and attractive. They belong to three tribes, namely, Alpinieae, Zingibereae, and Hedychieae.[2]

Etlingera Giseke is a genus of the tribe Alpinieae. *Etlingera* species are tall forest plants with larger species growing up to 6 m in height.[2-3] Inflorescences are raised above the ground in the *Phaeomeria* group or just appearing at soil level in the *Achasma* group.[4-5] The varying shades of pink and red colours of bracts and flowers make *Etlingera* species very attractive plants. About 15 *Etlingera* species have been recorded in Peninsular Malaysia.[5] They have traditional and commercial uses as food, condiment, medicine, and ornament.[6]

Etlingera elatior (Jack) Smith or torch ginger grows up to 5-6 m tall forming clumps.[3,5] Rhizomes are stout (3-4 cm in diameter), strongly aromatic and found just below ground level. Leaves are entirely green (up to 80 × 18 cm) with young leaves sometimes flushed pink (Figure 1). Petioles are 2.5-3.5 cm in length. Borne on stalks protruding from the ground, inflorescences are large and attractive with showy bracts which are pink to red and sometimes white (Figure 2). Young inflorescences have a spear-like head. Crushed leaves and inflorescences emit a distinctive pleasant sour fragrance. The species is native to Malaysia and Indonesia. Synonyms of *E. elatior* are *Alpinia elatior, Elettaria speciosa, Nicolaia elatior, Nicolaia speciosa*, and *Phaeomeria speciosa.*

Plants of *E. elatior* are widely cultivated in Southeast Asia. Farms in Australia and Hawaii are cultivating the species and selling its inflorescences as cut flowers commercially.[2] Young inflorescences are a compulsory ingredient of sour curry and other spicy dishes. In Malaysia, the hearts of young shoots, inflorescences and fruits are consumed by the indigenous communities as condiment, eaten raw or cooked as vegetable.[7] A decoction of fruits is used to treat earache while a decoction of leaves is applied for cleaning wounds.[8] Leaves are also used by post-partum women and mixed with other aromatic herbs in water for bathing to remove body odour.

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Figure 1: Plants of *Etlingera elatior*

Figure 2: Inflorescences of *Etlingera elatior*

PHYTOCHEMISTRY

The phytochemistry of *E. elatior* has received some attention in recent years. From the rhizomes, two new and six known compounds of diarylheptanoids, labdane diterpenoids, and steroids have been isolated.[9] The known compounds were demethoxycurcumin, stigmast-4-en-3-one, stigmast-4-ene-3,6-dione, stigmast-4-en-6β-ol-3-one, 5α,8α-epidioxyergosta-6,22-dien-3β-ol, and 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one. The new compounds were 1,7-bis(4-hydroxyphenyl)-2,4,6-heptatrienone and 16-hydroxylabda-8(17),11,13-trien-16,15-olide.

Phytochemical screening of methanol extracts of *E. elatior* inflorescences showed the presence of flavonoids, terpenoids, saponins, tannins and carbohydrates.[10] Phenolic, flavonoid, anthocyanin and tannin contents of inflorescences were 361 mg GAE/100 g, 763 mg QE/100 g, 5.1 mg CGE/100 g, and 468 mg CE/100 g, respectively.[11] The phenolic content of inflorescences of 1.2 mg/100 g was attributed mainly to quercetin.[12] From the leaves of *E. elatior*, flavonoids of kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside, and quercetin 3-rhamnoside have been reported.[13] Other phenolic compounds isolated from the leaves were caffeoylquinic acids of 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), and 5-*O*-caffeoylquinic acid methyl ester, and flavonoids of isoquercitrin, quercitrin, and (+)-catechin.[14-15]

Chlorogenic acid (CGA) in leaves of *E. elatior* (294 mg CGA/100 g) was significantly higher in content than flowers of *Lonicera japonica* or Japanese honeysuckle (173 mg CGA/100 g), the commercial source. A protocol to produce a standardised extract of CGA with 40% w/w purity from leaves of *E. elatior* was developed.[14] Freeze drying of leaves of *E. elatior* followed by extraction with 30% ethanol, and sequential fractionation using Diaion HP-20 and Sephadex LH-20 yielded a CGA extract with 40% w/w purity. CGA fractions had antioxidant, antibacterial, and tyrosinase inhibition properties. The entire fractionation process took only 6.5 h, using gravity flow. From 50 g of leaves, the final yield of CGA extract was 0.2 g (0.4%). CGA content of the standardised extract from leaves (40%) of *E. elatior* is 1.6 times that of commercial extracts from honeysuckle flowers (25%). Inflorescences of *E. elatior* had flavonoid content consisting of 286 and 21 mg/kg of kaempferol and quercetin, respectively.[16]

Composition of essential oils varied with different parts of *E. elatior*.[17] Major components of leaves, stems, flowers and rhizomes were (*E*)-β-farnesene, β-pinene, 1,1-dodecanediol diacetate, cyclododecane, and (*E*)-5-dodecane. Essential oils isolated from inflorescences of *E. elatior* revealed that the main compounds were dodecanol (alcohol), dodecanal (aldehyde), and α-pinene (terpenoid).[18-19] Major constituents of oils from leaves of *E. elatior* have been reported to be β-pinene (24.9%) and 1-dodecene (24.3%),[20] and to be sesquiterpenes comprising (*E*)-farnesene (13.6%) and (*E*)-caryophyllene (8.6%).[21]

PHARMACOLOGY

Antioxidant activity

Analysis of diarylheptanoids isolated from rhizomes of *E. elatior* using the ferric thiocyanate assay showed that

their antioxidant activity is higher than that of α-tocopherol.[9] Lipid peroxidation inhibition of diarylheptanoids ranged from 92-94% compared to 70% of α-tocopherol. The radical scavenging activity of methanol extracts from inflorescences of *E. elatior* (76.3%) was reported to be comparable to that of butylated hydroxytoluene (86.8%).[10]

There are several publications on the antioxidant properties (AOP) of leaves of *Etlingera* with emphasis on *E. elatior*. AOP were evaluated in terms of total phenolic content (TPC), radical scavenging activity expressed as ascorbic equivalent antioxidant capacity (AEAC), and ferric reducing power (FRP). Of five *Etlingera* species screened, *E. elatior* had the highest TPC, AEAC, and FRP.[22] Values were 3550 GAE/100 g, 3750 mg AA/100 g, and 20 GAE/g, respectively. Screening of AOP of leaves of 26 ginger species belonging to nine genera and three tribes showed that *Etlingera* had the highest values followed by *Alpinia* and *Hedychium*.[23] *Boesenbergia*, *Curcuma*, *Elettariopsis*, *Kaempferia*, *Scaphochlamys*, and *Zingiber* had much lower values. The outstanding AOP of *Etlingera* species were attributed to their size and growth habitat. Plants of *Etlingera* are the largest among the gingers and can grow up to 6 m in height. They grow in gaps of disturbed forest and are continually exposed to direct sunlight. The other genera are small- to medium-sized herbs. Larger ginger plants growing in exposed forest sites have stronger AOP than smaller plants growing in shaded sites.

Leaves of *E. elatior* which had the highest TPC and AEAC were eight times higher than rhizomes.[23] Leaves had significantly higher TPC, AEAC, and FRP than inflorescences and rhizomes.[22] Ranking was in the order: leaves > inflorescences > rhizomes. It has been reported much greater concentrations of flavones and flavonols occur in leaves which are exposed to sunlight.[24] Only trace amounts were found in unexposed parts below the soil surface which include roots and rhizomes.

Leaves of highland populations of *Etlingera* species were found to have higher TPC and AEAC than lowland counterparts.[22] Values of *E. elatior* in the highland were 3550 mg GAE/100 g and 3750 mg AA/100 g compared to 2390 mg GAE/100 g and 2280 mg AA/100 g in the lowland, respectively. Higher altitudes seem to trigger an adaptive response in leaves of *Etlingera* species. The stronger AOP of highland over lowland plant populations might be due to environmental factors such as higher UV-B radiation and lower air temperature.[25-27]

The effects of different drying methods on the AOP of leaves of *E. elatior* have been reported.[28] All methods of thermal drying (microwave, oven, and sun drying) of leaves resulted in drastic declines in TPC, AEAC, and FRP. Many studies have reported losses in antioxidant properties of plant samples following thermal treatments. Loss in AOP of heat-treated samples has been attributed to thermal degradation of phenolic compounds, loss of antioxidant enzyme activities and degradative enzymes.[29-30] Declines are often accompanied by the loss of other bioactive properties.[31]

Of the non-thermal drying methods (air and freeze drying), significant losses were observed in air-dried leaves of *E. elatior*.[28] Declines in AOP resulting from air drying could be due to enzymatic degradation as the process was carried out at room temperature and takes several days for samples to dry. There were significant gains in TPC (21%), AEAC (31%), and FRP (26%) for freeze-dried leaves compared to fresh leaves. After one week storage, AOP of freeze-dried leaves of *E. elatior* remained significantly higher than those of fresh leaves as control. TPC and antioxidant activity of freeze-dried inflorescences have been reported to be nine and eight times that of fresh samples.[32]

There is no thermal degradation in freeze drying and neither does the process allow degradative enzymes to function.[28] Furthermore, freeze drying is known to have high extraction efficiency because ice crystals formed within the plant matrix can rupture cell structure, which allows exit of cellular components and access of solvent, and consequently better extraction.[33] The HPLC chromatogram of leaves of *E. elatior*, which showed greater amounts of minor compounds following freeze drying, supported this inference.[28] Freeze drying remains the best method of drying foods as the quality of freeze-dried products is comparable to fresh products.[34]

Antibacterial activity

Ethanol extracts from inflorescences of *E. elatior* displayed antibacterial activity.[35] Minimum inhibitory concentration (MIC) was 200 μg/ml against *Pseudomonas aeruginosa*, 400 μg/ml against *Bacillus megaterium*, and 800 μg/ml against *Escherichia coli*. Methanol extracts inhibited *Staphylococcus aureus*, *Bacillus thuringiensis*, *E. coli*, *Bacillus subtilis*, and *Proteus mirabilis* with MIC ranging from 1.56 to 50.0 mg/ml.[10] Leaves of *E. elatior* exhibited moderate inhibition against Gram-positive bacteria of *Bacillus cereus*, *Micrococcus luteus*, and *S. aureus* with no activity against Gram-negative bacteria of *E. coli*, *P. aeruginosa*, and *Salmonella choleraesuis*.[22] Essential oils from *E. elatior* leaves inhibited methicillin-resistant *S. aureus* (MRSA) with no activity on *P. aeruginosa*, *S. choleraesuis*, and *B. subtilis*.[20] Rich in sesquiterpenes (24.5%), leaf oils of *E. elatior* inhibited Gram-positive *B. cereus*, *M. luteus*, and *S. aureus* with MIC values of 25, 6.3, and 50 mg/ml, respectively.[21]

Antifungal activity

Twelve Thai medicinal plants were tested for antifungal activity against *Colletotrichum gloeosporioides*.[36] Hexane extracts

from young inflorescences of *E. elatior* demonstrated high inhibitory activity of mycelial growth with EC_{50} value of 804 μg/ml. When screened against a broad range of human pathogenic fungi, ethanol extracts of fruits and rhizomes of *E. elatior* did not show any antifungal activity.[37]

Tyrosinase inhibition activity

Methanol leaf extracts of five *Etlingera* species were analysed for tyrosinase inhibition (TI) activity using the modified dopachrome method with L-DOPA as substrate.[23] TI was strongest in leaves of *E. elatior* (55%) which was significantly higher than leaves of *Hibiscus tiliaceus* (44%) used as positive control. TI values of the other four *Etlingera* species ranged from 22-49%.

Cytotoxic activity

Of the diarylheptanoids, labdane diterpenoids, and steroids isolated from rhizomes of *E. elatior*, stigmast-4-en-3-one and stigmast-4-en-6*β*-ol-3-one displayed high anti-tumour activity using the Epstein-Barr virus (EBV) activation assay.[38] Ethyl acetate extracts showed strong cytotoxic activity against CEM-SS and MCF-7 cell lines using the methyl thiazole tetrazolium (MTT) assay. IC_{50} of rhizome extracts was 4.0 and 6.3 mg/ml, respectively, compared to tamoxifen with IC_{50} of 30 and 15 μM, respectively.

Ethanol extracts from *E. elatior* leaves and inflorescences are cytotoxic to HeLa cells.[35,39] The cytotoxic dose (CD_{50}) was in the range of 10 to 30 μg/ml. Methanol leaf extracts did not exhibit cytotoxic effect on normal WRL-68 (human liver) and Vero (African green monkey kidney) cells.[15]

Screening of 38 Thai edible plants for activity toward EBV activation in Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) showed that leaf and stem extracts of *E. elatior* were moderately active while rhizome extracts were weakly active.[40]

Hepatoprotective activity

The protective activity of ethanol extracts of *E. elatior* inflorescences against hepatotoxicity induced by lead acetate in male Sprague-Dawley rats has been reported.[41] Treatment with the extract significantly reduced hepatic lipid hydroperoxides and protein carbonyl content in the serum, increased antioxidant enzyme levels in the liver, and decreased lead levels in the blood. The study concluded that the hepatoprotective effect against lead toxicity in rats may be attributed to the powerful lead chelating ability of the extract. A related study examined the effect of *E. elatior* extracts on the bone marrow of male Sprague-Dawley rats exposed to lead acetate toxicity.[42] It similarly concluded that the species has a powerful antioxidant effect which protects bone marrow oxidative damage induced by lead acetate.

CONCLUSION

Plants of *E. elatior* are tall ginger plants that are widely cultivated for their inflorescences which have traditional and commercial uses as food, condiment, medicine, and ornament. From the rhizomes, compounds of diarylheptanoids, labdane diterpenoids, and steroids have been isolated. Phytochemical screening of inflorescences showed the presence of flavonoids, terpenoids, saponins, tannins, and carbohydrates. From the leaves, flavonoids and caffeoylquinic acids have been reported. Leaves of *E. elatior* had the strongest AOP out of 26 ginger species screened. Leaves had significantly stronger AOP than inflorescences and rhizomes with highland populations have higher values than lowland counterparts. Heat drying methods adversely affected the AOP of leaves while freeze drying enhanced their values. The various plant parts of *E. elatior* have antibacterial, antifungal, tyrosinase inhibition, cytotoxic, and hepatoprotective properties. With strong antioxidant and other pharmacological properties, the species has great potential to be developed into functional and other health products. To date, this report represents the most comprehensive review of the phytochemistry and pharmacological properties of *E. elatior*.

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Applications of Photosensitizer in Therapy

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ABSTRACT

Photosensitization can be defined as a process in which a reaction to normally harmless radiation is induced by the introduction of a specific radiation-absorbing substance (photosensitizer) that causes another component (substrate) to be changed by the radiation. Photosensitivity is characterized by phototoxic and photoallergic effects. Drugs and chemicals may interact with UV to induce photosensitivity. Photosensitive disorders may be classified as those entirely caused by solar exposure and the photoaggravated disorders. Those in the former category include polymorphic light eruption, hydroa vacciniforme, actinic prurigo, solar urticaria and chronic actinic dermatitis. Photosensitivity can be diagnosed by photo test, photo patch test and photo drug test. Recently the photodynamic therapy (PDT) is used for the treatment of cancers. There are various photosensitizers such as photofrin, foscan, 5-Aminolevulinic acid (5-ALA) etc which used in photodynamic therapy. Photosensitizers are also used to treat vitiligo, microbial infections and acne.

Key words: Photosensitizer, Juvenile spring eruption, Photodynamic therapy, Vitiligo, Acne.

INTRODUCTION

The term photosensitivity is used to describe any cutaneous reactions to light. Photosensitivity reaction occurs when a photosensitizing agent in or on the skin reacts to normally harmless doses of UV or visible light. It is classified as phototoxic or photoallergic reaction.[1,2,3,4,5] Phototoxic reaction results from direct damage to tissue caused by a photoactivated compound. Photoallergic reactions are cell-mediated immune responses to a photoactivated compounds. Phototoxicity is much more common than photoallergic reaction. Phototoxicity is an irritation of the skin occurs after exposure to UV light. Photoallergy is an allergic reaction of the skin to UV light. Both reactions occur in sun-exposed areas of skin including the face, neck, hands and forearms. A widespread eruption suggests exposure to a systemic photosensitizer whereas a localized eruption indicates a reaction to a locally applied topical photosensitizer. Acute phototoxicity is characterized by an exaggerated sunburn reaction with erythema, edema, blistering, weeping and desquamation that occurs within minute to hours of light exposure. Photoallergic reaction resemble allergic contact dermatitis, their onset is delayed by as long as 24-72 hours after exposure to the drug and light.[6]

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PHOTOSENSITIZATION MECHANISM

Phototoxicity

Various compounds especially those which have at least one resonating double bond or an aromatic ring that can absorb radiant energy cause direct damage to tissues which results in phototoxic reactions. Most compounds having those bonds and rings are activated in between wavelengths of UV-A (320-400 nm) range, although some compounds have peak absorption within the UV-B or visible range.[7] On exposure to UV rays a transient redness appears in few minutes. The major erythema response of skin to UV rays is delayed, beginning 2-6hrs after exposure and reaching a maximum in 12-24 hrs and then subsides over next few days. This delayed erythema response is sunburn and histologically it is characterized by appearance of sunburn cells (SBC). SBCs appear in 24-28 hrs after exposure and by 72 hrs, form a continuous band in stratum corneum. UV-B rays are more potent in inducing formation of SBCs than UV-A rays.[8]

Sunburn is the major cause of phototoxic reactions which occurs due the formation of hyperactive species of oxygen. Photoactivation of a compound results in the excitation

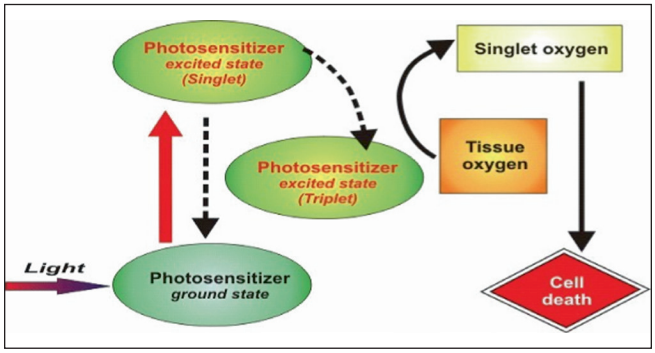

Figure 1: Mechanism of Phototoxicity[12]

of electrons from the stable singlet state to an excited triplet state. As excited-state electrons return to a more stable configuration, they transfer their energy to oxygen, leading to the formation of reactive oxygen intermediates. Reactive oxygen intermediates such as a singlet oxygen, superoxide anion, and hydrogen peroxide that can damage cell membranes and DNA.[9] Such functional molecules have been successfully applied in photodynamic therapy (PDT) of cancerous tumours. Signal transduction pathways that lead to the production of proinflammatory cytokines and arachidonic acid metabolites are also activated. The result is an inflammatory response that has the clinical appearance of an exaggerated sunburn reaction.[10,11]

Photoallergic reactions

Cell mediated immune responses are responsible for photoallergic reactions in which the antigen is a light-activated drug. Photoactivation results in the development of a metabolite that can bind to protein carriers in the skin to form a complete antigen.[13] The reaction proceeds specifically, through langerhans cells and other antigen-presenting cells which take up the antigen and then migrate to regional lymph nodes. In those locations, the langerhans cells present the photoallergen to T lymphocytes that express antigen-specific receptors. The T cells become activated and proliferate, and they return to the site of photoallergen deposition. In the skin, the T cells orchestrate an inflammatory response that usually has an eczematous morphology if the photoallergen is applied topically or the characteristics of a drug eruption if the photoallergen is administered systemically. These are mediated by immunological pathways and require prior sensitization to drug and UV-rays. It is usually seen in a small number of individuals. Clinical effects range from acute urticarial reactions to papular lesions appearing 24 hrs after exposure. UV radiation may react with photosensitising agent to form a stable hapten. The photoproduct then combines with protein carrier to form an antigen. Radiation can also alter tissue protein, enabling it to act as a carrier for either the photosensitizing agent itself or its photoproduct.[14,15] Photoallergy also differs from phototoxicity histologically. Photoallergic responses are uncommon, usually manifests as a pruritic eczematous eruption and consist clinically of immediate urticarial or delayed papular lesions. The immediate urticarial lesions show very little other than some edema and vasodilatation. The delayed papular reactions present a dense perivascular round-cell infiltrate in the dermis which is characteristic though not diagnostic of these responses.[16,17,18]

Symptoms and Diagnostic Tests

Human body shows different symptoms that depend upon the age of the patient. There are several indications of photosensitivity which are characterized as follows depending on the age of the patient.

Childhood symptoms

Lesions on ears in spring (juvenile spring eruption), itchy lesions on V area of neck or elsewhere (polymorphous light eruption), burning pain, increased protoporphyrin levels in red blood cell (Erythropoietic protoporphyria), lesions on bridge of nose (Actinic prurigo), scar formation (Hydroa vacciniforme).[19]

Adulthood symptoms

Females with itchy lesions in V area of neck (Polymorphous light eruption), all sun-exposed areas, positive phototest results (Drug-induced photosensitivity), (lesions appear within 5-10 min and disappears within 1-2 h, urticaria on phototesting (Solar urticaria), anti−RO/SS-A antibodies, skin immunofluorescence, phototesting with late readings (Lupus erythematosus), porphyrin determinations (Porphyria cutanea tarda).[19]

Old age symptoms

There are various old age symptoms such as persistent redness of face in elderly man (Chronic actinic dermatitis), all sun-exposed areas, positive phototest results (Drug-induced photosensitivity), CD4+ cells on histological examination (Cutaneous T-cell lymphoma), creatine level in 24-h urine (Dermatomyositis).[19]

Diagnostic tests

Various photosensitivity tests are conducted to determine the level of photosensitivity. These tests are performed after various symptoms of photosensitivity are observed. Ultraviolet radiation is divided into ultraviolet A (UVA) (operative wavelength of 320 to 400 nm), ultraviolet B (UVB) (operative wavelength of 290 to 320 nm) and ultraviolet C (UVC) (operative wavelength of 100 to 290 nm). UVA, UVB and visible light are most frequently used for the diagnosis purpose.

1) Photo test: The most widely conducted photo test is exposure to UVB irradiation in the minimal dose that causes

erythema in 24 hours (minimal erythema dose; MED). The average dose for ethnic Japanese is 60 to 100mJ/cm^2. When the MED is low, involvement of a photosensitive disease is suspected.[20]

2) Photo-patch test: The photo-patch test is conducted to examine the influence of rays when a chemical substance is placed on the skin. 24 to 48 hours after a material that is suspected of causing photosensitive disease is applied on the skin, the site is exposed to UV rays. If reddening or swelling occurs within 24 hours, the test is considered to be positive for such disease.[20]

3) Photo-drug test: The influence of radiation in the presence of a chemical substance can also be examined by photo-drug test. A drug that is suspected of causing a photosensitive disease is taken orally instead of topically. The photo-drug test is generally used for diagnosis of drug-induced hypersensitive diseases.[20]

Photosensitizers

Photosensitizers are the agents that may leave skin vulnerable to UV exposure causing erythema, itching, scaling, rashes or inflammation. These substances combined with UV light also may contribute to other health problems including skin cancer, photoaging and allergic reactions. It can be divided into following main groups:

Photodynamic Agents

Photodynamic agents are naturally occurring or may be synthetic pigments and dyes, which require oxygen for their action e.g. erythrosin, rhodamin, hypericine, Bengal rose, anthracene, acridine dye, methylene blue, quinine, buckwheat and porphyrin. These substances photo-oxidize terpenene, blood serum protein and cause haemolysis. They are topically inactive but on intradermal injection cause immediate photoreaction of short durations. Erythema produced by photodynamic compound appears immediately after irradiation and disappear after a few hours.[21]

Photosensitizing Agents

Photosensitizing agents do not require oxygen for reaction. These photosensitizing agents include furanocoumarins and their derivatives e.g. psoralen, xanthotoxin, bergapten, isobergapten and imperatorin. These compounds neither cause photo-oxidation of terpenene or haemolysis, nor photooxidize blood serum protein to any appreciable extent, but provoke dermatitis characterized by erythema after latent period of few hours and last several days succeeded by increased pigmentation on epicutaneous application and intradermal injection. These compounds have therapeutic value in leucoderma.[21]

Metallotetrapyrolics particularly porphyrins, azaporphyrins that includes gallium in central pyrrolic core have phototherapeutic application in photodetection and phototherapy of target tissues. These compounds are also used for treatment and detection of cardiovascular disorder.[22]

Drug Induced Photosensitivity

Cutaneous drug eruptions are one of the most common types of adverse reaction to drug therapy, with an overall incidence rate of 2-3% in hospitalized patients.[23,24,25] Almost any medicine can induce skin reactions, and certain drug classes, such as non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics and antiepileptics, have drug eruption rates approaching 1-5%.[26]

Photosensitivity Management

Prevention of photosensitivity reactions is mainly based upon patient education. Patients should be well educated

Table 1: List of Drugs that Induces Photosensitivity[27]

Drug classifications	Drugs
Muscle relaxant	Afloqualone
Psychoactive	Chlorpromazine, promethazine, diazepam, carbamazepin, imipramine
Antifungal agent	Griseofulvin, flucytosine, itraconazole
Antibacterial agent	Nalidixic acid, enoxacin, ofloxacin, ciprofloxacin, lomefloxacin, sparfloxacin, fleroxacin, tosufloxacin, tetracycline, doxycycline
Antihistamine	Diphenhydramine, mequitazine
Antiinflammatory	Ketoprofen, tiaprofenic acid, suprofen, piroxicam, ampiroxicam, actarit, diclofenac, naproxen
Antihypertensive agent	Hydrochlorothiazide, trichlormethiazide, meticrane, clofenamide, tripamide, metolazone, furosemide, tilisolol HCl, pindolol, diltiazem HCl, nicardipine HCl, nifedipine, captopril, lisinopril
Antipodagric	Benzbromarone
Antidiabetic	Tolbutamide, chlorpropamide, glibenclamide, carbutamide, glymidine sodium
Prostatomegaly therapeutic agent	Tamsulosin
Lipid-lowering drug	Simvastatin
Antitumor agent	5-FU, tegafur, dacarbazine, flutamide
Photochemistry therapeutic agent	8-Methoxypsoralen, trioxypsoralen, hematoporphyrin derivative
Antirheumatic	Sodium aurothiomalate, methotrexate
Vitamin	Etretinate, pyridoxine, Vit. B_{12}

to minimize sun exposure. Additional light protection can be provided by the use of UV-A protective sunscreens and physical barriers such as clothing. Sunscreens that provide UV-A coverage are dioxybenzone, avabenzone, titanium dioxide, zinc oxide. Patients should be counseled to avoid sources of high-intensity light like tanning beds. Some reactions may be dose related, a decrease in dose may be considered to help minimize the reaction. A mild reaction like sunburn may be easily handled with skin protectants and topical or systemic analgesics.[28] Patients may also be benefited from application of cooling creams or gels. Antibacterial creams may be necessary to prevent infection, if patients have blisters that are broken.[29] Oral or topical corticosteroids are used to handle severe reactions.[30] Drugs like antihistamines may also prevent pruritus associated with reactions.

