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Therapeutic Plants of Ayurveda; A Review on Anticancer

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ABSTRACT

Cancer is actually a group of many related diseases that all have to do with cells. Cells are the very small units that make up all living things, including the human body. There are billions of cells in each person's body. Cancer happens when cells that are not normal grow and spread very fast. Normal body cells grow and divide and know to stop growing. Over time, they also die. Unlike these normal cells, cancer cells just continue to grow and divide out of control and don't die when they're supposed to. A number of synthetic anticancer drugs are available in practice, however their effectiveness does not hold true with the entire range of population suffering from this disorder. Moreover the side effects and the drug interactions are major restrictions in its clinical utility. On the other hand, herbal medicines are now attracting attention as potential sources of anticancer agents are widely used across the globe due to their wide applicability and therapeutic efficacy coupled with least side effects, which in turn has accelerated the scientific research regarding the anticancer activity. In this overview we have summarized the current research advancements on plants belongs to different families like Apocyanaceae, Taxaceae, Beriberidaceae, Solanaceae, Cupressaceae etc. having anticancer activities along with their other activities.

Key words: Cancer, Anticancer herbal medicines.

INTRODUCTION

An attempt has been made to review some medicinal plants used for the prevention and treatment of cancer in India and foreign countries. Place of these plants have been collected from the literature. The extracts or decoctions of these are generally used. The medicinal plants contain several phytochemicals such as vitamins (A, C, E, K), carotenoids, Terpenoids, flavonoids, polyphenols, alkaloids, tannins, Saponins, enzymes, minerals, etc. These phytochemicals possess antioxidant activities, which prevent or can be used in the treatment of many diseases, including cancer. Herbal drugs are also known to have good Immunomodulatory properties. These act by stimulating both non-specific and specific immunity. Over the past decade, herbal medicines have been accepted universally, and they have an impact on both world health and international trade. Hence, medicinal plants continue to play an important role in the healthcare system of a large number of the world's population. Traditional medicine is widely used in India. Even in USA, use of plants and phytomedicines has increased dramatically in the last two decades. A National Centre for Complementary and Alteranative Medicine has been established in USA. The herbal products have been classified under 'dietary supplements' and are included with vitamins, minerals, amino acids and 'other products intended to supplement the diet'. Use of plants as a medicinal remedy is an integral part of the South African cultural life. It is estimated that 27 million South Africans use herbal medicines from more than 1020 plant species. In fact, there are several medicinal plants all over the World, including India, which are being used traditionally For the prevention and treatment of cancer.

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Alstonia scholaris

Alstonia scholaris, commonly known as sapthaparna, has been used for centuries in Ayurvedic medicine for treatment of various disorders. The objective of this study was to investigate the possible chemopreventive and anti-oxidative properties of this medicinal plant on two-stage process of skin carcinogenesis induced by a single application of 7, 12-dimethyabenz(a)anthrecene (100 lg/100 ll acetone), and two weeks later, promoted by repeated application of croton oil (1% in acetone/thrice a week) till the end of the experiment (16 weeks) in Swiss albino mice. The tumor incidence, tumor yield, tumor burden and cumulative number

Table 1: Medicinal plants with anticancer activity and their beneficial properties