Role of Photosensitizers in the Therapy

Vitiligo

Vitiligo is an idiopathic acquired pigmentary disorder characterized by loss of melanin formation which is the main pigment in mammalian skin, hair and eyes with subsequent development of white patches. Photochemotherapy is one of the most successful treatment of vitiligo.[31,32,33,34,35] El Mofty in 1948 introduced modern photochemotherapy of vitiligo with psoralen and UVA (PUVA).[36] 8-methoxy psoralens (8-MOP) and 4, 5, 8-trimethylpsoralen (TMP) are the most commonly used psoralens, both systemically as well as topically. TMP produces fewer side effects and better pigmentation than 8- MOP. Oral psoralen photochemotherapy requires longer UV-A exposures than those required for topical therapy.[37]

Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is an emerging modality for the treatment of neoplastic and non-neoplastic diseases.[38,39] It is based on the concept that light irradiation can change an inert substance into an active one. PDT involves the interaction of a specific light sensitive agent, so-called photosensitiser and a particular type of light. The photosensiting agent is injected into the bloodstream is absorbed by cells all over the body but remains in or around the tumour cells for a longer time than it does in normal cells. Approximately 24-72 hours after injection, when most of the agent has left normal cells but remains in cancer cells, the tumour is exposed to laser light which can be directed through fiber optic to deliver the proper amount of light to areas inside the body. The light energy is absorbed by the photosensiting agent that causes a chemical reaction and produces an active form of excited singlet oxygen.[40] These reactive oxygen species (ROS) have a very short lifetime but are extremely reactive and usually induce a phototoxic reaction that kills nearby tumour cells. In addition to directly killing tumour cells (cell death by necrosis or by adoptosis), PDT appears to shrink or destroy tumors by damaging blood vessels in the tumor (vascular shutdown), thereby preventing the cancer from receiving necessary nutrients[41] or may activate the immune system to attack the tumor cells.

Advantages

- Selective tumor destruction with normal tissue preservation.
- Limited damage to surrounded tissue.
- Lack systemic toxicity.
- It can be targeted very precisely.
- It is less invasive approach than surgery.
- Unlike radiation, it can be repeated several times at the same site if necessary.
- Usually performed in an outpatient procedure.
- It may result in less scarring.
- Well accepted cosmetic results.

Treatment of Intracranial Cancers

Malignant gliomas are one of the most invasive intracranial tumors which are difficult to eradicate surgically and carry a dismal prognosis. Their cure is mainly dependent on radical and complete local excision. The main causes of failure to eradicate them are their inability to visualize and detect them, the presence of the blood brain barrier and the low tolerance of brain tissue to ionizing radiation. Photodynamic detection and photodynamic therapy offers an excellent chance of visualizing tumor nests and targeted destruction of the remaining tumor cells safely followed by surgical excision which may results in the survival of patients suffering from these invasive tumors.[42] PDD/PDT is safe treatment for invasive intracranial tumors and well tolerated by most patients. Intracavity irradiation after surgical excision of high grade gliomas is the most favored method of brain tumor PDT.[43] PDD maximizes surgical tumor resection leading to better prognosis and prolonged survival while, PDT gives significant improvement in survival of patients with malignant gliomas who have dismal prognosis. Therefore, the majority of patients treated so far had recurrent malignant disease with very poor prognosis.

Photosensitizers used in PDT of Cancer

Photofrin

Photofrin is a first generation photosensitiser which is a hematoporphyrin derivative (HpD) are used most commonly for the treatment of bladder cancer, esophageal cancer, gastric cancer and cervical cancer. U.S. food and drug administration (FDA) has approved the porfirmer sodium or photofrin for use in PDT to treat or relieve symptoms of esophageal cancer.[44] When the cancer obstructs the esophagus or when the cancer cannot be satisfactorily treated with laser therapy. Porfirmer sodium can also used to treat

non-small lung cancer in patients for whom the usual treatments are not appropriate.[45] It is mainly activated by diode laser light at 630nm. The irradiation dose is 100-200 J/cm^2.[46]

Foscan (temoporfin)

Foscan is a more potent photosensitiser than photofrin and ALA. It has been approved for the treatment of head and neck cancer.[47] It is activated at 652 nm wave length. The irradiation dose is as low as 10 J/cm^2.

5-Aminolevulinic acid (5-ALA)

5-amino levulinic acid (ALA) is a second generation photosensitizer which is hydrophilic zwitter ion at physiological pH. ALA was approved for treatment of actinic keratosis and basal cell carcinoma of skin. Recently, it was introduced as a new drug for PDT of bladder cancer[48] and to be used in a diagnostic procedure (photodynamic diagnosis [PDD] or ALAinduced fluorescence endoscopy, [AFE]).[49,50] It has an advantage of possibility of topical administration.[49] ALA is an initial substrate of heme biosynthesis. 5-ALA formed *in vivo* in mitochondria by condensation of glycine and succinyl CoA (catalyzed by ALA-synthase). Subsequent reactions produce protoporphyrin IX (PpIX) which is converted to heme using ferrochelatase and Fe. Heme inhibits synthesis of 5-ALA. Excess administered 5-ALA passes through abnormal epidermis and converts to PpIX which is then accumulates with minimized amount of ferrochelatase. ProtoporphyrinIX (PpIX), is an active compound, which accumulates in tumor cells and can be activated by violet-blue light (375-440 nm) for PDD and diode laser light (635 nm) for PDT. Depth of tissue penetration is 7-15 mm and the irradiation dose required for PDT is 100 J/cm^2 and skin photosensitivity continues for 7-10 days after ingestion.[51] It can be given 3-4 hours before induction of anaesthesia in a mixture of non-fizzy orange juice at 15-20 mg/Kg bodyweight in PDD/PDT of brain tumors.[52,53,54]

Recent Trends in Therapy

PDT Acne Treatment

Acne vulgaris commonly called acne is a common human skin disease characterised by plugged pores (blackheads and whiteheads), pimples, follicular papules or comedones, pustules and even deeper lumps (cysts or nodules) that occurs on the face, neck, chest, back, shoulders and the upper arms. The term nodulocystic have been used to describe severe cases of inflammatory acne.[55] Cystic acne affects deeper skin tissue than does common acne. When severe, acne can lead to serious and permanent scarring. It can occur most commonly during adolescence, affecting more than 96% of teenagers and often continues into adulthood. Acne develops as a result of blockages in follicles, enlargement of sebaceous glands and an increase in sebum production occurs with increased androgens (male sex harmones). In these conditions the naturally occurring largely commensal bacteria i.e. Propionibacterium acnes can cause inflammation, leading to inflammatory lesions (papules, infected pustules, or nodules) in the dermis around the microcomedo or comedone, which results in redness and may result in scarring or hyperpigmentation.[56,57]

PDT is a procedure that treats active and resistant acne that combines a special light activated solution which targets and destroys acne activity. This treatment can also diminish older acne scars, leaving the skin with a much smoother appearance. Intractable acne on the body can be extensively treated by ALA based PDT (ALA-PDT).[58,59] ALA can be administered both topically as well as orally.[60] Kimura et al performed an experiment in which the total number of acne patients was 51. A 10mg/kg B.W. of ALA was administered orally to the patients then, after 4 hours acne lesions were exposed to polychromatic visible light from a metal halide lamp. The wavelength of a light ranges from 540 to 800nm. In one session, the total light energy dose was 60-80 j/cm^2 for the body. All patients undergo two sessions of PDT and no other treatments received after PDT or during the follow-up period. The study concluded that PDT-ALA was considered to be effective for the treatment of moderate to severe acne.[61]

Antimicrobial Photodynamic Therapy

Bacterial infection plays an important role in the development of necrosis in the dental pulp and the formation of periapical lesions, therefore, the main goal of endodontic treatment is the elimination of bacterial infection and associated inflammation in the pulpal tissue and also the mechanical removal of damaged tissue found inside the root canal that acts as a growth medium for microbes.[62] Garcez et al[63] performs an experiment in which ten single rooted freshly extracted human teeth were inoculated with stable bioluminescent Gram-negative bacteria (Proteus mirabilis and Pseudomonas aeruginosa) to produce 3-day biofilms in prepared root canals. Bioluminescence imaging was used to quantify bacterial burdens. A conjugate between polyethylenimine and chlorin(e6) as the photosensitizer (PS) can be employed in PDT and diode laser light (660-nm) delivered into the root canal via a 200-m fiber, and this was compared and combined with standard endodontic treatment using mechanical debridement and antiseptic irrigation. After the success of experiment, they concluded that endodontic therapy alone can reduced bacterial bioluminescence by 90% while, PDT alone can reduced bioluminescence by 95%. The combination can reduced bioluminescence by >98%, and the bacterial re-growth observed 24 hours after treatment was much less for the combination than for either single treatment.

Future Prospects of PDT

Recently new photosensitizers are being developed by several pharmaceutical companies that increases the number of choices for the treatment of cancers that are previously treated with photofrin but extend the indications as well. An example is the application of PDT with Benzoporphyrin Derivative-Monoacid Ring A (BPD-MA) for treatment of age-related macular degeneration and possibly for rheumatoid arthritis, the possible use of Tin Etiopurpurin (SnET2) and mTHPC (foscan) for prostatic diseases, the topical use of ALA or its methyl ester for dermatologic superficial lesions and perhaps the application of PDT for treatment of coronary artery diseases. However, the real challenge in the future is gaining physician acceptance of PDT as a viable treatment modality.[64]

CONCLUSION

Photosensitivity is a skin reaction (i.e. rash) that occurs after exposure to ultraviolet (UV) radiation from the sun or an artificial light source. Photosensitivity can be caused by various agents including cosmetics, perfumes, certain medications, and even the sunscreen that is meant to protect your skin. Phototoxic reaction results from direct damage to tissue caused by compounds that are activated by light. Photoallergic reactions are cell-mediated immune responses to a photoactivated compounds. Phototoxicity is much more common than photoallergic reaction. Cutaneous lupus erythematosus represents an autoimmune disease characterized by photosensitivity, apoptosis of keratinocytes and an inflammatory infiltrate in superficial and/or deep compartments of the skin. Recent findings in cutaneous LE study suggest an amplification cycle with UV-injury inducing apoptosis and necrosis of keratinocytes which in turn results in the production and release of a first set of chemokines and the presence of extracellular self-DNA. Subsequently, a first wave of effector memory T cells as well as PDC is recruited to sites of UV injury and may be activated via different pathways.

Drugs which are essentially used for treatment of various ailments have various side effects one of the major side effects is photosensitization that further induces phototoxicity and photo allergy. The diagnosis of the toxic effects of UV rays can now be easily detected by various types of tests. Photosensitizers that induce photosensitivity are now being used in PDC therapy for treating cancers. The efficacy of photosenstizers is also utilized in the Antimicrobial therapy and treatment of acne and vitiligo. The PDT is going to be the futuristic trend for treatment of various disorders like rheumatoid arthritis and to treat certain diseases like prostatic diseases, dermatologic superficial lesions and coronary artery diseases.

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Review: A Rare Medicinal Herb *Zizyphus xylopyrus* (Retz.) Willd

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ABSTRACT

In the present scenario, demand for the herbal products is growing exponentially through out the world and various pharmaceutical sectors are currently conducting extensive research on plant materials for their potential medicinal value. This inclination seems to be a result of people all over the world looking for various alternative system of medicine. The research of blending traditional knowledge with modern experimental methodology for testing the efficacy and safety of herbal drugs is increasing. Keeping this point of view the present work has been presented to explore the efficacy and medicinal values of a rare medicinal plant known as *Zizyphus xylopyrus* (Retz.) Willd (ZX). The available literatures on this plant though very less, divulges that it contains many phytoconstituents like, Flavonoids, Tannins, Saponins, Terpenoids, etc. which when pharmacologically tested proved to be medicinally significant. In vast research, all these phytochemicals has shown their variety of medicinal values, more potent compared to available allopathic medicines in some cases. Apart from that, ZX has shown its promising effect even in the Central Nervous System. Though much of scientific work has not been done on this plant, so in this context it needs to be heeded in all the medicinal and pharmaceutical concerns.

Key words: Zizyphus, Flavonoids, Pharmacology, Phytochemistry.

INTRODUCTION

Variety of reasons has been cited for the need of the studying medicinal plants. Most of the traditional knowledge about medicinal plants was in the form of oral knowledge that had been lost with persistent invasions and cultural adaptations. There was no uniform or standard procedure for maintaining the inventory of this plant and the knowledge about their medicinal properties. There is a prevalence of using plants and plant based products in various contemporary and traditional system of medicine, without any written documentation or regulation. Therefore it is essential that such uses of natural products be documented and studied for systemic regulation and wide spread application.[1] Zizyphus is a genus of about 40 species of spiny shrubs and small trees in the buckthorn family Rhamnaceae. The leaves are alternate, entire, with three prominent basal veins, and 2-7 cm long; some species are deciduous, others evergreen. The flowers are small, inconspicuous yellow-green. The fruit is an edible drupe, yellow-brown, red, or black, globose or oblong, 1-5 cm long, often very sweet and sugary, reminiscent of a date in texture and flavor.[2,3]

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PLANT PROFILE

Biological Source

The drug consists of the whole dried herb of *Zizyphus xylopyrus* (Retz.) Willd. (Family: Rhamnaceae)[4]

Geographical Source

The plant is found throughout North-Western India, Pakistan and China.[4]

Classification

- Domain : Eukaryota – eukaryotes
- Kingdom : *Plantae* Haeckel
- Subkingdom : *Viridaeplantae*
- Phylum : Magnoliophyta
- Subphylum : *Euphyllophytina*
- Infraphylum : *Radiatopses*
- Class : *Magnoliopsida*
- Subclass : *Rosidae*

Figure 1: Whole plant of *Z. xylopyrus*

- Superorder : *Rhamnanae*
- Order : *Rhamnales*
- Family : *Rhamnaceae*
- Genus : *Ziziphus*
- Specific epithet : *xylopyra*[5]

Common Names

- Hindi - Kat-ber, Gote, Kakor, Ghont
- Sanskrit - Ghonta, Ghontphala
- Tamil - Kottei
- Telgu - Gotte
- Maharastra - Goti, Bhorgotti, Kantegoti
- Oriya - Got, Gotoboro, Kantabohul
- Bot. Name - *Zizyphus xylopyrus* (Retz.) Willd.
- Old names - *Ziziphus Xylopyra* Willd.
- Synonym - *Rhamnus Xylopyrus* Willd.
- Parts used - Leaves, Bark, Flower, Seeds.[4]

Macroscopic Characters

A large, straggling shrub or a small three, armed with spines, up to 4 m. in height. Branches, inflorescence and fruits covered with short, grayish tomentum; leaves broadly elliptic, obovate or orbicular, serrulate, glabrous, dark above and covered with soft or pale tomentum beneath, oblique; flowers in compact chymes; fruits globose, 3-, rarely 2-, or 4- celled, with usually a seed in each cell, very hard and woody. The tree often forms impenetrable thickets when young. The tree is a light demender, growing typically in the open situations, but is capable of standing in a light degree of shade. It withstand drought well.[6]

Microscopic Characters

A transverse section of the fruit reveals a thick cuticle followed by epidermis consisting of unevenly arranged rounded cells; scattered thick-walled, uniseriate, multicellular trichomes present on epidermis; mesocarp with three zones - narrow outer and inner zones of small, compactly arranged parenchyma cells; a third wide middle spongy zone composed of thin walled parenchyma cells, lacunated and containing scattered vascular strands; endocarp consisting of thick walled stone cells, narrow fibres and a few lacunae, some stone cells containing prismatic crystals of calcium oxalate up to 12 µ in size; occasional inroads of mesocarp into the endocarp also seen; epidermis and a few outer layers of mesocarp adjacent to it contain abundant brown substances.

A section through the testa shows radially elongated, narrow, transluscent cells, followed by a subepidermal zone of crushed, thin walled, parenchyma cells demarcated inside by a reddish brown lining. A section through the cotyledons shows an outermost epidermal layer of small, squarish cells and a ground tissue composed of rectangular thin walled, prominently nucleated cells rich in fixed oil.

Powder - Thick walled uniseriate, multicellular, 200 to 260 µ long trichomes; fibres (upto 50 µ in width) and angular stone-cells with radial canals and circular striations, 40 to 170 µ in size are seen- tissue fragments of epidermis in surface view present.

Identity, Purity and strength

- Foreign matter not more than 1 per cent, Appendix 2.2.2.
- Total Ash Not more than 12 per cent, Appendix 2.2.3.
- Acid-insoluble ash not more than 1 per cent, Appendix 2.2.4.
- Alcohol-soluble extractive not less than 3 per cent, Appendix 2.2.6.
- Water-soluble extractive not less than 2 per cent, Appendix 2.2.7.

TLC

Thin layer chromatography of the alcoholic extract on silica gel 'G' plate (0.2 mm thick) using chloroform : methanol (95:5) as mobile phase shows on spraying with methanolic: sulphuric acid reagent and on heating the plate for ten minutes at 110°C spots at Rf. 0.24 (Pink), 0.39 (Pinkish orange), 0.48 (Yellow), 0.61 (Pink), 0.71 (Blue).[6]

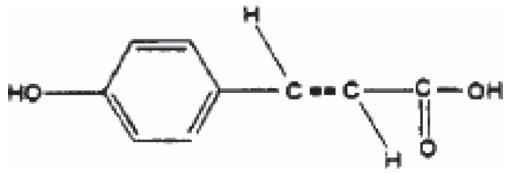
Chemical Constituents

The major chemical constituents found in this plant are:

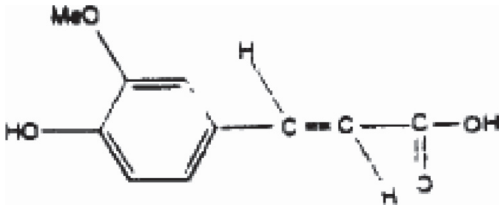
- Leaves are used for fodder an analysis of the leaves obtained from Tamil Nadu was as follows (on dry weight basis) ash, 12.82; Ca 2.61; total N_2 0.93; carbohydrate, 44%. The leaves contain Quercetin and Quercitrin.[7,8,9]

- Flowers the benzene fraction of ethanolic extract of flowers of ZX was shown to have E-4-hydroxy cinnamic acid: p-coumaric acid, (E)-4- hydmxy - 3-methoxy cinnamic acid: ferulic acid, 5,7,3',4'-tetra hydroxy-3-0-a-L-rhamnosyl flavone: quercitrin, 5,7,3',4'-tetrahydroxy 3-O-P-D-galactosyl flavone: hyperoside, kaempferol 3-O-rutinoside and Rutin which were isolated with the help of column chromatography. The entire chemical constituents were characterized with the use of UV, 1 H NMR, IR and or via the use of authentic samples.[8]
- Bark contains Tannins (7.2%), d-7, 3', 4'-trihydroxyflavan-3, 4-diol and oleanolic acid.[10,11] It also contains Cyclopeptide alkaloids namely Amphibine H, Nummularine- K,[11] Two new 13-membered cyclopeptide alkaloids, xylopyrine-A and xylopyrine-B have been isolated from the bark *Zizyphus xylopyra*, and their structures established by spectral and chemical evidences.[13]
- Root Bark reported to contain two flavonoids namely Kempferol-4'-methylether and Kempferol[14]
- The bark and wood of ZX was found to contain Betulinic acid (1%), However the wood of this plant does not show appreciable amount of either triterpines or leuco-anthocynidins.[14]
- Fruit contains Catechol-type of tannins (8-12%). Fruits were also reported to have Oleanolic acid,[15,16] l-leucocyanidin, 3, 3', 4-tri-O-methyl-ellagic acid.[10,11] The

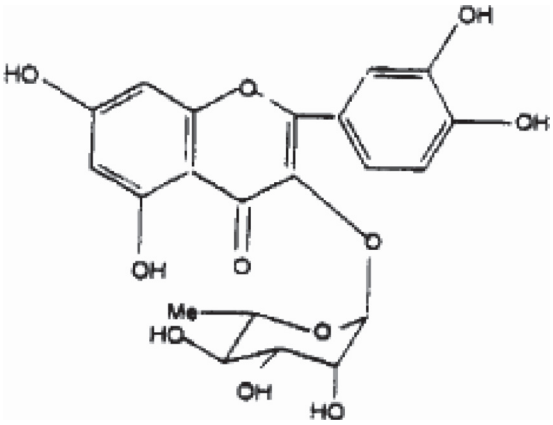
Flowers



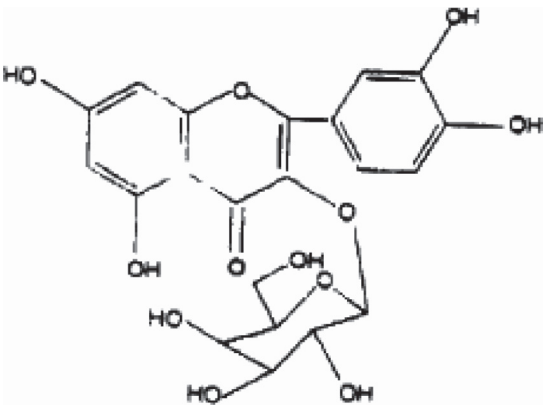
(E) - Paracoumaric acid



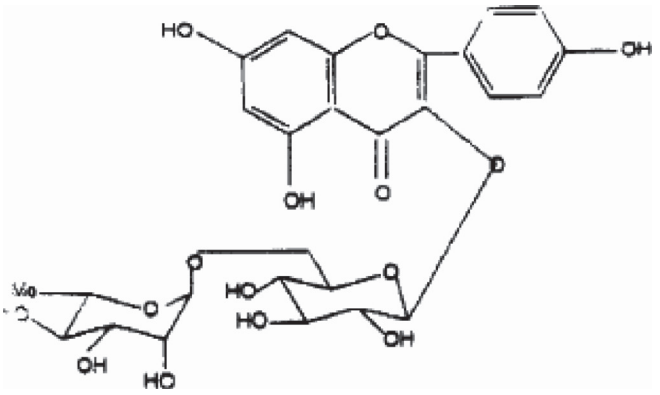
(E) - Ferulic acid



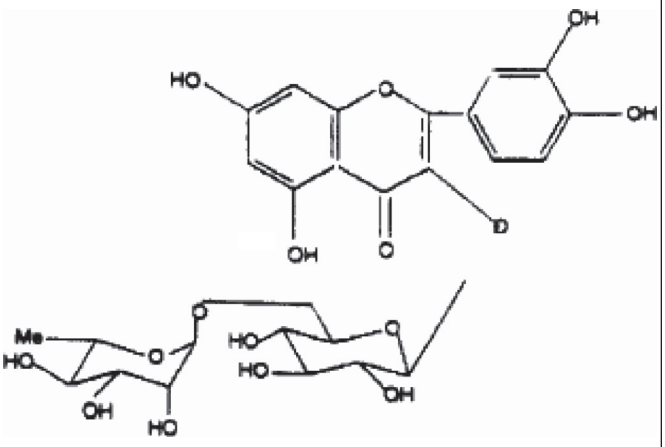
Quercetin 3-O-α-L-rhamnopyranoside



Quercetin 3-O-β-D-galactopyranoside



Kaempferol 3-O-β-D-rutinoside



Quercetin 3-O-β-D-rutinoside

Structures of Compounds isolated from *Ziziphus Xylopyrus*

pulp of the fruit contains reducing sugars, sucrose, citric acid, carotene, vitamin C and tannins.[6]

- Seeds unsaponifiable matter (0.8%) consists of a Sterol, insoluble mixed fatty acid found to contain Myristic, Linoleic and Oleic acid.[17]
- Stem wood is reported to have triterpenoids, lupeol, betulinic acid and a new triterpenoid designated as isoceanothic acid.[18]

Non Medicinal uses

Propagation of Lac

ZX is one of the chief hosts for the propagation of Lac. Shellac is prepared from the stick-lac, a resinous substance secreted on the twig of the plant by an insect *Tachardia lacca*. Shellac is the most satisfactory material for the manufacture of photographic records. It is a high-grade insulator and is extensively used in the electrical industry. It is the principal Spirit-Varnish resin yielding a tough film with a smooth finish. Shellac is also used in making sealing wax, drawing inks, some water colors and Nitro Cellulose lacquers, for sizing papers, for stiffening felt hats and in India for various ornamental purposes.[19,20,21]

Changes isozyme patterns of peroxidase and polyphenol oxidase by VAM fungi

The efficacy of six Vesicular Arbuscular Mycorrhizae (VAM) species viz. *Acaulospora morrowae* Spain & Schenk, *Gigaspora margarita* Beker & Hall, *Lomus fasciculatum*

(Thaxt. Sensu. Gerd.) & Troppe, *G. macrocarpum* Tul. & Tul. *Scutellospora calospora* (Nicol. & Gerd.) Walker and Sanders and *Sclerocytes rubiformis* Gerd. & Thorppe. Collected from the Rizospheres of ZX was evaluated for the enhancement of NR (nitrate reductase), GS (glutamine synthetase), PPO (poly phenol oxidase), PRO (per-oxidase), GDH (glutamine dyhydrogenase) activities and protein, Phenolics and Catectin contents in this fruit tree. Culturing was done under Glass house conditions and analysis was done 180 days after inoculation. All fungi showed beneficial effects with *Scutellospora calospora* seemed to be best promoting of all biological parameters.[22]

Traditional uses

This plant is widely used in Turkish folk medicines as a potent Sedative. The leaves (2 1/2) are chewed for 15 days as well as fruit is used in urinary troubles,[23] the roasted seed powder paste is applied over the chest for reliving pain after cough and colds.[24,25] In Ayurveda this plant is used as an Antidote especially against Snake bite and lizard poisoning. The bark is used as an Astringent.

This plant is also used as Dental sticks for teeth cleaning. In various parts of Orissa this plant is used in diarrhoea.[26] The fruit decoction of this plant is used as develop sterility in women for birth control in some parts of Rajasthan, India.[27] Roots of this plant crushed with *Calotropis gigantea* (Linn.) R.Br. (Jillaedu) stem barks, *Erythroxylum monogynum* Roxb. (Devadaari) and *Pterocarpus marsupium* Roxb. (Yegisha), and 10-12 dry chilies is administered for 2-3 days with one liter of water once daily in asthma by Lambadas or in the eastern Ghats region of Andhra Pradesh by the local tribal inhabitants and herbal practitioners.[28]

Medicinal uses

Antinociceptive, Anticonvulsant and Anti-inflammatory activity

The ethanolic extract of bark, was administered in graded doses (200-1000 mg/ kg body weight) intraperitoneally (ip) in albino mice of either sex (15-30 g) Antinociceptive activity of ZX was determined by radiant heat rat tail hot wire technique using a Techno analgesiometer. Anticonvulsant activity of ZX was determined against supramaximal electroshock seizure and the Anti-inflammatory activity of ZX was determined against oedema produced by subplantar injection of 1% carrageenin in saline. The results obtained suggest that ZX might possess central nervous system (CNS) depressant activity. The present study suggests that alcoholic extract of the bark of Zizyphus zylopyrus possesses antinociceptive, anticonvulsant and anti-inflammatory properties.[29]

Antidepressant effect

The antidepressant effect of ZX was examined using two behavioral models, the forced swimming test (FST) in rats and tail suspension test (TST) in rats. Ethanolic extract when administered at an acute dose of 50 mg/kg of body weight ($P < 0.01$) reduced the immobility time by 10 and 15 seconds as compared to the immobility time of control in both the screening models. Similarly Ethyl acetate fraction of ethanolic extract reduced latter by 30 and 35 secs. The aqueous ppt. fraction of ethanolic extract showed the best activity, reducing the immobility time by 50 and 60 secs. in both the tests. These results showed that after standard i.e. Imipramine HCl (30 mg/kg), the ppt. fraction is potent amongst all the studied drugs. The present study clearly demonstrated that *Zizyphus xylopyrus* exerts an antidepressant effect in these two behavioral models. It may be due to presence of various kinds of flavonoids.[30]

Antibacterial activity

The bacterial strains E. coli, B. subtilis, P. aeruginosa and S. aureus (multi-drug resistant strain) were used to evaluate the preliminary screening of seeds of ZX water extract, minimum inhibition concentration (MIC) and minimum bacterial concentration (MBC) with streptomycin and tetracycline used as reference antibiotics. Finally it was concluded that the later extract of ZX does not possess any antibacterial activity against all of the tested bacterial strains *in vitro*.[31]

Properties and Action

Rasa : Madhura, Katu, Kasaya
Guna : Laghu
Virya : Usna
Vipaka : Katu
Karma : Visaghna, Vatakaphahara

Important Formulations

Aragvadhadi Kvatha Churna

Therapeutic Uses

Dustavrana, Jvara, Kandu, Kustha, Prameha, Raktavikara, Vrana, Savayathu, Nadivrana, Vamana

Subscribe Dose - 3-6 g.[6]

FUTURE ASPECTS

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasonings, beverages, cosmetics, dyes, and medicines. The World Health Organization estimated that more then 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components.

Zizyphus xylopyrus (Retz.) Willd is not studied to a greater extent but, whatever the available literature suggests it has the potential to be a very good cure of various diseases. The phytochemial studies on this plant suggests that it contains variety of phytochemicals like Flavonoids, Tannins, Terpenoids, etc. Flavonoids belong to a group of polyphenolic compounds, which are classified as flavonols, flavonones, flavones, flavanols, flavan-3-ols and isoflavones according to the positions of the substitutes present on the parent molecule. Flavonoids of different classes have several pharmacological activities. Flavonoids have also been known to possesss biochemical effects, which inhibit a number of enzymes such as aldose reductase, xanthine oxidase, phosphodiesterase, Ca+2-ATPase, lipoxygenase, cycloxygenase, *etc.* They also have a regulatory role on different hormones like estrogens, androgens and thyroid hormone. In view of their wide pharmacological and biological actions, they seem to be having a great therapeutic potential.[32] Apart from that many of the Zizyphus species has shown to possess numerous pharmacological action and ZX is one amongst them.

Zizyphus xylopyrus (Retz.) Willd is one of the rare medicinal herbs which are yet to prove its medicinal efficacy. But to be medicinally effective it has to undergo various scientific approaches and every phytochemical and pharmacological concern has to be revealed. The present work on this plant is an effort to investigate all the aspects which can give light on the medicinal aspects of later. Though not much work has been done on this plant but we have tried to investigate every possible literature available on this plant. The above work depicts that if this plant is seriously studied scientifically it can prove to be a wonder herb for various ailments existing.

ACKNOWLEDGEMENT

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High-Performance Thin-Layer Chromatographic Fingerprints of Two New Macrocyclic Spermine Alkaloids (Budmunchiamines) from *Albizia amara*

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ABSTRACT

Many of the species of *Albizia* are used traditionally for various diseases like astringent, treating piles, diarrhoea, gonorrhoea, leprosy, leucoderma, erysipelas and abscesses. In traditional medicine, *Albizia amara* is used to heal stomach ailments and also used as tonic, anti dandruff.

The flowers have been applied to boils, eruptions, swellings, also regarded as an emetic and as a remedy for coughs and malaria. The plant under study has been used since ages by folk because of its rich medicinal values. All the parts of the plant have an ethno medicinal importance. In present paper; the fingerprint of high-performance thin-layer chromatography (HPTLC) was developed to identify species in detail. The unique properties of the HPTLC fingerprints were analyzed. The pattern of the HPTLC images of the leaves of *Albizia* spp. and the different ratios of the chemical distribution can directly discern the species.

Key words: *Albizia amara*; High performance thin layer chromatography; Budmunchiamines; Fingerprint.

INTRODUCTION

Albizia amara (Fabaceae/Leguminosae) seeds used in the traditional and siddha system as an astringent, treating piles, diarrhoea, gonorrhoea, leprosy, leucoderma, erysipelas, mouth ulcers and abscesses. The flowers have been applied to boils, eruptions, swellings, also regarded as an emetic and as a remedy for coughs and malaria. Alkaloids such as budmunchiamines have been demonstrated to be the major efficient component *Albizia amara* as anti tumor, anti bacterial.[1]

Concerning quality assessments of herbal medicine, conventional analysis based on GC, HPLC and TLC always focus on qualitative and quantitative determination of individual or several known components but fail to evaluate chemical properties comprehensively and effectively. In recent years, chromatographic fingerprint profiling has shown to be more convenient and effective for quality assessment of herbal materials, especially when there is a lack of authentic standard substances for the identification of the entire active component present in these complex natural products. Although HPLC dominated the chromatographic fingerprint literature, the unique feature of picture-like image of HPTLC coupled with digital scanning profile is more and more attractive to the herbal analysts to construct the herbal chromatographic fingerprint by means of HPTLC.[2] In the present study, the fluorescent HPTLC fingerprint of budmunchiamines (Alkaloids) in *Albizia amara* was developed with advanced instrumental planar chromatographic facilities, and the corresponding digital scanning chromatographic profiles were generated with self-developed software. This HPTLC fluorescence image coupling with the scanning profile provided adequate information and parameters for comprehensive identification and assessment of the species in order to use the herbal drug properly.

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MATERIALS AND METHODS

Apparatus and reagents

HPTLC was carried out with a Camag TLC system (Camag, Muttenz, Switzerland) fitted with a WinCATS 1.2.3 software. Samples were applied with a Camag automatic TLC

sampler 4 (ATS 4) and developed in twin-trough glass chamber (24.5 cm × 8cm × 22.5 cm). A ReproStar 3 with VideoStore 2 documentation software (Camag, Muttenz, Switzerland) was used for the imaging and archiving the TLC chromatograms. TLC digital scanning software used to transfer plate image to digital scanning plot was developed by our research group. HPTLC precoated plates, silica gel Merck 60, 20 cm × 10 cm were used (Merck, Darmstadt, Germany, code: OB456076). All chemicals and solvents were of analytical grade and used as obtained.

Materials

The leaves of *Albizia amara* were collected and authenticated from medicinal garden of Medicinal plants Revitalisation and Rehabilitation Centre, Sevaiyur, Tamilnadu and authenticated by Dr. S. Jha, Professor, Birla Institute of Technology, Mesra, Ranchi, India where voucher specimens were submitted according to morphological characteristics.

Preparation of sample solution

The crude drugs were dried under shade for 4-6 days. Then the dried materials were milled to powder. This powdered material was again dried in the oven at 40°C for 4 h and used for extraction followed by concentration, screening, TLC, HPTLC and column chromatography of extracts. The coarsely dried powdered leaves were extracted with Petroleum Ether (60°-80°) cold maceration for 72 h, and hot percolation by 90% methanol about 72 h. The extracts were recovered and concentrated to dryness.

Chromatography

Standard and sample solutions were applied bandwise (band length 6mm, 70 nL/s delivery speed, track distance 6 mm, distance from left edge 12 mm and low edge 8 mm) to the HPTLC plates. Then, the plate was desiccated in a vacuum trunk containing phosphoric anhydride (P_2O_5) for 2 h. After saturated for 15min with mobile phase vapor, the plates were developed for 85mm at ambient temperature of 16.5°C in a Camag twin-trough chamber with the low layer of chloroform–Diethylamine (88:22, v/v) as mobile phase. Visualization of the chromatogram was performed by spraying with Dragendorff reagent and heating at 105°C until the bands visible. The plate was observed immediately at UV (366 nm) cabinet and the HPTLC fluorescence image was documented.

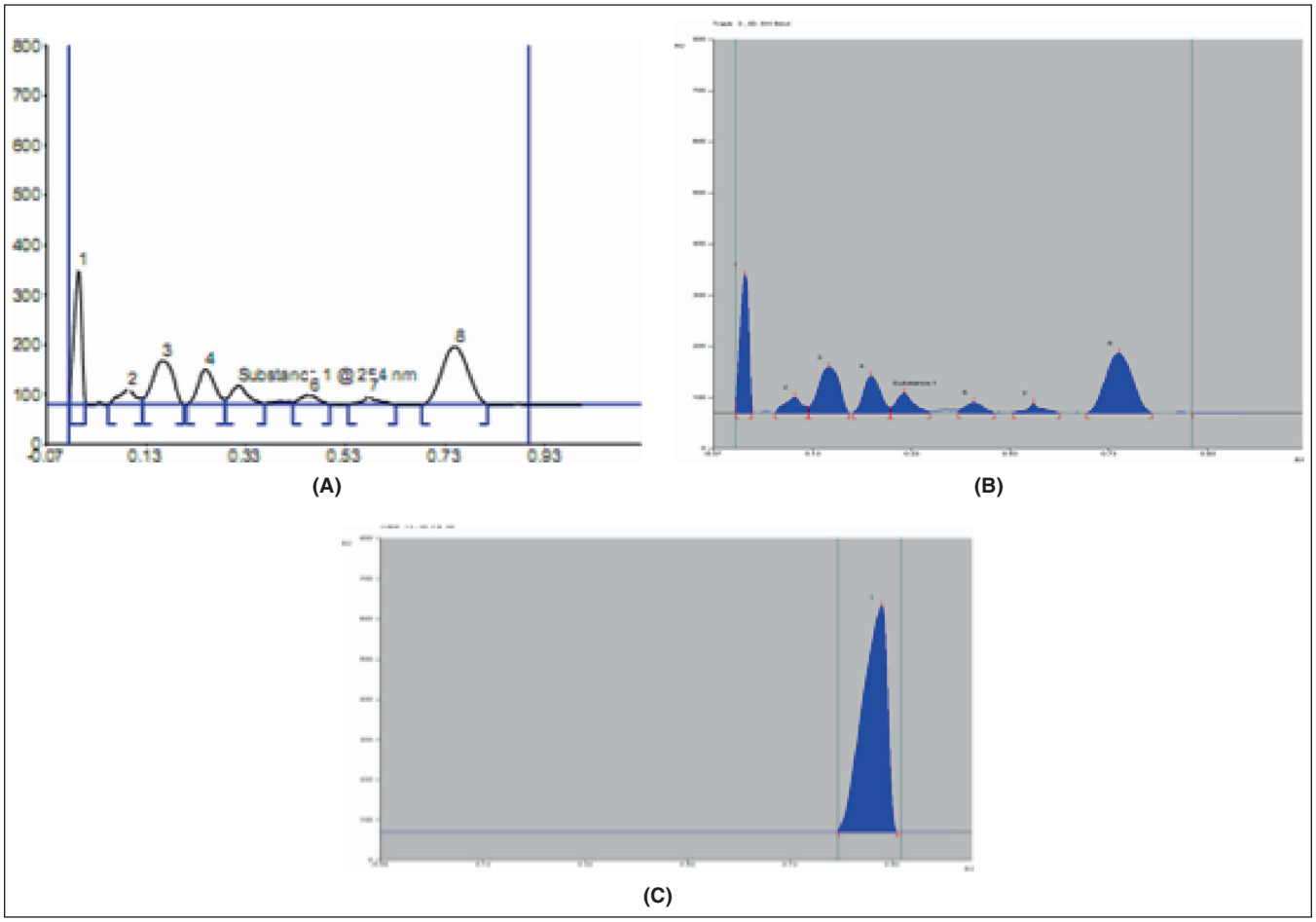

Figure 1 : (A), (B) HPTLC fingerprint of Petroleum ether extract
(C) HPTLC fingerprint of β-Sitosterol

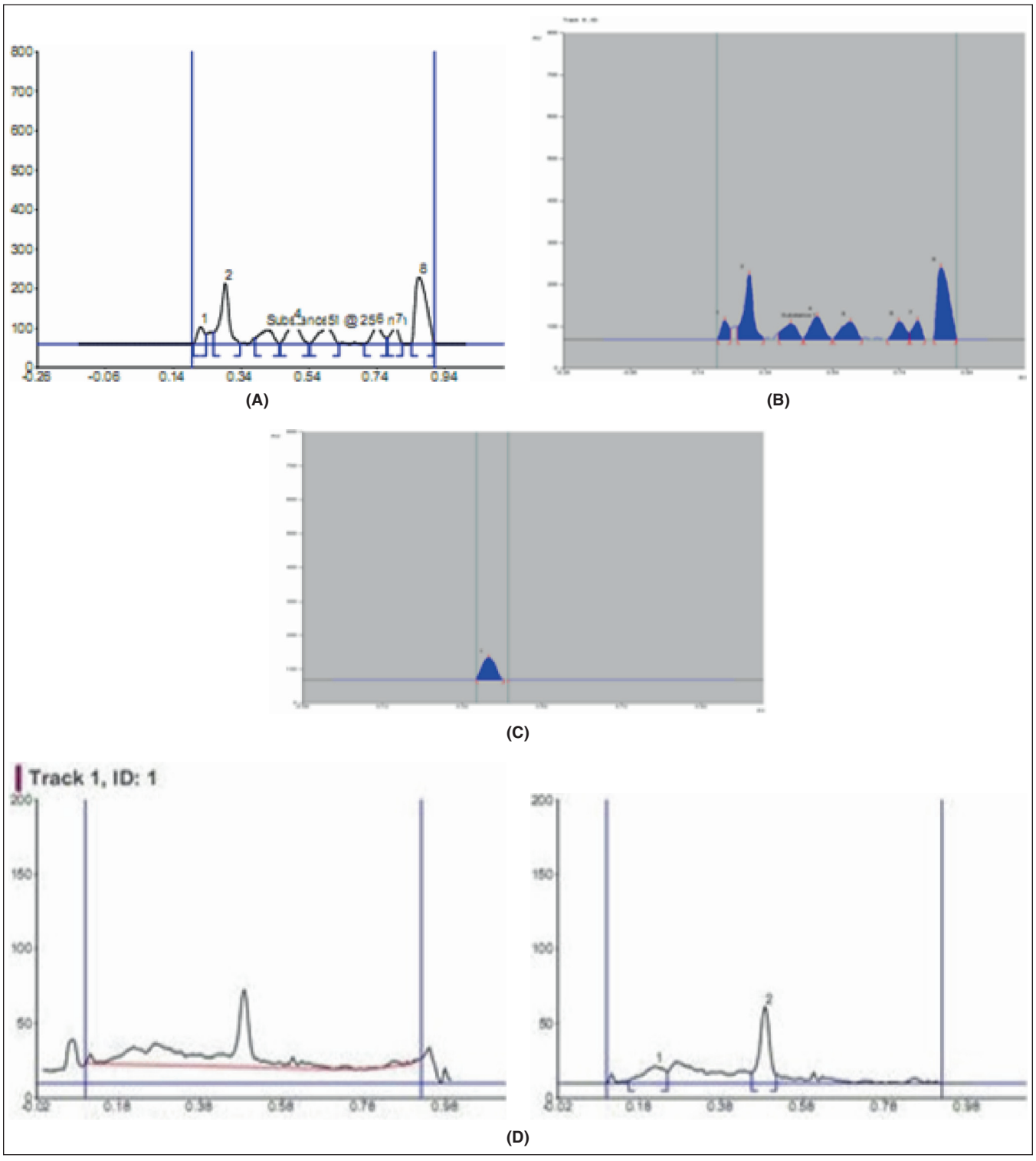

Figure 2: (A), (B) HPTLC fingerprint images of Methanolic extract
(C) HPTLC fingerprint of Budmunchiamine L6
(D) HPTLC fingerprint of 6~ ξ'-Hydroxybudmunchiamine-C

Isolation and purification of phytoconstituents

Petroleum ether and methanol extracts were subjected to column chromatography using gradient elution technique for the isolation of various phytoconstituents.[3,4,5,6]

By HPTLC analysis it was found that 8 compounds were present in petroleum ether extract. This extract was subjected to column chromatography using gradient elution technique over Silica-60-120 mesh (Merck), the spots were visualised

by spraying with anisaldehyde-sulfuric acid reagent followed by heating at 100°C for 3 min. column was packed with petroleum ether and eluted by gradient elution technique starting from petroleum ether (60-80°),chloroform, methanol ,ethyl acetate and water.

Chloroform: Ethyl acetate (60:40) yielded fraction V, after usual work up Compound SR-01 afforded 30 mg, TLC, Pet. Ether: Chloroform (5:3), R_f 0.8).

HPTLC suggested that 8 compounds were present in Methanolic extract. The MeOH extract were dissolved in 2% aqueous acetic acid (500 ml) and partitioned with $CHCl_3$, (500 ml). The aqueous acidic fraction was saved, and the $CHCl_3$, fraction was extracted with additional 2% acetic acid (500 ml). The aqueous fractions were combined, adjusted to a pH of 9.0 with Ammonium hydroxide and extracted with $CHCl_3$, (1 litre). The $CHCl_3$, fraction was recovered and concentrated to dryness in rotary evaporator.[1]

The basic substance was subjected to Column chromatography (CC) over Silica gel 60-120 mesh (Merck), the spots were visualised by spraying with dragendraff's reagent followed by heating at 100°C for 3 min. column was packed with petroleum ether and eluted by gradient elution technique starting from petroleum ether (60-80°),chloroform, methanol, ethyl acetate and water.

$CHCl_3$/MeOH/(60:40); yielded fraction 53-65(pooled together, Compound SR-02); $CHCl_3$/MeOH (20:80) yielded fraction 65-70 (pooled together, Compound SR-03). All fractions gave viscous liquids.

Compound SR-02, after rigorous purification (TLC, $CHC1_3$-Et_2NH-MeOH, 8:1:1, afforded isolate (R_f:0.53, 45 mg). after usual work up Compound SR-03 afforded 75 mg, TLC, $CHCI_3$-Et_2NH-MeOH, 8:1:1, R_f 0.45).

RESULTS

Compound SR-01 was a pale yellow to white crystalline powder isolated from petroleum ether extract responded to positive to Libermann - Burchard test for steroids, which was identified as β – sitosterol based on M.P, HPTLC, FT-IR and the literature available.[7]

The physico-chemical properties and the spectral data (UV, FT-IR, MS & ^{1}H-NMR) of the compound SR-02 were interpreted and compared with the Budmunchiamines L6 from *Albizia lebbeck*.[8,9]

The physico-chemical properties and the spectral data (UV, FT-IR, MS & ^{1}H-NMR) of the compound SR-03 were interpreted and compared with the 6~ ξ'-Hydroxybudmunchiamine-C from *Albizia schimperana*.[10]

DISCUSSION

Besides HPLC and GC, with the utilization of advanced instrument and data analysis system as well as optimized experimental operation, HPTLC is also feasible for development of chromatographic fingerprint profiling methods to determine complex herb extracts. Furthermore, the colorful picture-like HPTLC image provides extra intuitive visible color and/or fluorescent parameters for parallel assessment on the same plate. In present study, the proposed HPTLC fingerprint described how to construct a conjugated fingerprint of the alkaloid fraction. HPTLC images developed by two solvent systems. Such an approach makes the HPTLC plate efficiency extended and consequently enables the identification and quantifiable assessment between the two species more effective.[2]

CONCLUSION

In conclusion, the conjugated HPTLC fingerprint (Alkaloid Budmunchiamine) disclosed in detail that there are significant difference between the different extracts of leaves of *Albizia amara* not only in the content of alkaloids as described in literatures, but also in the whole alkaloids and peak-to-peak ratios expressed by the fingerprint patterns and its integrated area value.

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Pharmacognostic and Preliminary Phytochemical Studies on *Ruellia tuberosa* L. (Whole plant)

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ABSTRACT

Ruellia tuberoa L. of Acanthaceae family is a Minnie root medicinal, tropical plant widely distributed in south East Asia. In folk medicine, it has been used as diuretic, antidiabetic, antipyretic, analgesic, anti-hypersensitive, thirst-quenching, and antidotal agent. Recently this plant has been incorporated as a component in an herbal drink in Taiwan. However a very few chemical constituents and pharmacological activities have been reported for this species. As there is no pharmacognostic work reported on whole plant, the present investigation attempts to undertake to study the pharmacognostical and preliminary phytochemical studies along with HPTLC, fingerprinting analysis for whole plant of *Ruellia tuberosa* L. Preliminary organic analysis revealed the presence of tannin, flavonoid, steroid, triterpnoid and phenol in different extracts respectively. Physiochemical studies revealed that total ash is 13.53%, acid insoluble ash is 2.36%, alcohol soluble extractive value is 7.67%, water soluble extractive value is 24.78% and loss on drying at 105°C is 11.29%. These specific identities will be useful in identification and authentication of the raw drug.

Key words: *Ruellia tuberosa,* Phytochemical studies, Pharmacognosy, HPTLC.

INTRODUCTION

Ruellia tuberosa L. (Pattaskai) belong to the family Acanthaceae, is a Minnie root, tropical perennial plant with a hairy quadrangular stem growing up to a height of 6.5 cm. The leaves are simple, opposite elliptic about 5 cm in length. The plant only flowers after the start of the rainy season. It has thick finger like roots. The flower is bisexual and violet in colour. The capsule contain 7 to 8 seeds each burst and open with a bang when they get wet and the black seeds are hurdled away. The capsules are baton shaped and 3 cm in length and turn black with the age. Because of explosive behaviour of capsule name of the plant is Pattaskai in Tamil and cracker plant, blue bell, popping pod, waterkanon in English. This plant prefers semi shady moist condition.[5,6] *Ruellia tuberosa* L. is a tropical plant and widely distributed in South East Asia, in folk medicine, it has been used as diuretic, antidiabetic, antipyretic, analgesic, antihypertensive, thirst quenching, antinociceptive, anti-inflammatory and antidotal agent. It has also recently been incorporated as a component in an herbal drink in Taiwan[1,2,3,4,7,21] and traditionally used for reducing toxicity, healing urine tract inflammation.[8]

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MATERIALS AND METHODS

Fresh plant materials were collected from Tamilnadu and identified with the help of The Flora of Presidency of Madras[9,10] and a dried specimen (No: 00628) was deposited in museum of CSMDRIA Chennai. For microscopic study properly washed plant material was cut into desirable size and preserved in FAA, free hand sections were taken stained with the reagents used in pharmacognosy studies. Drawings were made with the help of camera lucida.[11, 22]

Powder of the dried whole plant of *Ruellia tuberosa* L. was used for chemical analysis: physicochemical studies like total ash, acid insoluble ash, water-soluble ash, alkalinity of water soluble ash content and loss on drying at 105ºC, crude fiber, water soluble extractive and alcohol soluble extractive were carried out as per WHO guidelines.[12] Preliminary phytochemical tests were done as per the standard methods.[13,14,15] The fluorescence behaviour of the powdered drug in the day light and ultraviolet light were done by moistening the powder in different solution and viewing under the light of different wavelengths in a UV-chamber.[10,16] The air dried and coarse powder of whole

plant was successively extracted with n-hexane, chloroform, ethyl acetate and alcohol and also aqueous separately using soxhlet apparatus, filtered, concentrated and made up to 10ml in a standard flask.[17]

HPTLC methodology by 0.200 gm of ethyl acetate extract was dissolved in 10 ml of ethyl acetate filtered and the solution was made up to 10 ml in a standard flask. 5, 10 and 15 µl of this solution were applied on (E.Merck) aluminum plate percolated with silica gel 60 F_{254} of 0.2 mm thickness, CAMAG Linomat IV applicator was used for applying the spot on plates. The plates were developed in toluene: ethyl acetate (6:2 v/v). The plate was visualized under in UV 254 and 366 nm. It was scanned using deuterium lamp provided with wincats software version 1.4.4. The air dried plate was then dipped in vanillin-sulphuric acid reagent and kept in oven at 105°C till the colour of the spots appeared. The photographs were taken.[15,18,19,20]

RESULT AND DISCUSSION

Macroscopy

Root: Rhizomatous or tuberous, fleshy, cylindrical, numerous from the root stock. Colour white, initially sweet in taste, gives slight tingling and bad taste later (Fig. 1C).