Plant name	Ayurvedic/common name	Anticancer and other beneficial effect in traditional medicine	Reference
Alstonia scholaris	Sapthaparna	Chemopreventive and anti-oxidative	1
Pipper nigrum	Pipper	Carminative, Stimulant, Anticancer, Stomachic	2
Withania somnifera	Ashwagandha	Anticancer, Sedative and Hypnotic, Gout, Hypertension	3
Cymbopogon flexuosus	Lemon grass oil	Anticancer	4
Saussurea lappa	Costus root	Anticancer, Bronchial asthma	5
Andrographis paniculata	Kalmegh	Anticancer, Hepatoprotective	6
Tanacetum gracile	Feverfew	Anticancer, Antipyretic	7
Abrus agglutinin	Abrin	Antitumor	8
Curcuma longa	Turmeric	Antioxidant, Antiinflammatory, Antiviral, Antibacterial, Anticancer	9
Saussurea costus	Kuth	Anticancer, Ulcer, Inflammatory diseases, Asthma	10
Crocus sativus	Saffron	Antitumor agent, Antispasmodic	11
Ficus benghalensis	Banyan tree	Malignant disease, Anti-inflammatory	12
Calotropis procera	Giant Swallow Wort	Antibacterial, Anthelmint, Anti-diarrhoeal, Ascaricidal, Schizonticidal	13
Cassia auriculata	Senna	Anticancer, Cathartic	14
Punica granatum	Anar	Anticancer, Anti-inflammatory	15
Carica papaya	Papain	Anticancer, Anti-inflammatory	16
Capsicum annum	Chillies	Anticancer, Stomachic, Carminative, Counter irritant	17
Vernonia amygdalina	Bitterleaf	Anticancer	18
Vitis vinifera	Grape	Anticancer, Hepatoprotective, Anemia	19
Ziziphus jujube	Jujube	Anticancer, Antianxiolytic, Antifungal, Antispastic, Cardiotonic, Antifertility	20
Passiflora incarnate	Passion flower	Anticancer, Antianxiety, Insomnia, Cough, Sexual dysfunction, Convulsion	21
Emblica officinalis	Amla	Anticancer, Hepatoprotective, Antioxidant, Antitumour	22
Poncirus trifoliata	Tang-ja-na-moo	Anticancer, Antioxidant	23
Hedychium spicatum	Kapur Kachari	Anticancer, Stomachic, Stimulant, Tonic,	24
Cynodon dactylon	Grass	Anticancer, Antioxidant	25
Tabernaemomtana divaricata	Chameli	Anticancer	26
Nigella sativa	Kalonji	Anticancer, Immunomodulator	27
Gingiber officinalis	Ginger	Anticancer, Antioxidant, Anti-inflammatory	28
Berberis vulgaris	Barberry	Anticancer, Antidiabetic, Heart failure	29
Sauromatum venosum	Voodoo lily	Anticancer	30
Eulophia compestris	Salam	Antitumurs	31
Ipomoea bahiensis	Krishnabija	Sarcoma cancer	32
Mangifera indica	Mango	Anticancer, Antibacterial, Antioxidant	33
Podophyllum emodi	Himalayan May-apple	Anticancer, Purgative, Bitter tonic	34
Polyalthia longifolia	Asoka	Anticancer	35
Tinospora cordifolia	Guduchi	Anticancer, Gout, Skin disease, Hepatoprotective	36
Bauhinia variegata	Kachnar	Antitumour	37
Thea sinensis	Tea	Anticancer, Diuretics, CNS stimulant	38
Phyllanthus amarus	Bhumyaamalaki	Anticancer	39
Bidens pilosa	Black jack	Anticancer, Antitumour	40
Solanum nigrum	Makoi	Anticancer, Antiviral, Anti-inflammatory	41
Vinca rosea	Periwinkle	Anticancer	42
Eurycoma longifolia	Pasak bumi	Anticancer	43
Coptidis rhizoma	Huanglian	Antineoplastic	44
Ximenia americana	Wild lime	Antineoplastic	45
Eruca sativa	Diplotaxis	Anticancer	46
Podophyllum hexandrum	Himalayan May-apple	Anticancer, Bitter tonic, Purgative	47
Taxus bacata	Europian yew	Anticancer	48
Cedrus deodara	Dvedar	Anticancer	49
Podophyllum hexandrum	Hymalayan mayapple	Anticancer	50
Scutellarria baicalensis	Baikal skullcap	Anticancer	51
Silybum marianum	Marian Thistle	Anticancer, Hepatoprotective	52
Panax ginseng	Panax	Anticancer, Immunomodulator,	53
Cynara cardunculus	Hathichuk	Anticancer	54
Radix sophorae	Flavescent sophora root	Anticancer	55
Sophora flavescens	Yellow sophora	Antitumour	56
Allium sativum	Garlic	Anticancer, Carminative, Expectorant, Stimulant, Disinfectant, Antihypertensive	57
Solanum lycopersicum	Tomato	Anticancer	58

Plant name	Ayurvedic/common name	Anticancer and other beneficial effect in traditional medicine	Reference
Leucaena leucocephala	White leadtree	Anticancer	59
Annonia reticulata	Vanya Kahu	Antiproliferative	60
Chlorophytum borivilianum	Safed musli	Antiproliferative	61
Rhodola imbricata	Rose root	Antioxidant, Anticancer	62
Ipomoea batatas	Shakarkand	Cancerchemoprotective	63
Hygrophila spinosa	Gokulakanta	Anticancer	64

of papillomas were found to be higher in the carcinogen treated control (without ASE treatment) as compared to experimental animals (ASE treated).[1]

Pipper nigrum

The first stereoselective total synthesis of new natural amide alkaloids 1-3 have been achieved from commercially available starting materials. Wittig olefination, Sharpless asymmetric dihydroxylation, epoxidation, a trans regioselective opening of 2,3-epoxy alcohol, Horner-Wadsworth-Emmons (HWE) olefination and amide coupling are the key steps. The amide alkaloids 1-3 are evaluated for their anticancer activity against colon (HT-29), breast (MCF-7) and lung (A-549) human cancer cell lines for the first time.[2]

Withania somnifera

Ashwagandha is an Ayurvedic shrub that forms a common ingredient of health supplements, tonics, and Indian home remedies designed to promote health and quality of life. Though sustained through experience and history, there are only a limited laboratory studies and experimental evidence to its effects. In our efforts to characterize Ashwagandha activities and their molecular mechanisms, we initially prepared leaf extract of Ashwagandha (i-Extract) that showed tumor-inhibitory activity. In the present study, we demonstrate that a major component of i-Extract and withanone (i-Factor) protected the normal human fibroblasts against the toxicity caused by withaferin A. We report here the isolation, structure elucidation, and biological properties of this compound, which showed good anti-inflammatory and anticancer activities.[3]