Stem: Woody to semi woody, hairy, branched, nodes swollen and slightly quadrangular in the young ones, internodes 5 to 7 cm long, up to 4 mm in diameter, lower basal somewhat spherical and bearing thin, slender, hard adventitious roots at the nodes, fracture short, colour pale green (Fig. 1A).

Leaf: Simple, opposite, decussate, petiolate, ridge joins opposite petioles, petiole 0.2 to 1.5 cm long, blade 5 to 9 cm long, 2 to 5 cm wide, elliptic or oblong or ovate or obovate, base tapering, margins entire or crenate, apex acuminate or acute or cuspidate or obtuse, hairy on veins. (Fig. 1A).

Flower: Arranged in cymes, sometimes cleistogamous, predominantly blue, actinomorphic or somewhat irregular, pedicellate, pedicel 0.4 to 0.8 cm long, perianth 2 -whorled; calyx 1.8 to 3 cm long, sepals 5, gamosepalous; Corolla 5 to 5.4 cm long, petals 5, tubular; stamens 4, adnate to the perianth, all alternating with the petals; anthers dorsifixed, dehiscing via longitudinal slits; ovary syncarpous, superior, 2 -celled; ovules 13 per cell in 2 rows; styles single, simple. (Fig. 1A).

Fruit: A dehiscent capsule, narrowing at ends, slightly flattened to cylindric with a septum at the centre, elliptic to linear-oblong, gland dotted when young, glabrous when fully mature, 1.7 to 3.2 cm long, 2.4 to 4 mm wide (Fig. 1B).

Microscopy

Root: TS is quadrangular in outline with winged corners. In the sections from young, the central pith is wider and is encircled by discontinuous ring of vascular bundles, unlike the older one where a continuous ring of xylem is seen. The detailed TS of mature stem shows a layer of epidermis covered with thin cuticle and few multicellular trichomes, hypodermis is formed by about 6 layers of regular square-like cells which are placed one above the other, cortex is narrow, major cortical cells are chlorenchymatous, endodermis is distinct; phloem is narrow, it is traversed with few isolated thin walled fibres, few layers of cambium lies underneath it; xylem is very wide, consist of few small sized, isolated, scattered vessels, tracheids, fibres and parenchyma, the major elements being of fibres. Pith is parenchymatous, occasionally embedded with few simple starch grains (Fig. 2A).

Figure 1: *Ruellia tuberosa* L.
A,B. Photograph from the natural habitat showing flower, stem and fruits; C. Tuberous roots

Stem: TS is quadrangular in outline with winged corners. In the sections from young, the central pith is wider and is encircled by discontinuous ring of vascular bundles, unlike the older one where a continuous ring of xylem is seen. The detailed TS of mature stem shows a layer of epidermis covered with thin cuticle and few multicellular trichomes, hypodermis is formed by about 6 layers of regular square-like cells which are placed one above the other, cortex is narrow, major cortical cells are chlorenchymatous, endodermis is distinct; phloem is narrow, it is traversed with few isolated thin walled fibres, few layers of cambium lies underneath it; xylem is very wide, consist of few small sized, isolated, scattered vessels, tracheids, fibres and parenchyma, the major elements being of fibres. Pith is parenchymatous, occasionally embedded with few simple starch grains (Fig. 2B).

Petiole: TS of petiole is almost rectangular in outline with two elongated winged projections at the corner with meristeles corresponding to the wings, under both the epidermis of the midrib lays the collenchymatous tissue, and an arc of centrally located meristele in the ground

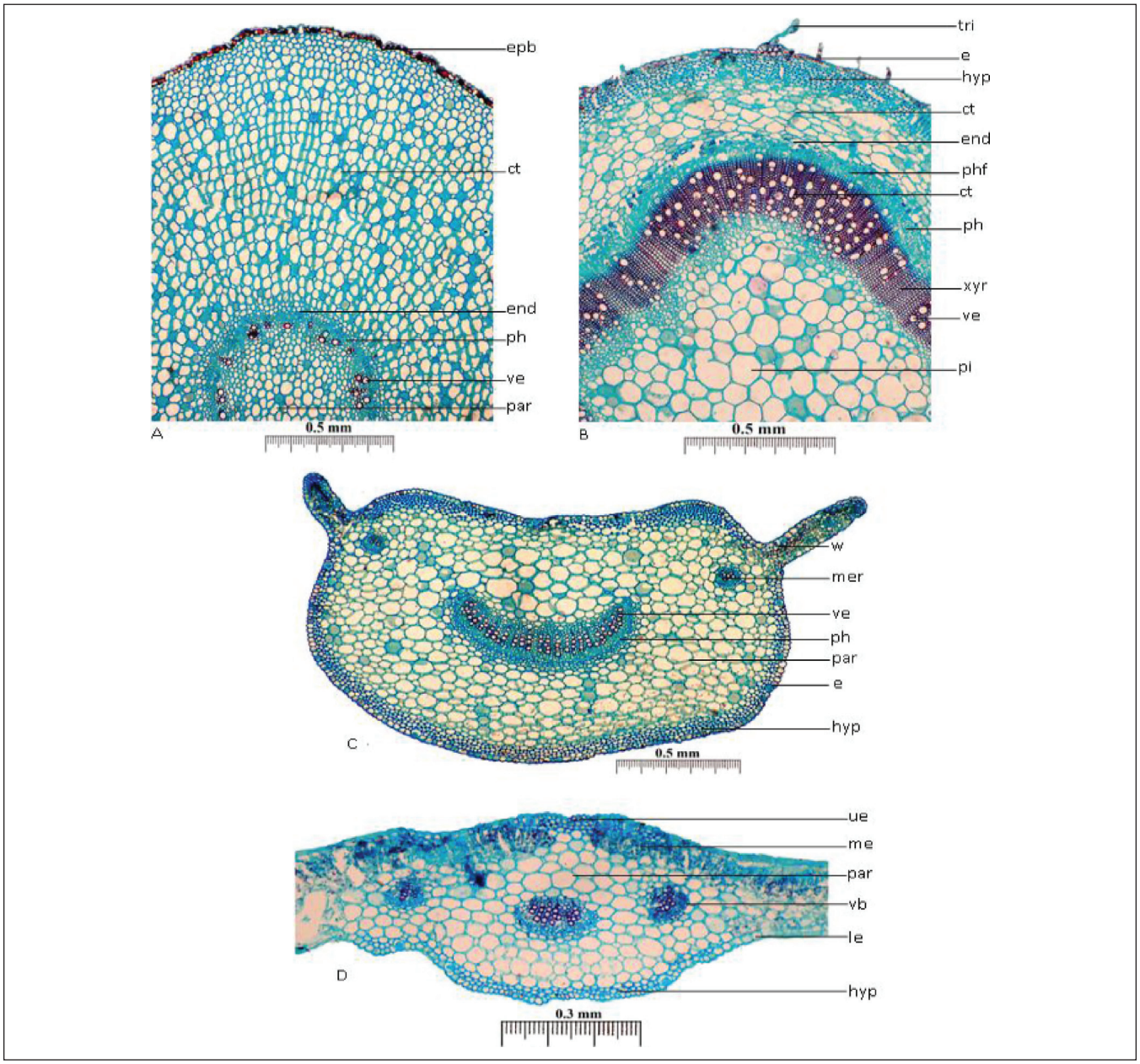

Figure 2: Detailed TS of *Ruellia tuberosa* L. whole plant.
A. Root; B. Stem; C. Petiole; D. Lamina passing through midrib. ct, cortex; e, epidermis; end, endodermis; epb, epiblema; hyp, hypodermis; le, lower epidermis; me, mesophyll; mer, meristele; par, parenchyma; ph, phloem; phf, phloem fibre; pi, pith; tri, trichome; ue, upper epidermis; vb, vascular bundle; ve, vessel; w, wing; xyr, xylem ray.

Table 1: Preliminary phytochemical test for different solvent extracts of *Ruellia tuberosa* L. whole plant

Test for	*n*-Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Alcohol Extract	Aqueous Extract
Steroid	+	+	+	+	+
Triterpenoid	+	+	+	+	+
Phenol	–	+	+	+	+
Flavonoid	–	–	+	+	+
Coumarin	–	–	–	–	–
Tannin	–	–	+	+	+
Furanoid	–	–	–	–	–
Alkaloid	–	–	–	–	–
Acid	–	–	–	–	–
Sugar	–	–	+	+	+

Present (+), Absent (–)

tissue. The detailed section shows epidermis bearing simple multicellular trichomes as mentioned above, few embedding cystoliths, followed by 3 to 5 celled thick collenchymatous tissue, the remaining cells being prenchymatous, an arc of well-developed centrally located conjoint collateral meristele of the midrib shows isolated phloem fibres, exhibiting cells of endodermis at places and 1 or 2 small rudimentary meristele far from its terminals (Fig. 2C).

Lamina: TS passing through middle of the midrib region of lamina is almost flat or slightly elevated on the upper side and broadly plano-convex at the lower side; few collenchymatous cells being located underneath of its both sides and palisade tissue under the upper epidermis only. Detailed TS shows upper and lower epidermis covered with thin cuticle, cells at places embedded stomata and few cystoliths, a layer of palisade runs under the upper epidermis, the remaining mesophyll tissue consists of 4 to 5 rows of spongy parenchyma, underneath both the epidermis of midrib lies few layers of collenchymatous tissue they being more celled in the upper elevated region; the ground tissue of the midrib is parenchymatous and is embedded with an arc of meristele (Fig. 2D).

Coarse powder of *Ruellia tuberosa* L. was soaked in n-hexane for 48 hrs the extract was filtered, the filtrate was concentrated and the last traces of solvent were removed in vacuum. Extraction was repeated with n-hexane and was then extracted twice with chloroform, ethyl acetate and alcohol respectively and successively (Yield were n-hexane 13.34%, chloroform extract 16.01%, ethyl acetate extract 39.85%, alcohol extract 56.63% and separate aqueous extract 63.86%). Results of preliminary phytochemical studies of various extracts are shown in Table-1. *n*-Hexane extract showed the presence of steroid and triterpenoids. Chloroform extract answered for steroid, triterpenoid and phenols. Ethylacetate extract and alcohol extract showed the presence of steroid, triterpenoid, phenols, flavonoids, tannin and sugars. Aqueous extract answered for steroid, triterpenoid, phenols, flavonoids, tannin and sugars. Coumarin, furanoid, alkaloid and acids are absent in all the extracts.

Table 2: Physico-chemical parameters of *Ruellia tuberosa* L. whole plant

Parameters	Results *n* = 3
Ash (%w/w)	13.53
Acid insoluble ash (%w/w)	2.36
Water soluble extractive (%w/w)	24.78
Alcohol soluble extractive (%w/w)	7.67
Los on drying at 105ºC (%w/w)	11.29

Table 3: HPTLC of ethyl acetate extracts of *Ruellia tuberosa* whole plant

Peak	$R_{f\,Value}$	Max %	Area %
1	0.05	1.87	8.44
2	0.08	1.36	0.93
3	0.16	2.36	3.05
4	0.20	1.34	0.74
5	0.25	9.45	7.17
6	0.27	5.42	3.63
7	0.31	4.19	4.56
8	0.39	0.75	0.72
9	0.44	1.34	1.18
10	0.46	1.31	0.67
11	0.50	1.18	1.01
12	0.57	0.81	1.02
13	0.62	3.82	3.36
14	0.66	1.11	0.85
15	0.73	4.97	4.25
16	0.77	8.30	6.78
17	0.81	1.97	2.85
18	0.86	8.46	8.79

Results of Physico-chemical parameters of whole plant powder of *Ruellia tuberosa* L. is shown in Table-2. Quantitative standards revealed that the ash content was 13.53% and acid insoluble ash was 2.36%. The water soluble extractive value was 24.78% it indicating the presence of sugar, acid and inorganic components. The alcohol soluble extractive value was 7.67% which shows the presence of polar and non polar secondary metabolites present in the plant materials. Loss and drying at 105°C was 11.29% revealing the moisture content in the plant.

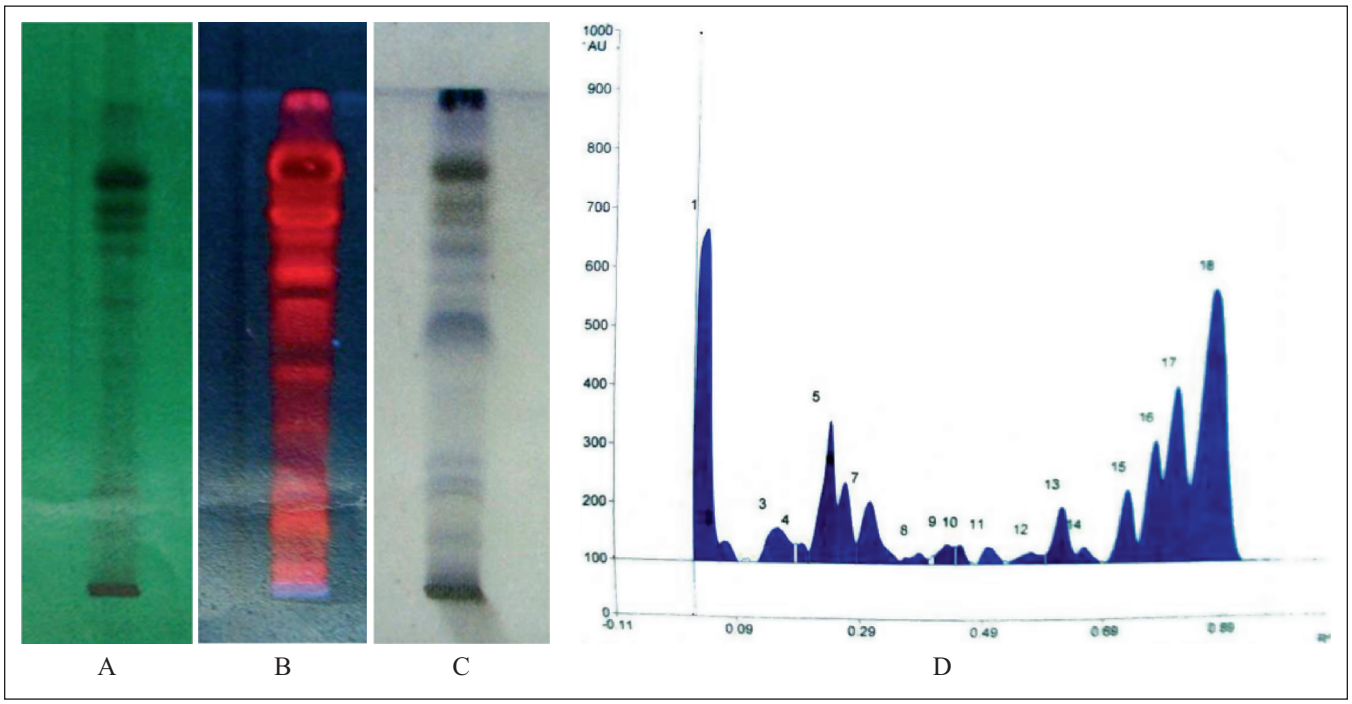

Figure 3: HPTLC of ethyl acetate extracts of *Ruellia tuberosa* L. whole plant.
A to C photo documentation at UV 254 nm; UV 366 nm and after dipping in Vanillin sulphuric respectively; D. Densitometric scan at UV 254.

Results of HPTLC studies shown in Table-3 and Figures 3: A, B, C, D. HPTLC finger print profile of the ethyl acetate extract showed 18 peaks. There were three major peaks at R_f 9.45 (7.17%), 8.30 (6.78%) and 8.46 (8.79%). Peaks with moderate concentration were observed as R_f 5.42 (3.63%), 4.19 (4.56%), 3.82 (3.36%), 4.97 (4.25%) the remaining peaks were in poor yield. At the point of application 0.05 also 8.44% remained.

CONCLUSION

The data generated from the present studies would help in the authentication of this whole plant when available both in dry and powder form. The microscopic features and the quantitative standards would be useful for laying down pharmacopoeial standards. The different spots observed in TLC and HPTLC finger print profile would be definitely useful in deciding the purity and quality of the drug, particularly of different batches. Morphology as well as various pharmacognostic aspects of whole plants were studied and described along with phytochemical, physicochemical, TLC and HPTLC studies in authentification for quality control.

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Pharmacognostic Study of Leaves of *Cordia rothii* Linn.

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ABSTRACT

Introduction: *Cordia rothii* Linn. syn. *Cordia sinensis*. (Family – Boraginaceae) is found commonly in India. Recent pharmacological findings indicate that its fruits possess significant activities like astringent, demulcent, diuretic, purgative, anti-ulcer, hepatoprotective, which comply with the claims made in the traditional medicinal texts. However, no conclusive pharmacognostic study of its leaves has been performed yet. Methods: The present investigation deals with the qualitative and quantitative microscopic evaluation of the leaf material and establishment of its quality parameters, including physicochemical and phytochemical evaluation. Results: Chief microscopic characters include vascular bundles having patches of perimedullary phloem and very long unicellular covering trichomes. Chief characters of powder include cylindrical shaped trichomes, calcium oxalate prisms, cluster crystals and xylem vessels in spiral shape. Conclusion: Such a study would serve as a useful gauge in standardization of the leaf material and ensuring quality formulations.

Key words: *Cordia sinensis*, Boraginaceae

INTRODUCTION

Cordia rothii Linn. syn. *Cordia sinensis*. (Family – Boraginaceae) is also known as (Gujrati) gundi , (Hindi) gondi, (Sanskrit) laghushleshmataka and (Sind) liai.[1] Its fruits are used traditionally in several disorders like bronchitis, ulcers, dysentery, diuretic, abscess, chronic fever.[2] The present investigation deals with the qualitative and quantitative microscopic evaluation of the leaf material.

MATERIALS AND METHODS

Collection and authentication of leaves

Leaves of *C. rothii* were collected from the herbal garden of R. K. College of Pharmacy, Rajkot in March, 2010. Herbariums and voucher sample were prepared and deposited in Department of Pharmacognosy, R. K. College of Pharmacy (Voucher no. RKCP/COG/09/2010). Authentication was done by the taxonomist of Department of Botany, Saurashtra University.

Pharmacognostic studies

Morphology of fresh leaves of *C. rothii* was studied. Photomicrography of stained and unstained transverse sections of fresh leaves was performed using Win DVR software. Leaf constants were established using camera lucida. The leaves were dried under shade, powdered to 60#, stored in airtight containers and used for powder study and quantitative microscopy (Table 1).[3-11]

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RESULTS AND DISCUSSION

Pharmacognostic study

Macroscopical characteristics

The leaves are 12-14 cm in length and 2.5-4 cm in width. The upper surface is dark green while lower surface is light green in color. It is a simple leaf containing reticulate venation and papery texture. It has entire margin with obtuse apex. The general outline is lanceolate and base is asymmetrical. The lamina surface is very rough. It has short petiole about 1.3 cm long and stipules are absent. The arrangement of leaves is sub opposite and they have characteristic taste and odor (Figure 1).

Microscopy: Transverse section

Lamina of the transverse section has no prominent hypodermis beneath the upper epidermis. Underlying the upper epidermis a double-layered, compact, radially elongated palisade cells followed by spongy mesophyll composed of 3-4 layers of loosely arranged parenchymatous cells with scattered calcium oxalate cluster crystals. Midrib consists of a well-developed collenchyma below upper epidermis and above lower epidermis. Ground

Table 1: Quantitative microscopy

Parameter	Mean value ± SD
Stomatal Index	
Upper surface	25 ± 0.5
Lower surface	28 ± 0.5
Stomatal Number	
Upper surface	345 to 360
Lower surface	367 to 385
Palisade ratio	6 ± 1
Vein islet number	7 ± 0.5
Vein termination number	5 ± 1
Cluster crystal diameter	12.88 µ – 23.39 µ – 37.2 µ
Length of covering trichome	32.55 µ – 54.85 µ – 88.15 µ

Number of observations = 10
SD = Standard Deviation

Figure 1: Leaves of *Cordia rothii*

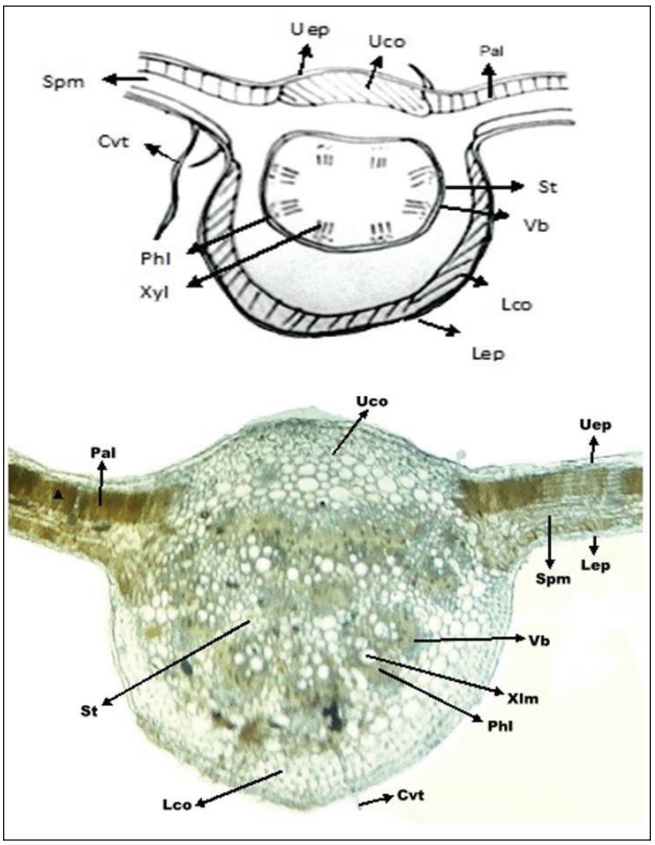

Figure 2: Diagrammatic and Detailed T. S. of leaf (X40)
(Uco, Upper Collenchyma; UEp, Upper Epidermis; Lco, Lower Collenchyma; Lep, Lower Epidermis; Pal, Palisade; Vb, Vascular bundles; Cvt, Covering Trichomes; Phl, phloem; Xlm, Xylem; St, Stele; Spm, Spongy Mesophyll)

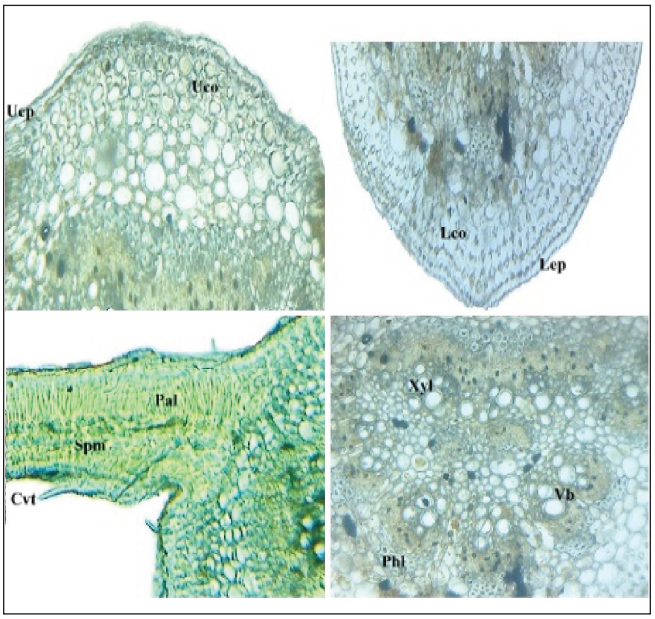

Figure 3: T. S. of leaf showing single enlarged portions (X400)
(Uco, Upper Collenchyma; UEp, Upper Epidermis; Lco, Lower Collenchyma; Lep, Lower Epidermis; Pal, Palisade; Vb, Vascular bundles; Cvt, Covering Trichomes; Phl, phloem; Xlm, Xylem; St, Stele; Spm, Spongy Mesophyll)

tissue consists of loosely arranged polygonal parenchymatous cells having calcium oxalate prisms and cluster crystals. Vascular bundle is very significantly arranged, having patches of perimedullary phloem. Starch grains are scattered throughout the ground tissue. Trichomes are covering, very long, unicellular and few having a cylindrical shape (Figure 2, 3).

Microscopy: Powder characteristics

It is a dark green powder with no distinct odor or taste. The important diagnostic features of the powder include parts of epidermis in surface view showing straight walled

epidermal cells and anisocytic stomata, xylem vessels with spiral shape or reticulate thickening, calcium oxalate cluster crystal, calcium oxalate prisms, starch grains and numerous covering trichomes, unicellular or cylindrical, narrow, conical, pointed (Figure 4).

CONCLUSION

The present work deals with the pharmacognostical evaluation of the leaves of *C. rothii*. Main microscopic characters include very long unicellular covering trichomes

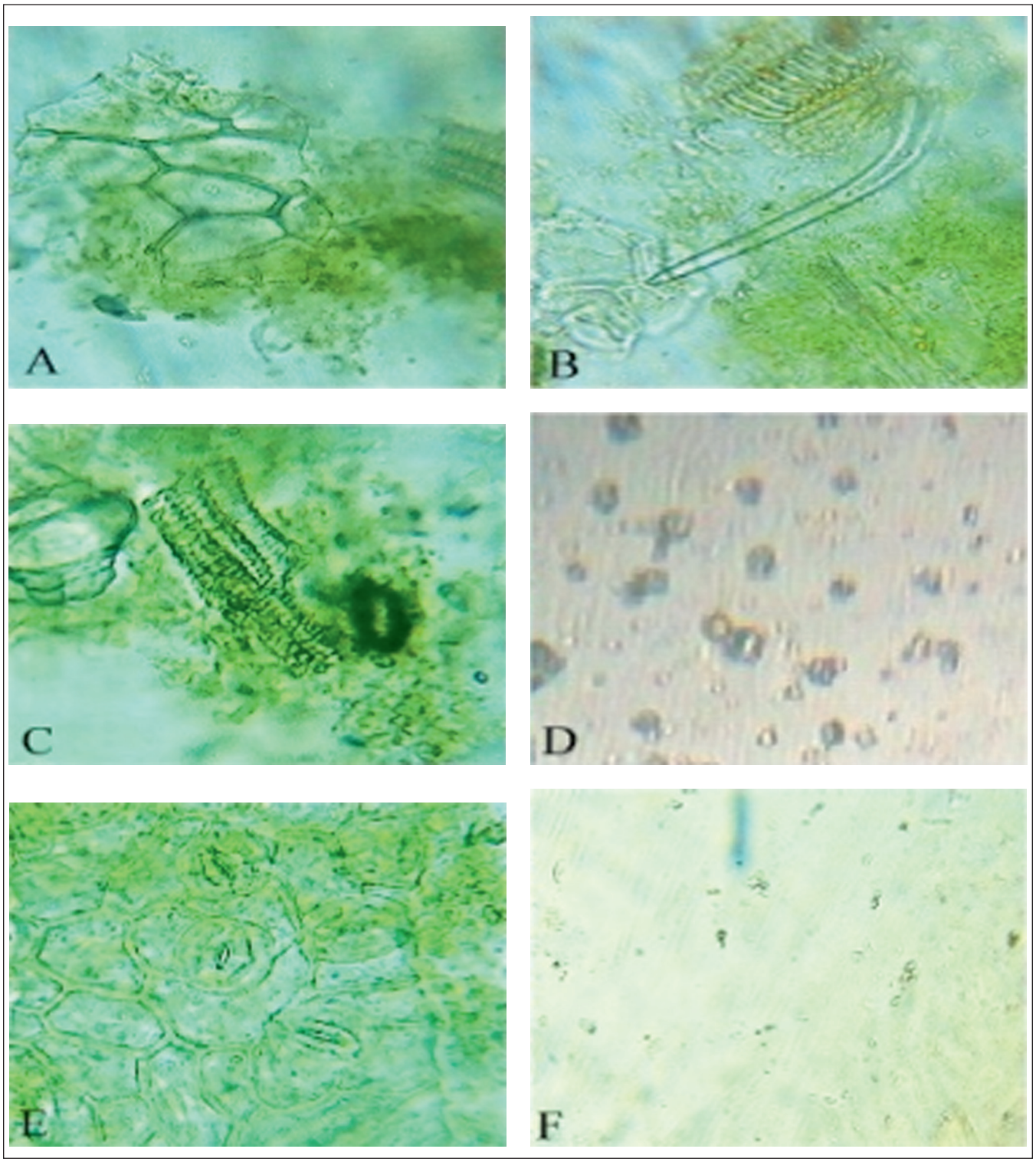

Figure 4: Powder study (X400)
(A, Epidermis in surface view; B, Covering trichome; C, Xylem vessels with reticulate thickening; D, Calcium oxalate crystals; E, Anisocytic stomata; F, Starch grains)

and very different vascular bundles having patches of perimedullary phloem. Diagnostic characters of powder include cylindrical shaped trichomes, calcium oxalate cluster crystals, calcium oxalate prisms, starch grains, anisocytic stomata and xylem vessels with spiral shape and reticulate thickening. Such a pharmacognostic study is useful for standardizing crude drugs and can be used to differentiate closely related species.

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Preliminary Pharmaceutical Characterization of Some Flowers as Natural Indicator: Acid-Base Titration

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ABSTRACT

Indicators used in titrations show well-marked changes of color in certain intervals of pH. Most of these indicators are organic dyes and are of synthetic origins. The present study was designated to evaluate the extracted flower petals of some easily available plants. A comparative study of *Delonix regia* Raf. and *Caesalpina pulcherrima* Swartz Obs. flower extracts with synthetic indicator viz. phenolphthalein and methyl red were carried out to evaluate the accuracy of flower extracts as acid-base indicator. The results indicated that flower extracts of these plants can be used as acid-base indicator in titration of strong acid with strong base because similar results were obtained by phenolphthalein and methyl red. In case of weak acid and weak base titration, the results obtained by the flower extract coincide with the results obtained by mixed indicator. The rationale behind using these natural indicators in preference to synthetic indicators is its easy availability, inertness, ease of preparation and cost effectiveness.

Key words: Natural indicator, Anthocyanins, Titration.

INTRODUCTION

Indicators are the substances whose solutions change colour due to changes in pH. These are called acid-base indicators. They are usually weak acids are bases, but their conjugate base or acid forms have different colours due to differences in their absorption spectra. They are also known as neutralization indicator.[1]

Delonix regia Raf. (Leguminosae) is also known as *Poinciana regia*. It is strikingly ornamental medium-sized tree, planted in avenues and gardens throughout India. It has a spreading crown of feathery foliage falls and the branches are nearly bare. The flowers are with panicles, varying in colour from deep crimson through scarlet orange to delicate salmon, appear in profusion in broad erect clusters along the branches, presenting a gorgeous appearance.[2] The seeds of plant are considered a source of low cost protein for use as food or feed. Flower extract showed high toxicity to larvae and pupae of the polyphagous pest, *Pericallia ricini*.[3]

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Caesalpinia pulcherrima Swartz Obs. (Caesalpiniaceae) is a shrub or small tree, obtained from America and cultivated throughout India. Flowers are arranged in racemes, which are very broad, the lower pedicels are 7.5-10 cm long. Calyx are 1.3-1.6 cm and glabrous. Petals are round, crisped, reddish yellow, with a very distinct claw, stamens are much exerted and filaments are bright red, 3-4 times than the length of the corolla. The leaves, flowers and seeds are largely used in Indian medicine. It is considered as tonic and stimulant. An infusion of the flowers is pectoral and febrifuge. It is usually prescribed in bronchitis, asthma and malarial fevers.[4]

Presence of color pigments was investigated as per IP and the tests are given in Table 1. Anthocyanins are characterized by Band- 275-280 nm (UV region). Actual color of extract is depending on number and position of hydroxyl and methoxy group. When these are fixed, the color then depends upon the pH and solvent.[5]

Hence the present vocation was attempted to appraise the flowers as a natural indicator.

MATERIALS AND METHODS

Materials

Fresh flowers were collected from the local market of Rajkot region, Gujarat, and they were authenticated

from NISCAIR, New Delhi, Ref No: NISCAIR/RHMD/Consult/2010-11/1468/66. All other ingredients were of analytical grade and purchased from Loba Chemie Pvt Ltd, Mumbai.

Method

The flowers were cleaned by distilled water and cut into small pieces and macerated for two hours in 25 ml of 90% ethanol. The extract was preserved in tight closed container and stored away from direct sun light[6].

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

Thin Layer Chromatography

Ethanolic extracts of flowers of *Delonix regia* Raf. and *Caesalpinia pulcherrima* Swartz Obs. were subjected to thin layer chromatography studies, to find the presence of Anthocyanins on support of chemical test[7].

RESULTS AND DISCUSSION

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

The flowers extract of *Delonix regia* Raf. and *Caesalpina pulcherrima* Swartz Obs. showed λmax in Ultra Violet region Table 3. The λmax suggested the presence of anthocyanins in the extract.

The flowers extract was screened for its use as an acid base indicator in various acid base titrations, and the results of this screening were compared with the results obtained by standard indicators methyl red, phenolphthalein and mixed indicator [methyl orange: bromocresol green (0.1:0.2) results are presented in Table 4.[8] The titrations of strong acid with strong base (HCl & NaOH), strong acid with weak base (HCl & NH_4OH), weak acid with strong base (CH_3COOH & NaOH), and weak acid with weak base (CH_3COOH and NH_4OH) were carried out using standard indicators and flowers extract. The results of these titrations are given in Table 4, 5 and 6. It could be due to these flavonoids, the sharp end point appeared in the above mentioned titrimetric analyses. The flowers extract of *Delonix regia* Raf. and *Caesalpina pulcherrima* Swartz Obs. were found to have Poly-Phenolic, flavonoids, anthocyanins and is pH sensitive. The end point determination of acid base titrations by the traditional indicators, compared with flowers extract indicator, it was observed that traditional indicators gave incorrect results due to addition of excess of titrant (base) after the neutralization reaction was completed, but flowers extract indicator has given sharp end point because solutions give sharp color change at the equivalence points. Further an attempt has been made to separate the present compound by performing Thin layer chromatography. The solvent system was selected on the basic of the pigments i.e. Anthocyanins. The results are tabulated in the Table 7 and Figure 1 and 2. The ethanolic extract of *Delonix regia* Raf in both the solvent system [Ethyl acetate: glacial acetic acid:

Table 1: Standard chart for phytochemical identification

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

Table 2: Technological characterization for analysis of chemical test

Sample	Poly-Phenolic compound		Flavonoid	Anthrocyanins		
	Color with $FeCl_3$	Color with Lead acetate	Shinoda test	Color with aq. NaOH (Blue violet)	Color with Conc. H_2SO_4 (Yellow orange)	Color with Mg-HCl (Red)
DRPI	+	+	+	+	+	+
CPPI	+	+	+	+	+	+

+; Presence of compound, DRPI: *Delonix regia*, CPPI: *Caesalpinia pulcherrima*

formic acid: water (100:11:11:26) and n-Butanol: glacial acetic acid: water (40:10:20)] showed single spot at R_f at 0.23 and 0.35, *Caesalpina pulcherrima* Swartz Obs. also showed single spot at R_f 0.22 and 0.32.

Table 3: Determination of UV Visible absorption

Sample code	UV λmax
DRPI	319
CPPI	275

Table 4: Technological characterization of acid-base titration using standard indicator

Titration (Titrant v/s Titrate)	Strength in moles	Indicator	Mean ± S.D.*	Color	pH
NaOH v/s HCl	0.1	MR	12.3 ± 0.12	Yellow to pink	12.32 – 5.77
	0.5	MR	11.2 ± 0.16	Yellow to pink	12.55 – 4.87
	1.0	MR	11.2 ± 0.15	Yellow to pink	12.63 – 3.30
HCl v/s NH4OH	0.1	PT	05.9 ± 0.01	Pink to colorless	10.50 – 6.74
	0.5	PT	06.6 ± 0.08	Pink to colorless	10.61 – 8.28
	1.0	PT	06.5 ± 0.16	Pink to colorless	10.98 – 8.29
CH3COOH v/s NaOH	0.1	MR	12.0 ± 0.11	Yellow to light red	12.33 – 6.01
	0.5	MR	11.9 ± 0.14	Yellow to light red	12.56 – 5.96
	1.0	MR	12.0 ± 0.09	Yellow to light red	12.67 – 5.99
CH3COOH v/s NH_4OH	0.1	MI	05.0 ± 0.05	Orange to green	03.25 – 4.52
	0.5	MI	05.6 ± 0.19	Orange to green	02.81 – 4.68
	1.0	MI	06.1 ± 0.17	Orange to green	02.86 – 4.73

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

Table 5: *Delonix regia* Raf. Flower as indicator

Titration (Titrant v/s Titrand)	Strength in moles	Indicator	Mean ± S.D.	Color	pH
NaOH v/s HCl	0.1	DRPI	12.1 ± 0.13	Green to colorless	12.78 – 5.80
	0.5	DRPI	11.5 ± 0.17	Green to colorless	13.05 – 2.04
	1.0	DRPI	05.6 ± 0.13	Green to colorless	13.03 – 0.70
HCl v/s NH_4OH	0.1	DRPI	04.2 ± 0.05	Green to pink	11.02 – 6.38
	0.5	DRPI	00.5 ± 0.15	Green to pink	10.23 – 1.66
	1.0	DRPI	06.5 ± 0.08	Green to pink	11.77 – 0.76
CH_3COOH v/s NaOH	0.1	DRPI	16.4 ± 0.16	Green to colorless	6.67 – 6.63
	0.5	DRPI	11.8 ± 0.18	Green to colorless	12.56 – 6.42
	1.0	DRPI	10.2 ± 0.12	Green to colorless	12.65 – 6.77
CH_3COOH v/s NH_4OH	0.1	DRPI	04.6 ± 0.04	Green to pink	11.12 – 5.69
	0.5	DRPI	00.8 ± 0.07	Green to pink	06.6 – 6.60
	1.0	DRPI	06.0 ± 0.03	Green to pink	11.21 – 5.64

*All values are mean ± S.D. for $n = 3$
HCl: Hydrochloric acid, CH_3COOH: Acetic Acid, NaOH: Sodium Hydroxide, NH_4OH: Ammonium Hydroxide, DRPI: *Delonix regia*

Table 6: *Caesalpina pulcherrima* Swartz Obs. Flower as indicator

Titration (Titrant v/s Titrand)	Strength in moles	Indicator	Mean ± S.D.	Color	pH
NaOH v/s HCl	0.1	CPPI	14.1 ± 0.12	Yellow to colorless	12.49 – 3.40
	0.5	CPPI	11.0 ± 0.19	Yellow to colorless	12.32 – 1.96
	1.0	CPPI	10.7 ± 0.15	Yellow to colorless	12.66 – 1.04
HCl v/s NH_4OH	0.1	CPPI	20.0 ± 0.08	Yellow to colorless	10.36 – 2.03
	0.5	CPPI	13.6 ± 0.05	Yellow to colorless	10.83 – 1.58
	1.0	CPPI	07.0 ± 0.03	Yellow to colorless	11.09 – 1.61
CH_3COOH v/s NaOH	0.1	CPPI	12.1 ± 0.13	Yellow to colorless	12.33 – 6.22
	0.5	CPPI	11.6 ± 0.17	Yellow to colorless	12.65 – 6.22
	1.0	CPPI	11.3 ± 0.08	Yellow to colorless	12.68 – 6.47
CH_3COOH v/s NH_4OH	0.1	CPPI	05.4 ± 0.16	Yellow to colorless	09.78 – 7.15
	0.5	CPPI	09.4 ± 0.05	Yellow to colorless	10.68 – 5.25
	1.0	CPPI	09.9 ± 0.06	Yellow to colorless	11.01 – 5.30

*All values are mean ± S.D. for $n = 3$
HCl: Hydrochloric acid, CH_3COOH: Acetic Acid, NaOH: Sodium Hydroxide, NH_4OH: Ammonium Hydroxide, CPPI: *Cesaphenia pulcherima*

Table 7: Thin Layer Chromatography of ethanolic extracts of *Delonix regia* Raf. and *Caesalpinia pulcherrima* Swartz Obs.

Sr. no	Extracts	Solvent system	Spraying agent	Number of spot	R_f Value	Color
1	Ethanolic of *Delonix regia* Raf.	Ethyl acetate: glacial acetic acid: formic acid: water (100:11:11:26)	Anisaldehyde-sulphuric acid	1	0.23	Pink
2	Ethanolic of *Delonix regia* Raf.	n-Butanol: glacial acetic acid: water (40:10:20)	Anisaldehyde-sulphuric acid	1	0.35	Blue-Violet
3	Ethanolic of *Caesalpinia pulcherrima* Swartz Obs.	Ethyl acetate: glacial acetic acid: formic acid: water (100:11:11:26)	Anisaldehyde-sulphuric acid	1	0.22	Pink
4	Ethanolic of *Caesalpinia pulcherrima* Swartz Obs.	*n*-Butanol: glacial acetic acid: water (40:10:20)	Anisaldehyde-sulphuric acid	1	0.32	Blue-Violet

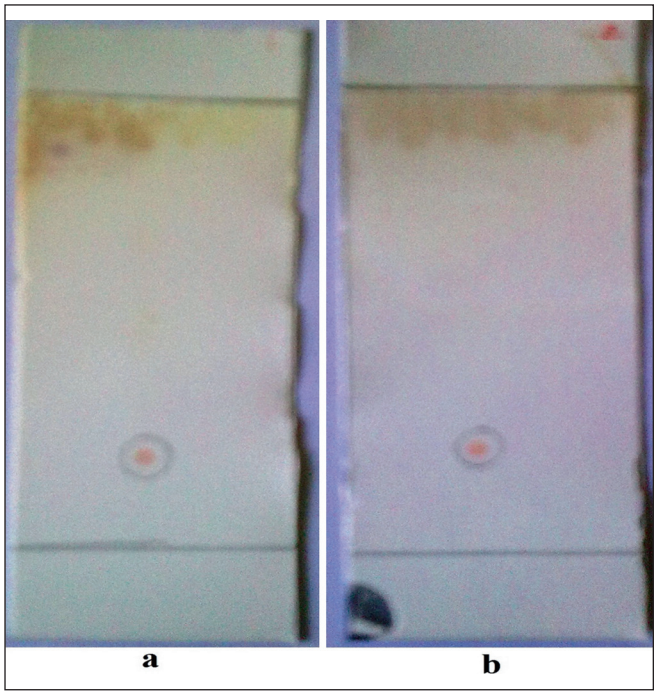

Figure 1: TLC of ethanolic extract of *D. regia* and *C. pulcherrima* in Ethyl acetate: glacial acetic acid: formic acid: water (100:11:11:26)

Figure 2: TLC of ethanolic extract of *D. regia* and *C. pulcherrima* in *n*-Butanol: glacial acetic acid: water (40:10:20)

Thus natural indicator employed in the acid base titrations was found economic, safe and an efficient alternative for traditional indicators. In comparison to this, chemical indicators were found more expensive and hazardous, which proves that flowers extract of *Delonix regia* Raf. and *Caesalpina pulcherrima* Swartz Obs. as a natural indicator is more worthy.

CONCLUSION

The results obtained in all the types of acid-base titrations lead us to conclude that, it was due to the presence of flavonoids sharp color changes occurred at end point of the titrations. We can also conclude that, it is always beneficial to use *Delonix regia* Raf. and *Caesalpina pulcherrima* Swartz Obs. flowers extract as an indicator in all types of acid base titrations because of its economy, simplicity and wild availability.

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Analysis of Fatty Acid in *Anisomeles* Species by Gas Chromatography-Mass Spectrometry

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ABSTRACT

Seven fatty acids identified from the methanol extract of *Anisomeles indica* L., and *Anisomeles malabarica* L. R. Br. Ex Sims aerial parts. The extracted fatty acids were methyl-esterified and then analyzed by GC-MS. The chemical composition of the fixed oil from *A. indica* and *A. malabarica* were investigated together here for the first time. The relative contents of the fatty acids were calculated with Area normalization. Seven fatty acids amounting to 77.778% in *A. indica* and 68.027% in *A. malabarica* of the total contents detected. The major finded fatty acids in *A. indica* were Palmitic acid (23.334%), stearic acid (22.749%), lignoceric acid (21.54%) and, in *A. malabarica*, Palmitic acid (35.252%), stearic acid (21.43%). The results the content of fatty acids was abundant in *Anisomeles* species, and it had a great range of potential utilities and a prospect of development in foods medical and health cares.

Key words: fatty acid, *Anisomeles indica*, *Anisomeles malabarica*, chemical component, GC-MS analysis

INTRODUCTION

Anisomeles Linn. R. Br. one of the largest genera of the Lamiaceae, is a genus of herbs or under-shrubs, distributed in tropical Asia and Australia. India is one of the richest countries in the world in *Anisomeles* diversity. Three species found in India yet, *Anisomeles indica, Anisomeles malabarica* and *Anisomeles heyneana*. Out of these *A. indica* and *A. malabarica* were investigated for their biological activities.[1]

Anisomeles indica are used in folk medicine all over the India. It is popularly known as 'Jirnya' in northeastern part of India, where it receives widespread used as folk medicine, predominantly in the treatment of intestinal disorders and intermittent fever. *Anisomeles indica* have anti-microbial, astringent, carminative, ethanolic extract (50%) of the herb showed hypothermic activity and when burnt acts as a mosquito repellant. The essential oil present in the herb is useful in uterine affections,[2-3] recently the valued plant investigated for its herbaceous activity.[4] Whereas, *Anisomeles malabarica* useful in halitosis, epilepsy, hysteria, amentia, anorexia, dyspepsia, colic, flatulence, intestinal worms, fever arising from teeting children, intermittent fever, gout, swelling and diarrhea.[5]

To our knowledge, the entire side material base research has not report yet. This article attempts to expound it has some effective chemical composition from studying the fatty acids ingredient in its fat soluble matter.

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MATERIALS AND METHODS

Plant collection and identification

Plant material of the *Anisomeles indica* were collected from Toranmal (MS), and *Anisomeles malabarica* were collected from Dindigul (TN); India. The collection was carried out two times, mansoon & autumn season, to accurately reflect the actual composition of the plants. The identity of the plant material was verified by Prof. (Dr.) H.B Singh, Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi, India. Voucher specimen number HNSIPER/Herb-05 of *A. indica* & HNSIPER/Herb-06 of *A. malabarica* was deposited at the Institute level. They were dried at 40°C, powdered and pass through sieve #44.

Chemicals & reagents

All applied reagents were of the highest purity available and purchased from the Sigma–Aldrich Chemical Company.

Lipid extraction

The powdered samples (100 g) were extracted 2 × 8 h in a Soxhlet extractor with hexane. After cooling, kieselguhr was added to remove chlorophyll from the solution. After removing the kieselguhr, the solution was evaporated to dryness.

Isolation of unsaponifiable matter and fatty acids

Lipids (1 g) and 50 mL of 2 N KOH in EtOH 96% v/v were boiled for 1 h. After cooling, 50 mL water was added. This solution was extracted with 3 × 50 mL hexane. The top layers were washed with 50 mL of 50% v/v EtOH, dried over Na_2SO_4, and evaporated to dryness to give the unsaponifiable matter. The bottom layer was acidified to a pH of 1.5 with HCl, then extracted by 2 × 30 mL hexane. The hexane layers were washed with 50 mL water, dried over Na_2SO_4, and evaporated to dryness to give the fatty acids.[6]

Methylation of fatty acids

Fatty acids (50 mg) were converted to methyl esters by treatment with 1.7 mL isopropyl ether and 0.4 mL of trimethylsulfonium hydroxide 2 M in MeOH.[7]

Gas chromatography-mass spectrometry analysis

The GC (Shimadzu GCMS Q.P. 2010™) system coupled to Shimadzu Turbo Mass MS. Shimadzu GCMS Q.P. 2010™ 30 m × 0.25 mm × 0.25 µm BPX-5 (SGE) column was used with helium as the carrier gas 1ml/min. The oven program was kept at 50°C for 10 min, programmed to reach 325°C at a rate of 5°C / min, and 1 µl injection (split 1:10) at 280°C were made. Mass spectra were recorded at 70 eV. Mass range was m/z 40 to 250.

The methylated fatty acids diluted with chloroform and then injected in column. The quantification of the components was performed on the basis of their GC peak areas on the column.[8] Identification of the oil components was based on their retention indices determined by reference

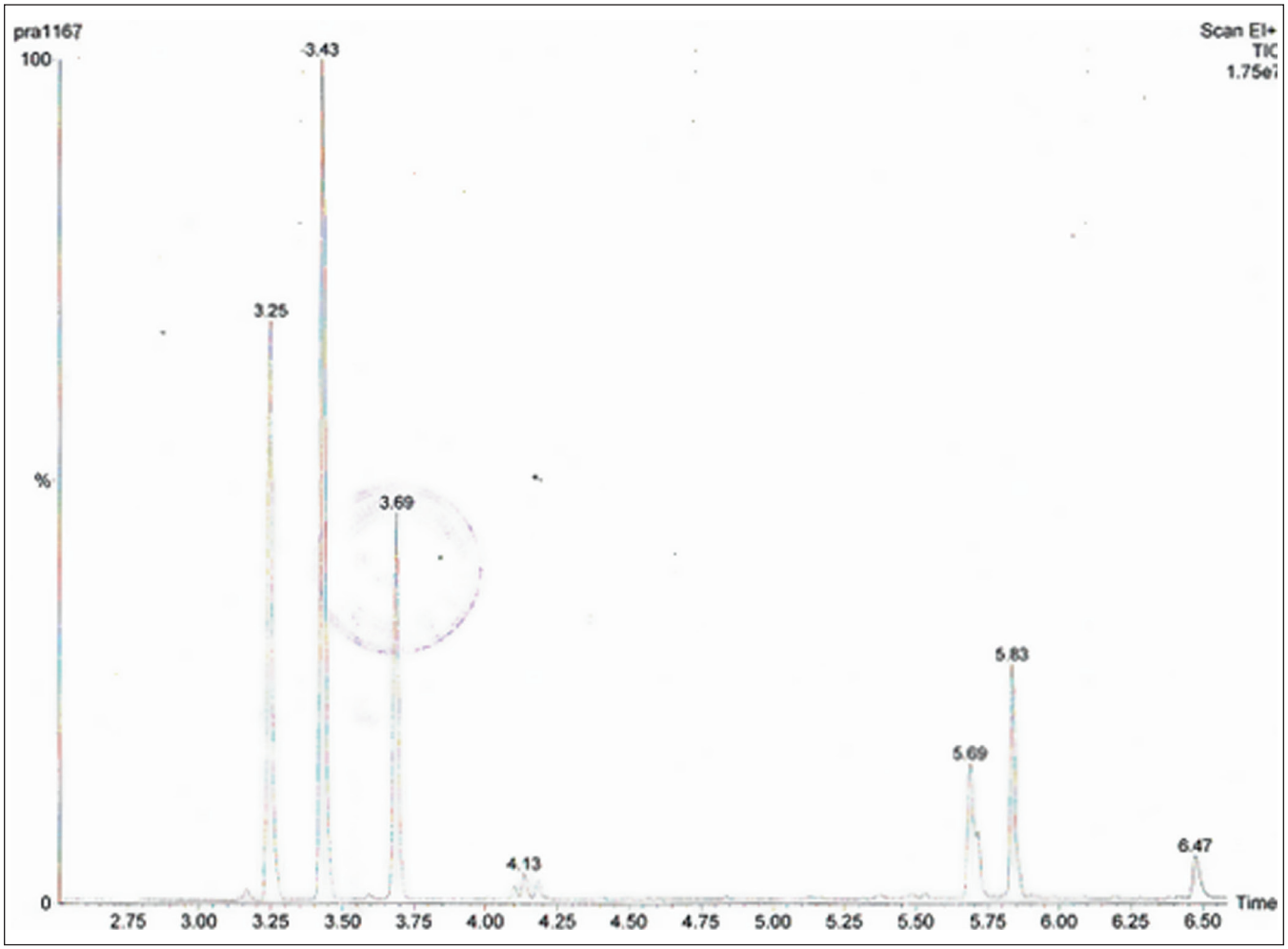

Figure 1: GC-MS Spectra for fatty acids in AIA

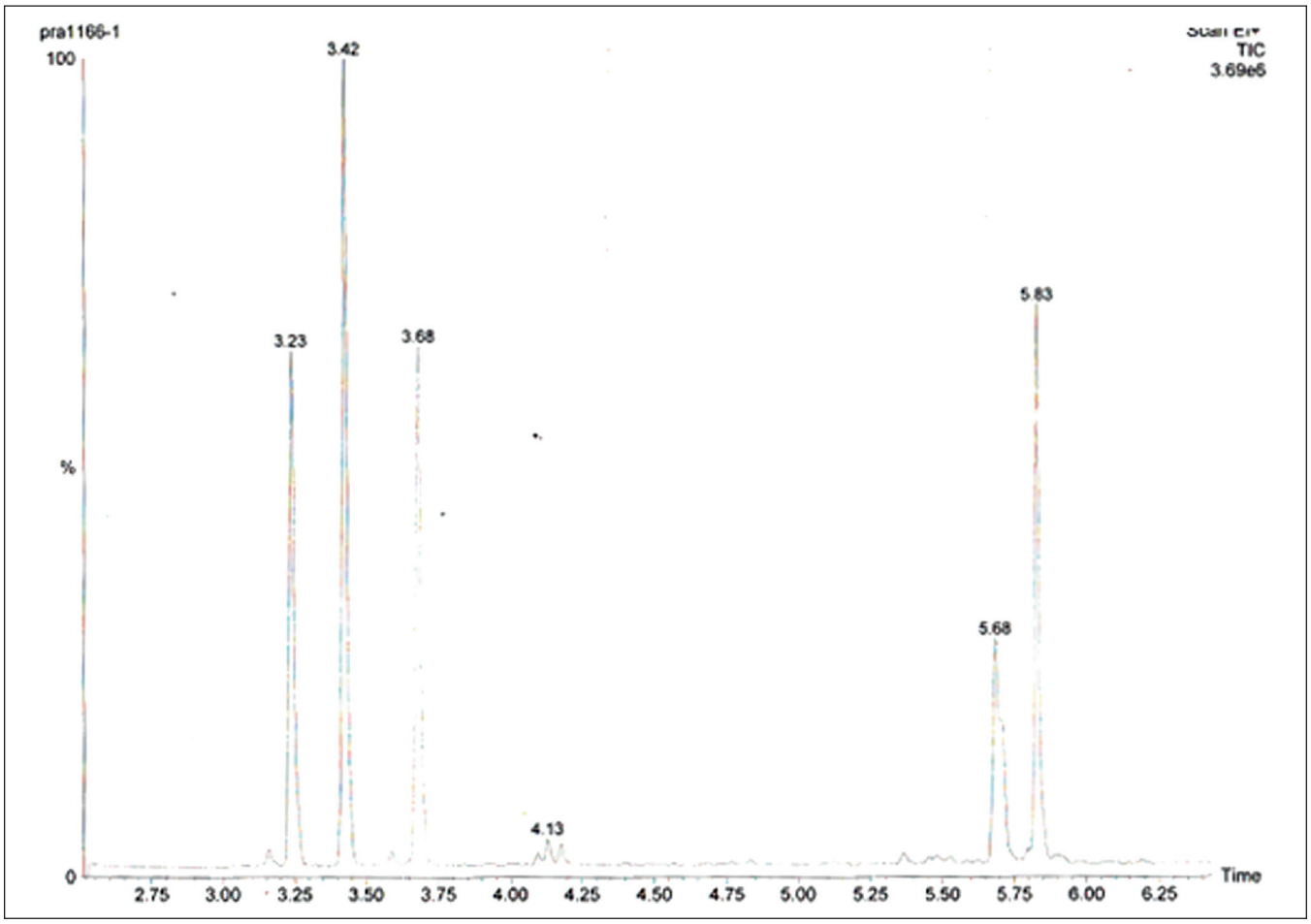

Figure 2: GC-MS Spectra for fatty acids in AMA

to a homologous series of *n*-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature, and stored on the MS library [NIST database 98/NBS 75K].

RESULTS AND DISCUSSION

The total lipid from *A. indica* (1.00% w/w) & *A. malabarica* (1.82% w/w) used for to get 0.54% & 1.22% w/w fatty acids respectively. The extracted fatty acids from *Anisomeles* species were methyl-esterified and then analyzed by GC/MS. Seven fatty acids detected were identified in *A. indica* & *A. malabarica*. The saturated fatty acids were Tridecanoic acid, Myristic acid, Palmitic acid, Stearic acid, Arachidic acid and Lignoceric acids. See Fig. 1, Fig. 2 and Table 1.

The main chemical component of the fatty acids in *Anisomeles* species was found Hexadecanoic (palmitic) acid. Hexadecanoic acid is the chief constituent of body, and it has effects of analgesia and anti-inflammation.[9] So Hexadecanoic acid is the effective component, too. The other main chemical component stearic (octadecanoic) acid, may help lower blood pressure in hypertensive patients, and also be useful to protect your heart.[10-11]

Table 1: GC–MS Data of Fatty Acids from AIA & AMA[a]

Rrt	Fatty acids	No. of Carbon atoms in fatty acids	% AUC	
			AIA	AMA
3.23	Tridecanoic acid	C-13	1.282	1.062
3.42	Myristic acid	C-14	1.239	1.421
3.68	Palmitic acid	C-16	23.334	35.252
4.13	Stearic acid	C-18	22.749	21.43
5.68	Arachidic acid	C-20	7.634	4.462
6.47	Lignoceric acids	C-24	21.54	4.4

[a]Compounds are arranged in the elution order on GC. Relative retention times (Rrt). Data for fatty acids correspond to those for the methyl ester derivatives.

In short, there are abundant fatty acids in *Anisomeles* species, they are summed up and then will be the part of the potential basis in extracts. Safety studies of these plants should be carried out.

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Major Compounds and Antimicrobial Activity of Essential Oils from Five Iranian Endemic Medicinal Plants

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ABSTRACT

Background: Essential oils are one of the most active components which can be found in medicinal plants. These compounds which are made up of many different volatile compounds possess antimicrobial activity against various microorganisms and have been used since the earliest reported history. **Objective:** To investigate essential oils of five plant materials (*Artemisia sieberi, Cymbopogon olivieri, Haplophyllum tuberculatum, Salvia macrosiphon, Teucrium polium*) for antibacterial activities. **Materials and Methods:** The GC/MS analyses were carried out on herbal extract using a Hewlett–Packard 6890. For investigating the anti-microbial effect, *Streptococcus pneumoniae, Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis* were used. **Results:** Main components of each plant were: *Cymbopogon olivieri*: piperitone (67.79%); *Haplophyllum tuberculatum*: borneol (25.73%); *Salvia macrosiphon*: piperitone (33.16%); *Teucrium polium*: limonene (37.70%) and *Artemisia sieberi*: piperitone (34.05%). The best antimicrobial activity by agar diffusion method was respectively belonged to *Salvia macrosiphon*; *Artemisia sieberi* and the mixture that had more activity than positive standard. Agar dilution method was used to identify MIC and MBC for each essential oil and the mixture. Best results were: *Haplophyllum tuberculatum, Cymbopogon olivieri* and *Teucrium polium* that showed MIC and MBC in 5 µL concentration on *Streptococcus pneumoniae* and MIC in similar concentration on *Staphylococcus aureus*. *Salvia macrosiphon* and *Artemisia sieberi* showed MIC in 5 µL concentration on *Streptococcus pneumoniae*. The mixture had good activities on *Streptococcus pneumoniae* and *Staphylococcus aureus*. **Conclusion:** our results and previous works indicate that these essential oils have good antimicrobial activity and have potentials for future works in this field.

Keywords: *Artemisia sieberi, Cymbopogon olivieri, Haplophyllum tuberculatum, Salvia macrosiphon, Teucrium polium*

INTRODUCTION

Essential oils are one of the most active components which can be found in medicinal plants. These compounds which are made up of many different volatile compounds[1] possess antimicrobial activity against various microorganisms and have been used since the earliest reported history.[2] Moreover antifungal, antiviral, insecticidal, food preservation and antioxidant properties are reported from essential oils.[3-6] *Haplophyllum tuberculatum* Forssk. (Rutaceae) (Sodabi Jonubi in Persian) is used as a remedy for headaches and arthritis. The juice is also applied in some countries as a wart removal, skin discoloration, infections and parasitic diseases.[7]

Teucrium polium L. (Lamiaceae) (Krishk) is traditionally used for different therapeutic purposes such as gastrointestinal disorders,[8,9] hypertension, convulsion, infection, inflammation, rheumatism and diabetes mellitus.[10] It is also used to relieve pains during pregnancy.[11] *Salvia macrosiphon* Boiss. (Lamiaceae) (Gol Honokeh) has antimicrobial activity.[12] In the Iranian folk medicine debility, heart disturbances in pregnancy and phlegmasia after childbirth are reported that can be treated by this plant.[11] Insecticidal activity of *Teucrium zanonii* is also proven.[13] In Iranian traditional medicine leaves and roots of *Cymbopogon olivieri* Boiss. (Astraceae) (Dereimeh) are widely used for treatment of stomachache and as an antiseptic.[14] Repellent activity against mosquito vectors of malaria and

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antipyretic effects have been also reported from India.[15] Moreover it is one of the main components of cold preparations in Iranian folk medicine.[9] Antimalarial[16] and antibacterial effects has been reported for several genus of *Artemisia*.[17,18] *Artemisia sieberi* Besser (Poaceae) (Dermaneh) is used in cold preparations and also has been reported as an anthelminthic medicine.[9] On the other hand its antimicrobial, antidiabetic, poison antidote, antihypertensive and emmenagogue effects have been noted in the literature.[19] The combination of all these plants is used as a remedy for after delivery infections. Considering the long time use of above medicinal plants in Traditional Iranian Medicine, in this study essential oils of these plant materials were investigated for antibacterial activities.

MATERIALS AND METHODS

Plant materials: Plant materials (*Haplophyllum tuberculatum*, *Teucrium polium*, *Salvia macrosiphon*, *Cymbopogon olivieri* aerial parts and *Artemisia sieberi* flowers have been collected from late May until the end of June 2008 in Evaz of Larestan (south of Fars province, Iran). Plant materials were identified and voucher specimens deposited in Shiraz Faculty of Pharmacy herbarium.

Essential oil isolation: Plant materials were air dried at room temperature, powdered (25 g) and subjected to hydrodistilation (250 ml water) for 4 h using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia.[20] Mixture was made by mixing similar amount of each essential oil.

GC-MS analyses: The GC/MS analyses were carried out using a Hewlett–Packard 6890. The gas chromatograph was equipped with a HP-5M capillary column (phenyl methyl siloxan, 25 m × 0.25 mm i.d., Hewlett–Packard Part No. 190915.433, USA). The oven temperature was programmed from 50 C (3 min) to 250 C at the rate of 3 C/min and finally held for 10 min at 250 C. The carrier gas was helium with the flow rate of 1.2 ml/min. The mass spectrometer (Hewlett–Packard 5973, USA) was operating in EI mode at 70 eV. The interface temperature was 250 C; mass range was 30-600 m/z. Identification of components was based on a comparison of their RI and mass spectra with Willey (275) and Adams libraries spectra.[21]

Antimicrobial assay: For investigating the anti-microbial effect, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermidis* and standard agar diffusion method and agar dilution method were used.[22]

Blank discs in 6 mm diameter were used in disc plate method. A suspension containing 10^8 of bacteria per ml equivalent to 0.5 standard of McFarland's tube was prepared from each bacterial strain and was read by spectrophotometer. Then dilutions 1/10 and 1/100 and 1/1000 were prepared from the suspension of initial bacterial population. Desired bacteria were placed on the surface of the plates containing Muller Hinton agar medium (Merck, Darmstadt, De) disk blank. It was done by sterile forceps in a suitable distance of bacteria from each other and from the edge of the contaminate plate. Ten, 5 and 2.5 µl of essential oils and their mixture were poured on the plate environment. Gentamicin was applied as positive control. Plates were incubated at 37°C. Subsequently after 18-24 hours non-growth zone diameter was measured using a caliper.

Pour plate method was used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of essential oils. At first, a suspension of bacteria were made equivalent to McFarland's pipe number 0.5. Then dilutions 1/10 and 1/100 and 1/1000 were prepared enough with the dilution of about 200 colonies of bacteria on Muller agar plate. The desired dilution of bacteria was 1/1000. Twenty ml of sterile Mueller agar were poured in plates, and then 100, 50, 20, 30 and 10 µl of essential oil of each plant and mixture were added to the plates. After preparing the medium, 0.1 ml of suspension with dilution of 1/1000 bacteria was shed to each plate. The plates were placed in the incubator of 37°C for 24 hours. Each plate that indicates 10 percent of the initial colony amount, this concentration, will be provided as the MIC.

MBC was determined by removing the agar from MIC plate and placing it on a Mannitol Salt Agar (MSA, Merck) and incubated at 37°C for 18 to 24 hours. Unchanged of media color was defined as the lowest concentration of crude extract with no bacterial growth.[22]

RESULTS AND DISCUSSION

Essential oil of *Cymbopogon olivieri* was obtained with yield of 1.1% (v/w). The identified components account for 96.91% of total. The major components of essential oil were piperitone (67.79%), elemol (12.27%) and β-eudesmol (3.67%) (Table 1). Monoterpene hydrocarbons were 2.67%, Oxygen containing monoterpenes were 65.36%, Sesquiterpene hydrocarbons were 3.99% and Oxygen containing sesquiterpenes were 19.92%. Other reports show that main components of plant's essential oil are piperiton, α-terpinen, limonene, elemol,[19] α-3-carene and α-eudesmol[23] or torreyol and α-cadinol.[24] Disk diffusion method showed that *C. olivieri* have antimicrobial activity against all microorganisms and the best result was for *Streptococcus pneumoniae* where the effects of essential oil and gentamicin were similar. MIC and MBC tests showed that the best

Table 1: Chemical constitutes of essential oils

	RI[a]	*C. olivieri*	*S. macrosiphon*	*A. sieberi*	*H .tuberculatum*	*T. polium.*
α-Pinene	**939**		2.03		**14.00**	4.26
Camphene	954				6.75	
β-Pinene	979		3.28			3.68
Yomogi alcohol	999			2.42		
δ-2-Carene	1002		1.56			1.15
α-Terpinene	1017	2.91			1.74	
***p*-Cymene**	**1023**					**8.20**
Limonene	**1029**		1.46		1.50	**37.70**
m-Cymene	1055					2.53
1,8-Cineole	1031		1.86	**9.55**		
allo-Ocimene	1132					1.15
γ-Terpinene	1060			1.40		
Artemisia alcohol	1084			1.87		
Camphor	**1146**			**17.68**		
Borneol	**1169**			7.43	**25.73**	
Terpinene 4 ol	1177			4.04	2.70	
Verbenone	1205					1.73
Dodecane	1206					1.73
Piperitone	**1253**	**67.79**	**33.16**	**34.05**	4.49	
Bornyl acetate	**1289**		3.19	1.06	**18.07**	
γ-Cadinene	1314	1.35				
Maaliene	1382	1.75				
Tetradecene	1389					1.61
Tetradecane	1400					1.38
trans-Caryophyllene	1409	1.24				
β-Caryophyllene	1419		**26.04**		7.43	
α-Humulene	1455		2.4			
allo-Aromandendrene	1460					1.61
Pentadecene	1496					2.07
Valencene	1496		5.62			
Myristicine	1519	3.44		3.34		
7-*epi*-α-Selinene	1522		1.98			
γ-Cadinene	1523					1.49
2,4di-tetr-Butylphenol	**1541**					**10.81**
Elemol	**1550**	**12.27**	1.74	3.47		
Hedycaryol	1568					3.56
Spathulenol	1578			3.80		
Caryophyllene oxide	**1583**		**14.39**			
Hexadecene	1590					1.15
Hexadecane	1600					1.26
β-Eudesmol	**1651**	**3.67**				
α -Eudesmol	1654	2.49				
Valerianol	1658					3.91
Farnesol	1701				1.74	
Geranyl linalool isomer	1789				7.11	
Monoterpene hydrocarbons		2.67	7.25	9.13	23.99	51.00
Oxygen-containing monoterpenes		65.36	28.1	61.93	50.99	1.5
Sesquiterpene hydrocarbons		3.99	31.34	—	7.43	6.1
Oxygen-containing sesquiterpenes		19.92	14.03	6.06	1.74	20.5
Total		96.91	98.71	90.11	91.26	90.98

[a]The retention index of compounds on DB-5 column was also determined.

result was for *Streptococcus pneumoniae* and *Staphylococcus aureus*, respectively.

Some reports indicate sensitivity of some microorganisms like *Bacillus subtilis*, *Staphylococcus epidermidis*, *Escherichia coli* and *Klebsiella pneumoniae* to this essential oil.[14]

Essential oil yield for *Salvia macrosiphon* was 0.51 % (v/w). The identified components account for 98.71% of total. Major components of essential oil were piperitone (33.16%), β-caryophyllene (26.04%) and caryophyllene oxide (14.39%); (Table 1). Monoterpene hydrocarbons were 7.25%, Oxygen containing monoterpenes were 28.1%, Sesquiterpene hydrocarbons were 31.34% and Oxygen containing sesquiterpenes were 14.03%. Major components of essential oil in other reports are β-cubebene, cyperene, germacrene B, β-selinene, α-farnesene, γ-gurjurene and (+)-aromandendrene[25] or linalool, hexyl hexanoate, hexyl isovalerate, hexyle-2-methyl-

butanoate, hexyl octanoate and sclareol.[26] Disk diffusion method showed that *S. macrosiphon* has antimicrobial activity against all tested microorganisms and the best result was for *Streptococcus pneumoniae* where the effect of essential oil was better than gentamicin. MIC and MBC test showed that the best result was for *Streptococcus pneumoniae* and results for *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were similar. To the best of our knowledge no particular antimicrobial activity study for *Salvia macrosiphon* has been done.

Artemisia sieberi essential oil was obtained by 0.43% (v/w) yield and 90.11% of components were identified. Major compounds components were piperitone (34.05%), camphor (17.68 %) and 1, 8-cineole (9.55%) and their categories were monoterpene hydrocarbons 9.13%, Oxygen containing monoterpenes 61.93% and Oxygen-containing sesquiterpenes 6.06% (Table 1). Major components which have been derived from *A. sieberi* in other studies are 1,8-cineol, myrcene, eudesm-7(11)-en-4-ol, 4-tepinyl acetate, davanone and ϱ-cymene[27] or bornyl acetate[28] or α-pinene and comphene[29] or α-thujone, β-thujone, camphor, verbenol and ϱ-mentha-1,5-dien-8-ol[30] or terpinen-4-ol.[31] Table 2 shows that *Artemisia sieberi* has potent activity against *Streptococcus pneumoniae* when compared to control and *Staphylococcus aureus*. Minimum concentration in MIC and MBC tests were for *Streptococcus pneumoniae* (Table 3). Some popular microorganisms which are sensitive to *A. sieberi* are *Pseudomonas aeruginosa*,[32] *Listeria monocytogenes*, *Bacillus cereus* and *Streptococcus mutans*.[33]

Oil yield was 0.33% for *Haplophyllum tuberculatum* and 91.26% of oil components were identified (Table 1). Components of *Haplophyllum tuberculatum* essential oil were borneol (25.73%), bornyl acetate (18.07%) and α-pinene (14.00%); containing: 23.99% monoterpene hydrocarbons, 50.99% Oxygen containing monoterpenes, 7.43% sesquiterpene hydrocarbons and 1.74% Oxygen containing sesquiterpenes. Main components of the *Haplophyllum tuberculatum* in other reports are β-phellandrene, limonene, (2)-β-ocimene, β-caryophylene, myrcene and α-phellandrene[7] or γ-3-carene, linalyl acetate and α-terpineol.[34] Table 2 shows that *H. tuberculatum* has mild antimicrobial activity on microorganisms. MIC and MBC tests showed that the best result was for *Streptococcus pneumoniae* and *Staphylococcus aureus*, respectively (Table 3). *H. tuberculatum* inhibits the growth of *Escherichia coli*, *Salmonella coleraesuis*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[7]

Yield for *Teucrium polium* volaytile oil was 0.34% (v/w). Major components of essential oil were limonene (37.70%), 2,4di-tetr-Butylphenol (10.81%) and *p*-Cymene (8.20%) including Monoterpene hydrocarbons (51.00%), Oxygen containing monoterpenes (1.5%), sesquiterpene hydrocarbons (6.10%) and Oxygen-containing sesquiterpenes

Table 2: Antimicrobial activity of essential oil by agar diffusion method

Bacteria \ Plant	*Haplophyllum tuberculatum*			*Teucrium polium*			*Salvia macrosiphon*			*Artemisia sieberi*			*Cymbopogon olivieri*			Mixture			Control
	2.5 µL	5 µL	10 µL	2.5 µL	5 µL	10 µL	2.5 µL	5 µL	10 µL	2.5 µL	5 µL	10 µL	2.5 µL	5 µL	10 µL	2.5 µL	5 µL	10 µL	10 µL
Streptococcus pneumoniae	9	10	13	11	16	17	**17**	**21**	**22**	**17**	**20**	**21**	10	15	17	**11**	**16**	**19**	17
Escherichia coli	6	6	6	6	6	7	9	9	11	10	10	11	8	9	10	7	7.5	11	22
Klebsiella pneumoniae	6.5	6.5	8.5	6	7	7.5	8.5	9	9.5	6.5	9	10	7	8	8.5	6.5	8	8.5	20
Staphylococcus aureus	8	8.5	12	10	10.5	11	6	15	16	10	12	16.5	9	10	12	6	10	12	18
Staphylococcus epidermis	6	7.5	8.5	7.5	9	9.5	8	9.5	12.5	6.5	8	10	6.5	9	10	6	7	9	25

Table 3: Antimicrobial activity of essential oil by agar dilution method

Bacteria \ Plant	*Haplophyllum tuberculatum*		*Teucrium polium*		*Salvia macrosiphon*		*Artemisia sieberi*		*Cymbopogon olivieri*		Mixture	
	MIC µL	MBC µL	MIC µL	MBC µL	MIC µL	MBC µL	MIC µL	MBC µL	MIC µL	MBC µL	MIC µL	MBC µL
Streptococcus pneumoniae	5	5	5	5	5	10	5	10	5	5	5	10
Escherichia coli	–	–	50	100	10	20	10	20	20	30	30	50
Klebsiella pneumoniae	30	50	20	30	10	20	20	30	10	20	10	20
Staphylococcus aureus	5	10	5	10	10	20	10	20	5	10	5	20
Staphylococcus epidermis	20	30	10	20	20	30	20	30	20	30	20	30

(20.5%). Totally 90.98% of oil was identified (Table 1). Major reported components of essential oils of *Teucrium polium* are α- pinene, linalool, cariophylene oxide, β-pinene, β-cariophylene[35] or myrcene, germacrene D and α-cadinol[36] or 3β-hydroxy-α-muurolene[37] or ϱ- cymene.[38] Table 2 shows that *Teucrium polium* has mild antimicrobial activity on microorganisms. MIC and MBC tests indicated that the best result was for *Streptococcus pneumoniae* and *Staphylococcus aureus*, respectively (Table 3). The extract of *Teucrium polium* was effective on *Bacillus anthracis*, *Bordetella bronchiseptica* and *Salmonella typhi*.[39] Another study showed better effectiveness of this plant against *Staphylococcus epidermidis* when compared with gentamicin.[40]

Mixture of all these essential oils has antimicrobial activity against all microorganisms and the best result was for *Streptococcus pneumoniae* (Table 2). The best MIC and MBC of mixture was for *Streptococcus pneumoniae* and *Staphylococcus aureus*, respectively.

The best antimicrobial activity by agar diffusion method was respectively belonged to *Salvia macrosiphon*; *Artemisia sieberi* and the mixture that had more activity than positive standard.

Agar dilution method was used to identify MIC and MBC for each essential oil and the mixture. Best results were obtained for *Haplophyllum tuberculatum*, *Cymbopogon olivieri* and *Teucrium polium* that showed MIC and MBC in 5 µL concentration on *Streptococcus pneumoniae* and MIC in similar concentration on *Staphylococcus aureus*. *Salvia macrosiphon* and *Artemisia sieberi* showed MIC in 5 µL concentration on *Streptococcus pneumoniae*. The mixture has good effect on *Streptococcus pneumoniae* and *Staphylococcus aureus*.

Differences between our results and previous works may be due to differences between position and time of plant collecting that can lead to changes in constitutes. But overall, our results and previous works indicate that these essential oils have good antimicrobial activity and have potentials for future works in this field.

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Antioxidant and Antibacterial Properties of *Alpinia galanga*, *Curcuma longa*, and *Etlingera elatior* (Zingiberaceae)

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ABSTRACT

Antioxidant and antibacterial properties of methanolic extracts, non-polymeric phenolic fractions, and polymeric tannin fractions of leaves and rhizomes of *Alpinia galanga* and *Curcuma longa*, and leaves and inflorescences of *Etlingera elatior* were investigated. Antioxidant properties based on total phenolic content (TPC) and ascorbic acid equivalent capacity (AEAC) were screened using the Folin-Ciocalteu and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, respectively. Antibacterial activity based on minimum inhibitory dose (MID) was tested against Gram-positive *Staphylococcus aureus*, *Micrococcus luteus*, and *Bacillus cereus* using the disc-diffusion method. The effect of ethylenediamine tetraacetic acid (EDTA) on the antibacterial properties of extracts and fractions was also studied. Extraction yields ranged from 4.1-6.0%. Yields of non-polymeric phenolic (NP) fractions (66-92%) were much higher than that of polymeric tannin (PT) fractions (0.5-10%), suggesting that the former were the major compounds. Highest TPC and AEAC were observed in the PT fraction of *A. galanga* rhizomes, in the crude extract and NP fraction of *C. longa* rhizomes, and in the PT fraction of *E. elatior* leaves. Leaf extracts and fractions of *A. galanga* and *C. longa* did not show any antibacterial activity against *S. aureus*, *M. luteus*, and *B. cereus*. Rhizome extracts and fractions of *A. galanga* and *C. longa* had no inhibitory effect on *M. luteus* and *S. aureus*, respectively. PT fractions of *E. elatior* leaves and inflorescences displayed no antibacterial activity. With the addition of 0.01 mg/ml of EDTA, extracts and fractions of *A. galanga*, *C. longa*, and *E. elatior* showed moderate, weak, and strong responses, respectively. Strongest antibacterial activity was observed in the PT fraction of *A. galanga* rhizomes with MID of 0.06 mg/disc against all three bacterial species. PT fractions of *E. elatior* leaves and inflorescences displayed antibacterial activity with MID of 0.13 mg/disc, which showed no activity prior to the addition of EDTA. The effect of EDTA on the antibacterial activity of extracts and fractions of these three ginger species warrants further investigation.

Key words: Crude extracts, fractions, non-polymeric phenolic, polymeric tannin, leaves, rhizomes, inflorescences

INTRODUCTION

Gingers of the family Zingiberaceae are perennial herbs that produce aromatic rhizomes.[1] Ginger plants are widely used as spice, condiment, and traditional medicine. The ethno-medicinal uses of rhizomes and leaves of gingers have been reviewed.[2,3] Rhizomes of ginger plants are eaten raw or cooked as vegetables and used for flavouring food.[1] Species that are widely cultivated are *Alpinia galanga*, *Curcuma longa*, *Etlingera elatior*, and *Zingiber officinale*. Rhizomes of *Z. officinale* are used as additives and flavouring in the food and beverage industry. They are used in the production of beverages such as ginger beer, ginger ale, and ginger wine.[4] They are also widely used to make ginger bread, biscuits, cakes, puddings, and pickle. Rhizomes of *C. longa* are popular as a spice used in curries both for flavouring and colouring.[1] Rhizomes of *A. galanga* are used as spice for meat dishes. As traditional medicine, ginger rhizomes are consumed by women during ailment, illness, and confinement. Rhizomes are also taken as a carminative for relieving flatulence.

Leaves of ginger plants have also been used for food flavouring and in traditional medicine.[1] In Malaysia, leaves of *C. longa* are used to wrap fish before steaming or baking and as spice for curries. Leaves of *E. elatior*, mixed with other aromatic herbs, are used by post-partum women for bathing to remove body odour.[5] They are also used for cleaning wounds. A decoction of leaves of *A. galanga* is consumed to treat diarrhea.[6]

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Beside rhizomes and leaves, other plant parts of gingers are also consumed as food, spice, and condiment. Young inflorescences of *E. elatior* are an essential ingredient of sour curry dishes. In recent years, gingers have become popular ornamental plants as their flowers and foliage are colourful and attractive.[1] Species of *Alpinia*, *Curcuma*, *Etlingera*, *Hedychium*, *Kaempferia*, and *Zingiber* have been cultivated as horticultural plants for their attractive leaves and/or flowers.

Previous studies on the antioxidant and antibacterial properties of ginger species are confined to rhizomes.[7-9] Although leaves of ginger species have been used for food flavouring and in traditional medicine, little research has been done on their antioxidant properties until recent years. The antioxidant properties of ginger leaves have recently been reviewed.[10] Studies have shown that leaves of gingers have stronger antioxidant activity than rhizomes[11] and that leaves of highland ginger populations have higher antioxidant activity than lowland populations.[12] Drying of ginger leaves using thermal methods resulted in drastic declines in antioxidant activity.[13]

Screening of antioxidant properties of leaves of 26 species and nine genera of gingers showed that *Etlingera* species had the highest values followed by *Alpinia* species.[11] Of leaves of five *Etlingera* species studied, *E. elatior* had the strongest antioxidant properties.[12] Antioxidant properties of leaves of *E. elatior* were significantly higher than inflorescences and rhizomes. Leaves of *Etlingera* exhibited moderate inhibition against Gram-positive bacteria with no activity against Gram-negative bacteria. However, it is not known if the antioxidant and antibacterial activities of ginger plants are due to their non-polymeric phenolic (NP) or polymeric tannin (PT) constituents.

In this study, the antioxidant and antibacterial properties of methanolic extracts, NP fractions, and PT fractions of leaves and rhizomes of *A. galanga* and *C. longa*, and leaves and inflorescences of *E. elatior* were investigated. The effect of ethylenediamine tetraacetic acid (EDTA) on the antibacterial properties of extracts and fractions was also studied.

MATERIALS AND METHODS

Plant materials

Ginger species studied were *A. galanga*, *C. longa*, and *E. elatior*. Fresh leaves and rhizomes of *A. galanga* and *C. longa*, and inflorescences of *E. elatior* were purchased from the market. Leaves of *E. elatior* were collected beside the campus of UCSI University. Materials are wrapped in a plastic bag, kept in the refrigerator and brought to the laboratory for analysis the next day. Leaves, rhizomes, and inflorescences (100 g each) were cleaned and shredded into 0.2 cm strips using a pasta maker (IKEA Malaysia). Brief botanical descriptions and uses of the ginger species studied are shown in Table 1.

Extraction

Leaf, rhizome, and flower strips (100 g each) were transferred into a 1000 ml extraction flask and extracted with 500 ml of methanol, successively three times for 1 h each time. The mixture was swirled continuously at 120 rpm with an orbital shaker. Samples were then filtered using a vacuum filter. After filtration, the residues were transferred back into the extraction flask and extracted again with 500 ml methanol. After drying at 50°C using a rotary evaporator, the dried extracts were kept at –20°C in freezer for further analysis.

Fractionation

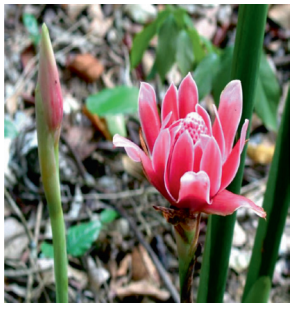
Tannins were fractionated following the procedure of column chromatography previously described.[15,16] Crude extract (2 g) dissolved in methanol (16 ml) was applied onto a chromatographic column (40 × 3 cm) packed with Sephadex LH-20 (GE Health, Sweden) and equilibrated with 100% (v/v) methanol. To obtain the NP constituents, the column was washed with 250 ml of 100% methanol. PT constituents were eluted from the column using 250 ml of 70% acetone. After evaporating at 50°C using a rotary evaporator, the fractions were tested for total phenolic content, and for free radical scavenging and antibacterial activities.

Antioxidant properties

Total phenolic content (TPC) of extracts and fractions was determined using the Folin-Ciocalteu (FC) assay.[11,12] Samples (300 µl in triplicate) were introduced into test tubes wrapped with aluminium foil, followed by 1.5 ml of FC reagent (10 times dilution) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min in the dark before absorbance was measured at 765 nm. TPC was expressed as gallic acid equivalent (GAE) in milligram per gram of extract. The calibration equation for GA (Fluka) was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$) where y is absorbance and x is mg/ml of GA.

Free radical scavenging (FRS) activity of extracts and fractions was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.[11,12] Different dilutions of samples (1 ml in triplicate) were added to 2 ml of DPPH (5.9 mg in 100 ml of methanol) in test tubes wrapped with aluminium foil. Absorbance (A) was measured at 517 nm after 30 min of incubation in the dark at room temperature. All measurements were made in triplicate using distilled water as blank. FRS activity of samples (%) was calculated

Table 1: Brief botanical descriptions and uses of ginger species studied

Species	Botanical description and use
 	*Alpinia galanga* (L.) Sw. (Greater galangal) Plants can grow up to 3 m tall. Rhizomes (2-4 cm diameter) are branched, light yellow, fibrous, and fragrant. Leaves are alternate, pubescent and oblong-lanceolate. Flowers are terminal, white, and fragrant. The species is widely cultivated in Southeast Asia for its rhizomes as spice. Shoots and inflorescences are eaten raw, and rhizomes are used in traditional medicine.
 	*Curcuma longa* L. (Tumeric) Plants can grow up to 1 m tall. Rhizomes (1-2 cm diameter) are branched, bright orange, and strongly aromatic. Leaves are oblong-lanceolate with an acute apex and aromatic. Inflorescences are terminal and erect, with pale green bracts and white flowers. The species is extensively cultivated in tropical Asia for its rhizomes as spice. Rhizomes are the source of turmeric which has a long tradition of medicinal use.
 	*Etlingera elatior* (Jack) Sm. (Torch ginger) Plants can grow up to 5-6 m tall, forming dense clumps. Rhizomes (3-4 cm diameter) are stout and pungent. Leaves (100-150 cm long) are lanceolate and green. Crushed leaves emit a pleasant sour fragrance. The species is native to Malaysia and Indonesia, and is widely cultivated in the tropics for its inflorescences as spice.

as $(A_{control} - A_{sample})/A_{control} \times 100$ and calculated as IC_{50}, the concentration of sample needed to scavenge 50% of the DPPH free radical. IC_{50} was then expressed as ascorbic acid (AA) equivalent antioxidant capacity (AEAC) using the equation of AEAC (mg AA/g of extract) = IC_{50}AA/IC_{50}sample $\times 10^5$. IC_{50} of AA used for calculation of AEAC was 0.00387 mg/ml.

Antibacterial activity

Antibacterial activity of extracts and fractions was measured using the disc-diffusion method.[12,14] Bacterial species tested were Gram-positive *Staphylococcus aureus*, *Micrococcus luteus*, and *Bacillus cereus*. Inoculums (100 µl) were spread evenly onto 20 ml Mueller-Hinton agar set in 90 mm Petri dishes using a sterile cotton swab. Sterilized paper discs (6 mm diameter) were impregnated with plant samples (2 mg per disc) using a micropipette and firmly placed onto the inoculated agar ensuring even distribution to avoid overlapping of zones. Streptomycin susceptibility discs (10 µg) were used as positive controls. After incubation overnight at 37ºC, the minimum inhibitory dose (MID) or minimum concentration of extract or fraction in mg/disc required to show a zone of inhibition was noted. The disc-diffusion method was repeated by adding 0.01 mg/ml of EDTA to the agar to enhance the antibacterial activity of plant samples.

Statistical analysis

All experiments were done in triplicate ($n = 3$) and results were expressed as means ± standard deviation (SD). Results were analyzed using the Turkey Honestly Significant Difference (HSD) one-way analysis of variance (ANOVA) software developed by Vassar College, New York State, USA. The significant difference was based on $P < 0.05$.

RESULTS AND DISCUSSION

Percentage yield

The percentage yield of methanolic extracts from leaves and rhizomes of *A. galanga* was 6.0 and 5.5%, from leaves and rhizomes of *C. longa* was 4.3 and 4.1%, and from leaves

and inflorescences of *E. elatior* was 4.1 and 4.8%, respectively [Table 2].

Methanol was chosen as the solvent for extraction as it is most suitable for extracting phenolic compounds from plant tissues and is able to inhibit the action of polyphenol oxidase.[17-19] Methanol is efficient in cell wall degradation, easy to evaporate, and able to prevent microbial growth. Based on TPC and AEAC, the extraction efficiency of four different solvents on leaves of *C. longa* and *E. elatior* showed that methanol was the most efficient followed by 50% aqueous methanol, ethyl acetate, and dichloromethane.[19] The extraction yield of three successive extractions of *A. galanga* leaves using methanol was 79, 15, and 6%.[20] TPC values were 492, 95, and 40 mg GAE/100 g.

The percentage yield of NP and PT fractions from leaves and rhizomes of *A. galanga* was 74, 0.5, 66, and 6.0%; from leaves and rhizomes of *C. longa* was 92, 7.4, 86, and 2.0%; and from leaves and inflorescences of *E. elatior* was 85, 10, 80, and 9.0%; respectively [Table 2].

All three species yielded much higher percentage of NP (66-92%) than PT (0.5-10%) constituents, suggesting that the former were the major compounds. An earlier study reported the tannin content in methanol rhizome extracts of eight ginger species varied from 1.2-18%.[21] Rhizomes of *Curcuma haritha* contained 1.8% tannin content.[22]

Antioxidant activity

Results of antioxidant properties (AOP) of crude extracts of *A. galanga*, *C. longa*, and *E. elatior* are shown in Table 2. In *A. galanga*, TPC of leaves was significantly higher than that of rhizomes. However, AEAC values were comparable. In *C. longa*, both TPC and AEAC of rhizomes were significantly higher than those of leaves. Values were 1.6 and 1.5 times higher in rhizomes than leaves. In *E. elatior*, both TPC and AEAC of leaves were significantly higher than those of inflorescences. Values of leaves were 7.7 and 5.3 times higher than inflorescences, respectively.

Earlier studies have reported that leaves of *A. galanga* had significantly higher TPC but significantly lower AEAC than rhizomes.[11,23] This study found that leaves and rhizomes of *A. galanga* had comparable AEAC. Comparing the AOP of leaves of five *Alpinia* species, TPC and AEAC values of *A. galanga* were the lowest.[24] The significantly higher TPC and AEAC of *C. longa* rhizomes than leaves have earlier been reported.[11] It is interesting to note that this trend in *C. longa* was unique among *Curcuma* as leaves of *C. aeruginosa*, *C. mangga*, and *C. zanthorrhiza* have significantly stronger AOP than rhizomes. An earlier study has reported that TPC and AEAC values were significantly higher in leaves than inflorescences of *E. elatior*.[12] Values were 12 and 14 times higher in leaves than in inflorescences, respectively.

AOP of NP and PT fractions of *A. galanga*, *C. longa*, and *E. elatior* are shown in Table 2. In leaves of *A. galanga*, TPC and AEAC of the NP fraction were comparable to those of the PT fraction. In rhizomes, values of the PT fraction were significantly higher than the NP fraction. In leaves of *C. longa*, TPC and AEAC of the PT fraction were significantly higher than the NP fraction and *vice versa* for rhizomes where values of the NP fraction were significantly higher than the PT fraction. In both leaves and inflorescences

Table 2: Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of extracts and fractions from *Alpinia galanga*, *Curcuma longa*, and *Etlingera elatior*

Species	Plant part	Extract/fraction	Yield (%)	TPC	AEAC
A. galanga	Leaves	Crude extract	6.0	62 ± 6.9^{a}	29 ± 1.2^{a}
		NP fraction	74	52 ± 3.4^{ab}	17 ± 0.1^{b}
		PT fraction	0.5	48 ± 6.2^{b}	17 ± 1.1^{b}
	Rhizomes	Crude extract	5.5	39 ± 3.4^{c}	31 ± 2.8^{a}
		NP fraction	66	32 ± 5.4^{c}	8.0 ± 0.8^{c}
		PT fraction	6.0	155 ± 16^{d}	143 ± 6.0^{d}
C. longa	Leaves	Crude extract	4.3	59 ± 2.1^{a}	39 ± 1.6^{a}
		NP fraction	92	57 ± 2.1^{a}	39 ± 2.2^{a}
		PT fraction	7.4	68 ± 1.0^{b}	60 ± 0.3^{b}
	Rhizomes	Crude extract	4.1	94 ± 3.9^{c}	59 ± 5.6^{bc}
		NP fraction	86	92 ± 6.3^{c}	53 ± 1.0^{c}
		PT fraction	2.0	28 ± 2.9^{d}	21 ± 3.4^{d}
E. elatior	Leaves	Crude extract	4.1	192 ± 16^{a}	242 ± 27^{a}
		NP fraction	85	204 ± 20^{a}	229 ± 5.3^{a}
		PT fraction	10	575 ± 79^{b}	616 ± 13^{b}
	Inflorescences	Crude extract	4.8	25 ± 2.2^{c}	46 ± 13^{c}
		NP fraction	80	22 ± 3.6^{c}	45 ± 4.9^{c}
		PT fraction	9.0	191 ± 14^{a}	236 ± 28^{a}

TPC in mg GAE/g extract and AEAC in mg AA/g extract are means ± SD (n = 3). ANOVA was analysed using the Tukey HSD test. For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$. ANOVA does not apply between species. Abbreviations: NP = non-polymeric phenolic, PT = polymeric tannin, GAE = gallic acid equivalent, and AA = ascorbic acid.

of *E. elatior*, TPC and AEAC of PT fractions were significantly higher than NP fractions.

In leaves of *C. longa* and *E. elatior*, AOP values of PT fractions were significantly higher than NP fractions. In leaves of *A. galanga*, both fractions had comparable values. AOP values of fractions of the other plant parts were somewhat varied. Values of the PT fraction were significantly higher in rhizomes of *A. galanga* and in inflorescences of *E. elatior*. In rhizomes of *C. longa*, values of the NP fraction were significantly higher. Generally, PT fractions have stronger AOP than NP fractions with the exception leaves of *A. galanga* and rhizomes of *C. longa*.

Tannins are polymeric phenolic compounds of intermediate to high molecular weight, ranging between 500 and 3000 Da.[25,26] Due to their higher molecular weight and greater degree of hydroxylation of aromatic rings, tannins exhibit strong antioxidant potential.[26] Their antioxidant potency depends on the number of phenolic hydroxyl groups and the degree of hydroxylation of aromatic rings. Polymeric tannins have been reported to be much more potent antioxidants than simple monomeric phenolics.[27] Tannins were 15-30 times more effective at quenching peroxyl radicals than simple phenolics, suggesting that they are important biological antioxidants. The basic mechanisms of antioxidant activity of tannins are free radical scavenging activity, chelation of transition metals, inhibition of prooxidative enzymes, and lipid peroxidation.[26,28]

It is generally believed that tannins are not absorbed by the gastro-intestinal tract in the human body due to their high molecular weight and their ability to form insoluble complexes with components of food such as proteins.[26,28] The molecular weight of a compound should be less than 500 Da to be considered orally active.[29] Though some studies have shown that the absorption of tannins is higher than it was assumed, there are still many questions concerning their bioavailability. Generally, absorption of tannins decreases with increasing polymerization.

Stronger AOP of the NP fraction of *C. longa* rhizomes may be attributed to curcuminoids, the major phenolic compounds.[30,31] Curcuminoids notably curcumin and demethoxycurcumin have been reported to possess potent DPPH radical scavenging activity.[32] The wide ranging radical scavenging, ferric ion reducing, and ferrous ion chelating activities of curcumin have also been studied. The presence of hydroxy and β-diketone groups in curcuminoids contributes to their potent AOP.[33,34] Other phenolic compounds with AOP found in rhizomes of *C. longa* included *p*-coumaric acid, vanillin, ferulic acid, and quercetin.[33,35]

Antibacterial activity

Results of antibacterial activity of crude extracts of *A. galanga*, *C. longa*, and *E. elatior* are shown in Table 3. Leaves of *A. galanga* and *C. longa* did not show any antibacterial activity against *S. aureus*, *M. luteus*, and *B. cereus*. Rhizomes of *A. galanga* showed inhibition against *S. aureus* and *B. cereus* with MID of 0.50 and 1.00 mg/disc, respectively. Rhizomes of *C. longa* strongly inhibited the growth of *M. luteus* and *B. cereus* with MID of 0.13 mg/disc. It is interesting to note that leaf and rhizome extracts of *A. galanga* and *C. longa*

Table 3: Antibacterial activity of extracts and fractions of *Alpinia galanga*, *Curcuma longa*, and *Etlingera elatior* using the disc-diffusion method

Species	Plant part	Extract/fraction	Minimum inhibitory dose (MID) in mg/disc		
			M. luteus	*S. aureus*	*B. cereus*
Alpinia galanga	Leaves	Crude extract	– (–)	– (1.0)	– (1.0)
		NP fraction	– (–)	– (–)	– (–)
		PT fraction	– (–)	– (–)	– (–)
	Rhizomes	Crude extract	– (1.0)	0.5 (0.5)	1.0 (0.25)
		NP fraction	– (–)	– (–)	– (0.5)
		PT fraction	– (0.06)	0.5 (0.06)	0.13 (0.06)
Curcuma longa	Leaves	Crude extract	– (–)	– (–)	– (–)
		NP fraction	– (–)	– (–)	– (–)
		PT fraction	– (–)	– (–)	– (–)
	Rhizomes	Crude extract	0.13 (0.13)	– (–)	0.13 (0.13)
		NP fraction	0.25 (0.25)	– (–)	0.25 (0.25)
		PT fraction	– (–)	– (–)	– (–)
Etlingera elatior	Leaves	Crude extract	1.0 (1.0)	1.0 (1.0)	0.5 (0.25)
		NP fraction	1.0 (0.25)	– (1.0)	1.0 (0.5)
		PT fraction	– (0.13)	– (0.13)	– (0.13)
	Inflorescences	Crude extract	1.0 (1.0)	2.0 (1.0)	1.0 (1.0)
		NP fraction	2.0 (1.0)	2.0 (0.5)	1.0 (1.0)
		PT fraction	– (0.13)	– (1.0)	– (0.13)

Concentration of extracts and fractions used was 2 mg per disc. Abbreviations: NP = non-polymeric phenolic, PT = polymeric tannin, *M.* = *Micrococcus*, *S.* = *Staphylococcus*, *B.* = *Bacillus*, and – = no activity. Figures in brackets are MID with 0.01 mg/ml ethylenediamine tetraacetic acid (EDTA) added to the agar.

were ineffective against *M. luteus* and *S. aureus*, respectively. Leaves and inflorescences of *E. elatior* inhibited the growth of all three bacterial species with MID ranging from 0.50 to 2.00 mg/disc. Strongest antibacterial activity was observed in the rhizome extract of *C. longa* with MID of 0.13 mg/disc against *M. luteus* and *B. cereus*.

Antibacterial activity of NP and PT fractions of *A. galanga*, *C. longa*, and *E. elatior* is shown in Table 3. Leaves of *A. galanga* and *C. longa* did not show any antibacterial activity. The PT fraction of rhizomes of *A. galanga* showed inhibition against *S. aureus* and *B. cereus* while the NP fraction had no activity. On the contrary, the NP fraction of rhizomes of *C. longa* showed inhibition against *M. luteus* and *B. cereus* while the PT fraction had no activity. The observation that all fractions of *A. galanga* and *C. longa* were ineffective against *M. luteus* and *S. aureus*, respectively, is noteworthy. NP fractions of leaves and inflorescences of *E. elatior* showed antibacterial activity with the exception of the PT fraction against *S. aureus*. Surprisingly, PT fractions showed no antibacterial activity. Strongest antibacterial activity was observed in the PT fraction of *A. galanga* rhizomes with MID of 0.13 mg/disc against *B. cereus*.

In support of this study, it was earlier reported that leaf extract of *A. galanga* did not have inhibitory effect on seven bacterial species including *S. aureus*, *M. luteus*, and *B. cereus*.[23] The antibacterial activity of *A. galanga* rhizomes has been well documented.[36-38] Rhizomes of *A. galanga* had strong inhibitory effect against *S. aureus*.[37,38] Using transmission electron microscopy, it was observed that the rhizome extract of *A. galanga* caused membrane damage and cytoplasm coagulation in *S. aureus*.[37] In this study, it was observed that the rhizome extract and fraction of *A. galanga* inhibited *S. aureus* but not *M. luteus*. On the contrary, an earlier study reported that *A. galanga* rhizome extract was non-inhibitive against *S. aureus* but had inhibitory effect against *M. luteus*.[23]

Results of this study showed that the growth of *S. aureus* was not inhibited by extracts and fractions of *C. longa* which contradicted earlier findings that *C. longa* rhizome extracts had inhibitory effect against *S. aureus*.[39] The disparity in findings could be due to different strains of bacteria used.

Leaves and inflorescences of *E. elatior* are known to have antibacterial activity. Leaves of *E. elatior* exhibited moderate inhibition against *S. aureus*, *M. luteus*, and *B. cereus* with no activity against Gram-negative bacteria of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella choleraesuis*.[12] Extracts of *E. elatior* flowers displayed antibacterial activity against *Bacillus megaterium*, *E. coli*, and *P. aeruginosa*;[40] and against *Bacillus thuringiensis*, *Bacillus subtilis*, *Proteus mirabilis*, *E. coli*, and *S. aureus*.[41]

Results of antibacterial activity of extracts and fractions using the disc-diffusion method with 0.01 mg/ml of EDTA added to the agar are shown in Table 3. This concentration was chosen because at 0.1 mg/ml of EDTA, there was no lawn culture of *M. luteus*. The addition of EDTA was to improve the antibacterial efficacy of extracts and fractions. Leaf extract of *A. galanga* showed antibacterial activity against *S. aureus* and *B. cereus*, with MID of 1.00 mg/disc, although the fractions remained inactive. Extract and the PT fraction of rhizomes inhibited the growth of all three bacterial species while the NP fraction of rhizomes inhibited *B. cereus*. EDTA did not have any effect on extracts and fractions of leaves and rhizomes of *C. longa*. Leaf extract and PT fraction of rhizomes remained inactive. Again, *S. aureus* is not susceptible to all extracts and fractions. All extracts and fractions of *E. elatior* inhibited the growth of all three bacterial species. Antibacterial activity of PT fractions of leaves and inflorescences, which showed no activity without EDTA, had been greatly enhanced with MID of 0.13 mg/disc. With the addition of 0.01 mg/ml of EDTA, strongest antibacterial activity was observed in the PT fraction of *A. galanga* rhizomes with MID of 0.16 mg/disc against *M. luteus*, *S. aureus*, and *B. cereus*.

Findings of this study showed that different plant and bacterial species respond differently to EDTA. With 0.01 mg/ml of EDTA, extracts and fractions of *A. galanga*, *C. longa*, and *E. elatior* showed moderate, weak and strong response, respectively. Noteworthy are the PT fractions of *E. elatior* which showed strong inhibitory effects. It can be seen that EDTA greatly enhanced the antibacterial activity of PT fractions of *E. elatior*.

Previous studies have also reported varying effects of EDTA on the antibacterial activity of plant samples. Adding 2 mM EDTA caused *P. aeruginosa* to be susceptible to leaf extracts of *Etlingera* species but the chelating agent inhibited the culture of *E. coli* and *S. choleraesuis*.[12] Adding 1 mM of EDTA rendered streptomycin ineffective against *S. choleraesuis* but enhanced the efficiency of the antibiotic against *P. aeruginosa*.[24] EDTA inhibited the culture of *B. cereus* but *S. aureus* grew prolifically.[42] It has bactericidal effect on *P. aeruginosa*[43] and *Staphylococcus epidermis*.[44] The chelating agent is known to enhance the effectiveness of antimicrobials and antibiotics, especially against Gram-negative bacteria.[45] It alters the structure of the outer membrane of Gram-negative bacteria by chelating divalent cations, weakening the outer membrane, and renders the entry of antibiotics.[43,46]

CONCLUSION

All three ginger species had much higher percentage yield of NP than PT constituents, suggesting that the former

were the major compounds. AOP of methanolic extracts and fractions from various plant parts of each species showed variable trends. Highest TPC and AEAC were observed in the PT fraction of *A. galanga* rhizomes, in the extract and NP fraction of *C. longa* rhizomes, and in the PT fraction of *E. elatior* leaves. Leaf extracts and fractions of *A. galanga* and *C. longa* did not show any antibacterial activity against *S. aureus*, *M. luteus*, and *B. cereus*. Rhizome extracts and fractions of *A. galanga* and *C. longa* had no inhibitory effect on *M. luteus* and *S. aureus*, respectively. PT fractions of leaves and inflorescences of *E. elatior* displayed no antibacterial activity. With the addition of 0.01 mg/ml of EDTA, extracts and fractions of *A. galanga*, *C. longa*, and *E. elatior* showed moderate, weak, and strong responses, respectively. Strongest antibacterial activity was observed in the PT fraction of rhizomes of *A. galanga* with MID of 0.16 mg/disc against all three bacterial species. Noteworthy was the strong inhibition of PT fractions of *E. elatior* leaves and flowers which showed no antibacterial activity prior to the addition of EDTA. The effect of EDTA on the antibacterial activity of these three ginger species warrants further investigation. A range of EDTA concentrations and more bacterial species including Gram-negative bacteria should be tested.

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Evaluation of Nephroprotective Activity of Fruits of *Ficus hispida* on Cisplatin-Induced Nephrotoxicity

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ABSTRACT

Traditional medicaments, chiefly obtained from plants have played a vital role in sustaining disease free human existence on this planet. In spite of overwhelming influence of modern science and tremendous advances made in the production of synthetic drugs, traditional medicaments designed as herbal drugs in different places in the literature have retained their place in the therapy. In this context the folk medicine *Ficus hispida* was studied for its nephroprotective activity. The nephroprotective effect of fruits of *Ficus hispida* (Moraceae) were investigated by acute toxicity studies, estimation of biochemical parameters and *in vitro* antioxidant studies. In our investigation methanolic extract showed significant nephroprotective activity than nephrocuration on cispaltin induced nephrotoxicity.

Key words: *Ficus fruits,* cisplatin, nephrotoxicity, free radical scavenging activity, nephroprotective effect.

INDRODUCTION

Nephrotoxicity comprises renal disorders produced by a wide range of drugs, disgnostic agents and chemicals. Nephrotoxicity is not an uncommon event, which can cause significant morbidity and can be easily overlooked. There are a growing number of hospitalized patients who develop drug-induced renal problems because increasing number of potent drugs has been added to the therapeutic arsenal in recent years.[1] Different classes of drugs[2,3,4] by virtue of immunological mechanisms or direct toxicity initiate certain stereotyped renal response. These drugs include antibiotics (aminoglycosides, tetracyclines, acyclovir, etc.,), chemotherapeutic and immunosuppressants (cisplatin, methotrexate, mytomycin, cyclosporine, etc.,) radio contrast agents and NSAIDs.

Search for nephroprotective agents has made man turn to alternative medicine. It is a well documented fact that a number of medicinal plants show beneficial effects in renal disorders.[5-11] It is suggested that the nephroprotective activity of the plants are due to their antioxidant potential.[12] Currently much interest is paid to medicinal plants with nephroprotective potential. The genus *Ficus* comprising of 700 species belongs to the family Moraceae. The plant *Ficus hispida* (FH) is a shrub or small tree having rough leaved fig and all parts are more or less hispid-pubescent. FH is commonly known as Kathumber (English), Katgular (Hindi), Verse-atti pandhu (Telugu), Peyatti (Tamil) and Kakadumbura (Sanskrit) is widely distributed in India, Sri Lanka, Myanmar and southern regions of the Republic China, in damp localities and in shady places.[13] All parts of *Ficus hispida* are bitter, cooling acrid and astringent. Indian Medicinal Plants (IMPs) mentions its use in "Kapha', dysentery, ulcers, biliousness, psoriasis, anaemia, piles, jaundice, hemorroage of the nose and mouth and blood diseases.[14]

Literatures documents its wide range of biological activities[15-20] such as antidiarrhoel, antitussive, anti-ulcer, antipyretic, anti-inflammatory, antioxidant, sedative, anticonvulsant, hepatoprotective and cardioprotective activities in leaves and hypoglycaemic activity in bark. Phytochemical studies revealed that the whole plant of *Ficus hispida* contains alkaloids (phenathroindolizidine and biphenyl hexahydroindolizine) and flavanoids (β-amyrin, hispidine, bergapten and β-sitosterol) lupeol acetate, β-sitosterol and β-amyrin acetate in bark whereas leaves contain steroids and terpenoids.[21]

The tribal people in Tirumala hills (Andhrapradesh, India) use FH fruits for renal disorders. Lack of experimental data for its nephroprotective activity, prompted us to study the nephroprotective activity of FH fruits against cisplatin induced nephrotoxicity.

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MATERIALS AND METHODS

Collection

Ficus hispida was collected from talakona forest of chittoor district, Andhra Pradesh, India, during september and it was authenticated by Dr Madhavashetty, Dept of Botany, SV University, Tirupati, India.

Extraction

The fresh fruits were cut into small pieces shade dried and coarsely powdered. The coarse powder (100 g) was extracted with hot methanol in a Soxhlet extractor for 20 h. The methanol was removed by distillation under reduced pressure and controlled temperature using rotary vacuum evaporator. 25 g of methanolic extract of *Ficus hispida* fruits were subjected to fractionation and yield of 10 g is obtained.

Chemicals

Cisplastin was purchased from Sigma Aldrich Co, St Couis, USA. Trichloroacetic acid, thiobarbituric acid, *o*-phosphoric acid, diacetyl monoxime, thio semicarbazide, sodium tungstate, sodium nitroprusside are purchased from Sd fine-Chem Ltd (India) and Merck (India). All the chemicals used are of analytical grade.

Preparation of test and standard solutions

The stock solutions of extracts of FH fruits and the standard antioxidants rutin, butylated hydroxyl anisole, α-tocopherol and anticancer drug cisplatin were dissolved in dimethyl sulphoxide (DMSO) separately and used for the *in vitro* antioxidant tests. The stock solutions were serially diluted with the DMSO to obtain lower dilutions.

Phytochemical screening

Preliminary phytochemical screening of different percentage of ethyl acetate fractions revealed the presence of fixed oil, fats, steroids, flavanoids, triterpenoids and alkaloids[22]. Liebermann-Burchard test, Shinoda's test[23] and Hirschohin test given positive results and confirms the presence of steroids, flavanoids and triterpenoids. Presence of alkaloids was confirmed by the positive results obtained from Mayer's, Dragendorff's, Wagner's and Hagner's tests. Presence of tannins was confirmed by ferric chloride test and purple colour produced by the Modified Borntrager's test indicates the presence of glycosides.

Animal model

The animal toxicity study was conducted on male Wister albino rats weighing 150-200 g. The animals were deprived of food for 24 h but allowed free access to water in the same ambience. The animals were housed under standard conditions of temperature (23ºC ± 1ºC) and were acclimatized to 12 h light. The animals were fed with commercial rat feed pellets (Gold Mohur pellets, Bangalore) and were given water ad libitium. The ethical clearance was obtained from institutional animal ethic committee.

EXPERIMENTAL PROTOCOL

Acute toxicity and gross behavioural studies

Acute toxicity studies were carried out for methanolic extract using Acute Toxic Method as described in OECD (Organization of Economic Co-operation and Development) Guidelines No. 423. Animals were given increasing doses of 30, 100, 300, 600 and 1000 mg/ kg, p. o. of the methanolic extract suspended in 2 % tween-80 solution. The animals were observed continuously for 2 h gross behavioural changes and intermittently once every 2 h and finally at the end of 24 and 72 h to note any toxic sign.

Treatment schedule

The Wister albino rats were divided into seven groups eight animals each.[24] Four groups served as control, cisplatin-treated, prophylactic control, curative control, one group of animals received prophylactic dose (500 mg/kg, p.o.) and other two group of animals received curative doses (250 and 500 mg/kg, p.o,).

Group 1 - Control: Rats received 2 % Tween 80 orally for 10 days.

Group 2 - Cisplatin treated: Rats were injected intraperitoneally with single dose of cisplatin (CP) 5 mg/kg, dissolved in 2 % Tween 80 on the first day of the experiment.

Group 3 - Prophylactic control: Rats were given 2 % Tween 80, p.o. for 10 days, on 11th day cisplatin 5 mg/kg, i.p.

Group 4 - Prophylactic activity: Rats were given extract of FH 500 mg/kg, p.o. for 10 days, on 11th day cisplatin 5 mg/kg, i. p.

Group 5 - Curative control: Rats were injected single dose of cisplatin 5 mg/kg, i. p. dissolved in saline on the 1st day of the experiment and normal saline is given on 6th day onwards.

Group 6 - Curative activity: Rats were injected single dose of cisplatin 5 mg/kg, i. p. dissolved in saline on the 1st day of the experiment and 6th day onwards crude extract of FH 250 mg/kg, p. o. for 10 days.

Group 7 - Curative activity: Rats were injected single dose of cisplatin 5 mg/kg, i. p. dissolved in saline on the 1st day of the experiment and 6th day onwards methanolic extract of FH 500 mg/kg, p. o. for 10 days.

At the end of the experimental period (i.e on 11th day of the experiment), the animals were anaesthetized with ether

and blood was withdrawn through heart puncture. The kidneys were immediately removed and rinsed with ice-cold saline. Serum was separated from blood collected for biochemical parameters. Nephroprotector activity of FH was screened by percentage change in body weight of each rat before and after treatment and also by estimating the blood urea nitrogen (BUN: Diacetyl monoxime method), serum total protein (S_{TP}: Biuret method), serum creatinine and urinary creatinine (SC and UC: Alkaline picrate method), WBC count, creatinine clearance (Cr_{Cl}) and urinary total proteins.[23]

IN VITRO ANTIOXIDANT ACTIVITY

Thiobarbituric acid test

Preparation of rat kidney homogenate

Albino rat kidney was removed and washed with ice-cold saline. The brain was homogenized in ice-cold buffer (pH 7.4) using Teflon homogenizer. The supernatant was stored at -70 ºC.

Assay

Lipid peroxidation assay was evaluated as the malonyldialdehyde (MDA) production according to the described method with some modifications.[26, 27] Lipid peroxidation was initiated by adding ferric chloride (100 µm) to a mixture containing the rat kidney homogenate (0.25 ml) and different concentrations of extracts in a total volume of 0.75 ml. The reaction mixture was incubated for 20 min at 37ºC and 20 % of the homogenate is taken in 1.5 % KCl. To 1 ml of homogenate, 20 % of 2.5 ml of trichloroacetic acid is added. The mixture is centrifuged at 3500 rpm for 10 min. The resulting pellet was dissolved in 2.5 ml of 0.05 M sulphuric acid and 3 ml of 0.38 % of thiobarbituric acid is added and incubated at 37ºC for 30 min. The contents were then extracted into 4 ml of n-butanol and absorbance was measured spectrophotometrically at 530 nm.

Nitric oxide scavenging activity

Nitric oxide is a free radical and scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Nitric oxide was generated from sodium nitroprussside and measured by the modified Griess Ilosvog reaction.[24,28] The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (1 ml), and extracts (0.4, 0.6, 0.8 and 1 mg/ml) or standard solution (1 ml) was incubated at 25ºC for 2 h. After incubation, 0.5 ml of the reaction mixture containing nitrite was removed; 1 ml of sulphanilic acid reagent (0.33 % in 20 % glacial acetic acid) was mixed and allowed to stand for 5 min for complete diazotization. Then 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min. The absorbance of chromophore formed measured at 542 nm. The experiment was repeated in triplicate and percentage scavenging effect was calculated.

Histopathological studies

Animals from each group were sacrificed on the day of withdrawal of blood and kidneys were isolated. The kidney sections were stained with hematoxylin and eosin and observed under light microscope.[24]

Reducing power

Different dose of methanolic extract FH were diluted to get 150, 250 and 400 µg/ml concentrations.[26,27] Test concentrations are mixed with phosphate buffer (2.5 ml, 0.02 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1 %). The mixture was incubated at 50ºC for 20 min. A portion of trichloro aceticacid (2.5 ml, 10 %) added to the mixture which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.05 ml, 0.1 %) was added and the absorbance was measured at 700 nm.

Statistical analysis

The data were expressed as mean ± SE, statistical analysis was performed by one-way ANOVA followed by post-hoc student-Neuman Keuls test. *P* values < 0.001 were considered as significant.

RESULTS

Acute toxicity

The acute toxicity showed no animal died even at 1000 mg/kg and hence the extract was treated as non-toxic. Therefore as per the CPCSEA guideline 420 it was thought that 1000 mg/kg is the LD_{50} cut off dose. Hence, 250 and 500 mg/kg were selected as test doses for further investigations.

Nephroprotective activity screening

Nephroprotective activity was assessed by measuring the following parameters

Body weight

Body weight was recorded in alternative days. The body weight of the animals receiving CP 5 mg/kg, i. p. decreased when compared to the control animals [table-1]. However, when the methanolic extract was given at prophylactic and curative doses the body weight increased significantly.

Biochemical parameters

Biochemical parameters BUN, SC, S_{TP} and Cr_{Cl} levels were increased significantly and also reduction in the WBC count is observed. Methanolic extract of FH reversed these effects moderate to significantly by the administration of 250 and 500 mg/kg of methanolic extract. The reports were given in the table-1 and 2.

Table 1: Effect of methanolic extract of fruits of *Ficus hispida* in cisplatin induced renal damage (values are mean ± SE of 6 replications)

Groups	Treatment regimen	Body weight (g)	BUN (mg/dl)	SC (mg/dl)	S_{TP} (mg/100 g)
1	Control	162.25 ± 1.11	21 ± 0.58	0.58 ± 0.14	6.91 ± 0.14
2	Cisplatin (5mg/kg, i.p)	154.75 ± 0.85*	41 ± 0.58*	1.3 ± 0.06*	13.78 ± 0.06*
3	Prophylactic control	127 ± 1.29	55 ± 1.39	1.4 ± 0.06	11.7 ± 0.1
4	Prophylactic regimen (500 mg/kg, p.o)	149.25 ± 1.11**	21.67 ± 1.89**	0.63 ± 0.07**	7.01 ± 0.07**
5	Curative control	104.75 ± 0.85	39 ± 0.58	1.1 ± 0.06	12.83 ± 0.08
6	Curative regimen (250 mg/kg, p.o)	127 ± 1.29**	22 ± 0.97**	0.63 ± 0.07**	7.7 ± 0.06**
7	Curative regimen (500 mg/kg, p.o)	129.25 ± 1.11**	21 ± 0.76**	0.59 ± 0.05**	7.1 ± 0.06**

BUN: Blood urea nitrogen; SC: serum creatinine; S_{TP}: serum total protein (Biuret method);
*P < 0.01 (compared to control); **P < 0.001 (compared to cisplatin treated group).
One way ANOVA followed by post hoc Student-Newman-Keuls Test.

Table 2: Effect of methanolic extract of fruits of *Ficus hispida* in cisplatin induced renal damage (values are mean ± SE of 6 replications)

Groups	Treatment regimen	Urine creatinine (mg/dl)	Urinary total proteins (mg/24 h)	Creatinine clearance (ml /h/100 g body weight)
1	Control	6.72 ± 0.17	7.07 ± 0.08	14.8
2	Cisplatin (5mg/kg, i.p)	13.78 ± 0.09*	13.78 ± 0.05*	6.9*
3	Prophylactic control	14.32 ± 0.06	16.33 ± 0.67	6.14
4	Prophylactic regimen (500 mg/kg, p.o)	6.17 ± 0.06**	7.17 ± 0.08**	14.77**
5	Curative control	11.9 ± 0.06	13.92 ± 0.12	8.65
6	Curative regimen (250 mg/kg, p.o)	6.75 ± 0.04**	8.3 ± 0.06**	11.78**
7	Curative regimen (500 mg/kg, p.o)	6.22 ± 0.05**	7.27 ± 0.07**	14.80**

*P < 0.01 (compared to control); **P < 0.001 (compared to cisplatin treated group).
One way ANOVA followed by post hoc Student-Newman-Keuls Test.

Lipid peroxidation

CP increases MDA level where as methanolic extract of FH showed modearately decreased MDA levels at the doses of 250 and 500 mg/kg. However prophylactic dose (500 mg/kg) of methanolic extract showed significant reduction in MDA level. Increased MDA [table-3] level by CP is reversed by methanolic extract of FH.

Histopathalogy

Intraperitoneal administration of CP (5 mg /kg) induced severe biochemical changes as well as oxidative damage in kidney. CP treated rat kidney exhibited marked congestion of the glomeruli with glomerular atrophy, desquamation of tubular epithelial cells and induced acute renal necrosis [Fig-1 (A-G)].

Nitric oxide scavenging activity

Methanolic extract of FH in 0.8 and 1.0 mg /ml showed significant (40 % and 49 % inhibition) nitric oxide scavenging activity [table-4].

Reducing power

Reducing power of the extract increased with the increase in concentrations (150, 250, 350 and 400 µg/ ml) of the methanolic extract of fruits of FH [table-5].

Table 3: Effect of methanolic extract of fruits of *Ficus hispida* on cisplatin induced lipid peroxidation in rat kidney homogenate (values are mean ± SE of 6 replications)

Groups	Treatment regimen	Absorbance	% Inhibition
1	Control	0.033 ± 1.74	—
2	Cisplatin (5mg/kg, i.p)	0.061 ± 1.74*	—
3	Prophylactic control	0.102 ± 0.002	—
4	Prophylactic regimen (500 mg/kg, p.o)	0.051 ± 0.007**	51
5	Curative control	0.13 ± 0.004	—
6	Curative regimen (250 mg/kg, p.o)	0.007 ± 1.74**	25
7	Curative regimen (500 mg/kg, p.o)	0.055 ± 0.008**	41

*P < 0.01 (compared to control); **P < 0.001 (compared to cisplatin treated group).
One way ANOVA followed by post hoc Student-Newman-Keuls Test.

DISCUSSION

The clinical use of cisplatin, a potent anticancer agent used in solid tumors of testes, ovary, breast, lungs, bladder, etc is limited by its renal toxicity.[30,31] Studies suggest that the

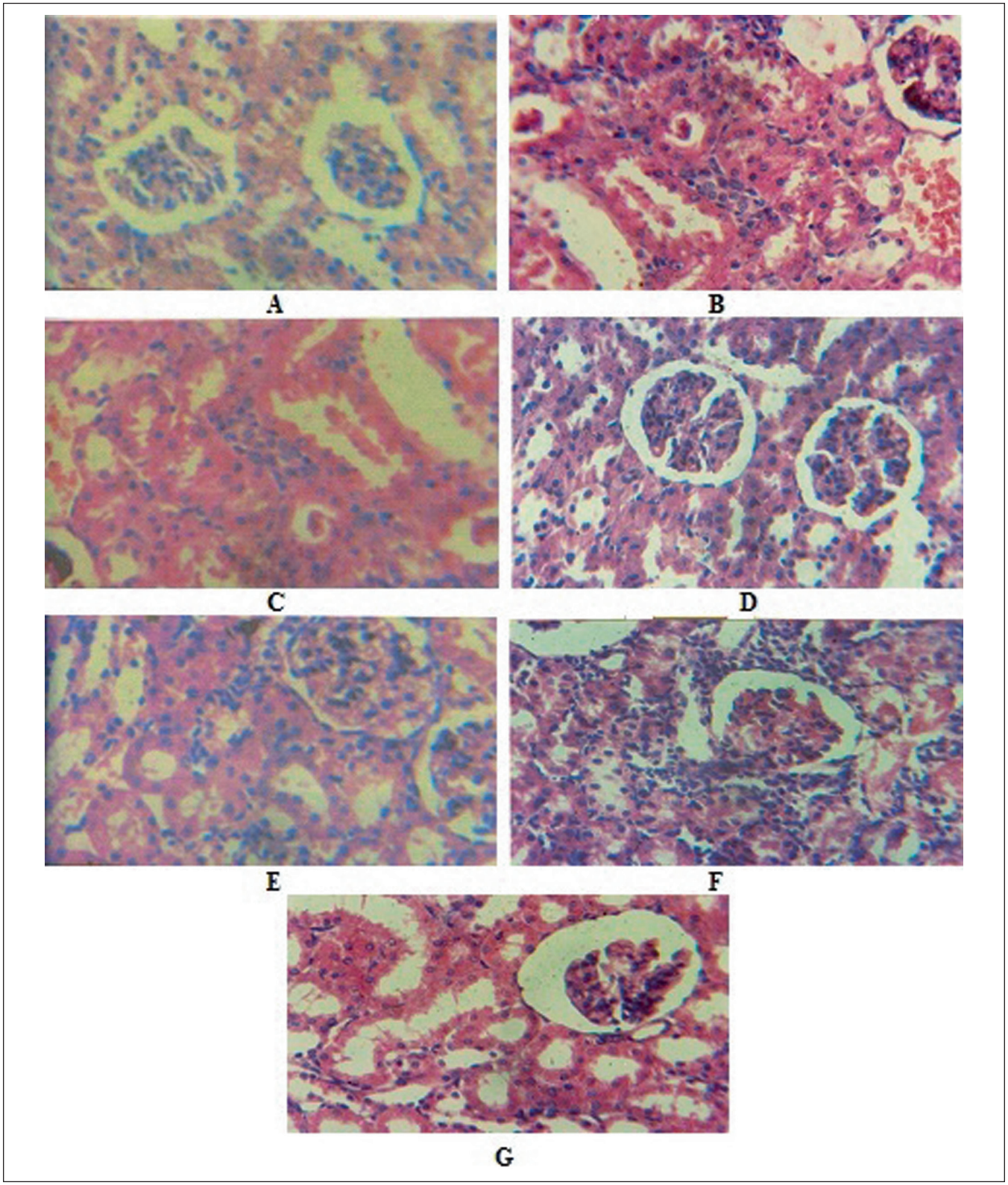

Figure 1: Histopathological study results of cisplatin induced nephrotoxicity in rat kidney.
A) Normal kidney showing normal organization of tubular epithelial cells and glomeruli, B) Cisplatin treated rat kidney showing infiltration of cells tubular congestion and glomerular atrophy, C) Rat kidney treated with cisplatin (Prophylactic control) showing glomerular congestion and congestion of inter-tubular blood vessels, D) Prophylactic group (500 mg/ kg, p.o) sshowing regenerative changes in glomeruli and tubules, E) Curative control showing congestion in glomeruli, F) Curative group (250 mg/ kg, p.o) showing normalicy of tubular epithelial cells and glomeruli, G) Curative group (500 mg/ kg, p.o) showing normalicy of tubular epithelial cells and glomeruli,

Table 4: Nitric oxide scavenging activity of methanolic extract of fruits of *Ficus hispida*

S. No.	Conc (mg/ml)	Absorbance	% Inhibition
1	Control	0.984 ± 0.007	—
2	0.4	0.746 ± 1.74*	24
3	0.6	0.672 ± 0.006	32
4	0.8	0.586 ± 0.008	40
5	1.0	0.501 ± 0.007	49

Sodium nitroprusside (5mM) was mixed with different concentrations of crude extract and incubated at 25°C for 2 hrs. Griess reagent was added and absorbance of chromophore formed was read at 546 nm. Control experiment was also carried out in a similar manner.

Table 5: Reducing power of methanolic extract of fruits of *Ficus hispida*

S. No.	Conc (mg/ml)	Absorbance	% Inhibition
1	Control	0.319 ± 0.005	—
2	150	0.242 ± 0.003	24
3	250	0.218 ± 0.008	32
4	350	0.192 ± 0.008	40
5	400	0.183 ± 0.006	43

Different doses of methanolic extract of *Ficus hispida* were mixed in 1 ml of distilled water so as to get 150, 250,350 and 400 µg/ml concentration. Then mixed with phosphate buffer and potassium ferricyanide and the mixture was incubated at 50°C for 20 min. To this trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. The upper layer of the solution was mixed with distilled water and $FeCl_3$ and the absorbance was measured at 700 nm.

free radicals play an important role in cisplatin induced nephrotoxicity[32, 33]. This renal damage occurs 1st hr after administration, hence protective agent must present in sufficient concentration to prevent the damage. This might explain the rationale for using nephroprotective agent, and screening methanolic extract of FH for the same. Biochemical parameters analysed from both control and CP treated animals confirms the role of CP in renal toxicity. Prophylactic dose of extract of FH (500 mg/kg, p. o) significantly decreases the elevation of BUN and SC in rats. Partial protection was observed in case of reduction of body weight. The renal clearance data was markedly improved by the administration of FH. Therefore it is important that the protective agent must be present in sufficient concentrations in the renal tissues before the damage occurs.

The methanolic extract was administered in two doses in the curative regimen (250 and 500 mg/kg, p. o.). The extract shown partial protection against CP induced renal and functional impairment. However, the protection is more significant at the higher dose (500 mg/ kg, p. o.). In the lipid peroxidation studies, the extract in curative group showed moderate protection against CP induced elevated levels of MDA. But prophylactic group showed increased protection compared to curative group. Microscopic examination of renal cortical part of CP treated animals exhibited marked congestion of glomeruli with glomerular atrophy and presence of casts in tubular epithelial cells indicating acute renal necrosis. In curative regimen (500 mg/kg), mild degenerative changes and congestion were observed. However, in 250 mg/ kg features of tubular necrosis persisted. In preventive regimen, congestion of the glomeruli was reduced; degeneration of tubular cells was not observed indicating significant protection against the injury. One of the proposed mechanism for CP induced free radical damage is by increasing the activity of calcium-independent nitric oxide synthase. Antioxidants modulate this free radical damage due to their interaction with biomolecules. Methanolic extract of fruits of FH showed moderate nitric oxide scavenging activity and significant reducing power indicating its antioxidant potential.

Analysis of all the parameters i.e., biochemical parameters, histopathological changes in kidney clearly shows that the methanolic extract of fruits of FH shown protection in both prophylactic and curative regimen; the protection is more significant in prophylactic regimen. The exact mechanism of the protection need to be established and that helps in the further development in the related studies. Further studies are required for isolation of active constituents and to understand the mechanism of protection.

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