Cymbopogon flexuosus

The essential oil from a lemon grass variety of Cymbopogon flexuosus was studied for its *in vitro* cytotoxicity against twelve human cancer cell lines. The *in vivo* anticancer activity of the oil was also studied using both solid and ascitic Ehrlich and Sarcoma-180 tumor models in mice. In addition, the morphological changes in tumor cells were studied to ascertain the mechanism of cell death.[4]

Saussurea lappa

The dried roots of Saussurea lappa, called costus roots, are used in the traditional system of medicine for the treatment of cancer. In our investigation for the anticancer constituents from the hexane extract of this plant, a new sesquiterpene was isolated along with the known compounds costunolide, beta-cyclocostunolide, dihydro costunolide and dehydro costuslactone.[5]

Andrographis paniculata

Herbal medicines are now attracting attention as potential sources of anticancer agents. Andrographis paniculata is a traditionally used anticancer herb in Indian and Chinese herbal medicine. Phytochemical investigation of the ethanol extract of the aerial parts of this herb resulted in the isolation of 14 compounds including flavonoids and labdane diterpenoids. This is the first isolation of compounds from a natural source, and the aerial parts of A. paniculata are a rich source for the molecule andrographolide (9, 1.375%, w/w).[6]

Tanacetum gracile

The essential oil of Tanacetum gracile (Accession no. AT-01 termed AT-01 in the manuscript), a cold desert alpine highly aromatic herb, has 40 constituents including lavendulol (21.5%), lavendulol acetate (1.7%), alpha-pinene (11.2%), 1,8-cineole (15.2%), CIS-beta-ocimene (6.9%), borneol (6.1%), limonene (5.1%) and chamazulene (3.7%). AT-01 was evaluated for its anticancer activity. It inhibited HL-60 cell proliferation with an IC (50) of 27 microg/mL.[7]

Abrus agglutinin

In our previous study, Abrus agglutinin showed antitumor activity both native and heat-denatured condition in mouse model. The purpose of this study is to explore the presence of anticancer peptide in agglutinin, and to elucidate the mechanism of its activity *in vitro*. A tryptic digested Abrus agglutinin peptide fractions obtained from 10-kDa molecular weight cut off membrane permeate (10 kMPP), was found to have selective antiproliferative activity (1-10 microg/ml) on several tumor cell lines *in vitro* without having any cytotoxic effect on normal cell lineswith dose of 100 microg/ml.[8]

Curcuma longa

Turmeric, derived from the plant *Curcuma longa*, is a gold-colored spice commonly used in the Indian subcontinent, not only for health care but also for the preservation of food and as a yellow dye for textilesSince the time of Ayurveda (1900 Bc) numerous therapeutic activities have

been assigned to turmeric for a wide variety of diseases and conditions, including those of the skin, pulmonary, and gastrointestinal systems, aches, pains, wounds, sprains, and liver disorders.[9]

Saussurea costus

Saussurea costus (Falc.) Lipschitz, *Saussurea lappa* C.B. Clarke is a well known and important medicinal plant widely used in several indigenous systems of medicine for the treatment of various ailments, viz. asthma, inflammatory diseases, ulcer and stomach problems. Sesquiterpene lactones have been reported as the major phytoconstituents of this species.[10]

Crocus sativus

The primary goal of this study was to propose saffron as a sustainable substitute crop with high added value in some Moroccan agricultural areas with low and erratic rainfalls, for their socio-economical development.[11]

Ficus benghalensis

This review explores medieval, ancient and modern sources for ethnopharmacological uses of *Ficus* species, specifically for employment against malignant disease and inflammation. The close connection between inflammatory/infectious and cancerous diseases is apparent both from the medieval/ancient merging of these concepts and the modern pharmacological recognition of the initiating and promoting importance of inflammation for cancer growth. Also considered are chemical groups and compounds underlying the anticancer and anti-inflammatory actions, the relationship of fig wasps and fig botany, extraction and storage of fig latex, and traditional methods of preparing fig medicaments including fig lye, fig wine and medicinal poultices.[12]

Calotropis procera

Despite significant progress in oncology therapeutics in the last decades, the urge to discover and to develop new, alternative or synergistic anti-cancer agents still remains. For centuries it has been known that the coarse shrub *Calotropis procera*is a very promising source of ascaricidal, schizonticidal, anti-bacterial, anthelmintic, insecticidal, anti-inflammatory, anti-diarrhoeal, larvicidal and cytotoxic chemicals. Different compounds like norditerpenic esters, organic carbonates, the cysteine protease procerain, alkaloids, flavonoids, sterols as well as numerous types of cardenolides have provided this plant for centuries with scientist's interest inducing activities.[13]

Cassia auriculata

The *in vitro* anti-cancer effect of *Cassia auriculata* leaf extract (CALE) was evaluated in human breast adenocarcinoma MCF-7 and human larynx carcinoma Hep-2 cell lines. CALE preferentially inhibited the growth of both the cell lines in a dose-dependent manner with IC_{50} values of 400 and 500 µg for MCF-7 and Hep-2 cells, respectively. The results showed the anti-cancer action is due to nuclear fragmentation and condensation, associated with the appearance of A_0 peak in cell cycle analysis that is indicative of apoptosis.[14]

Punica granatum

The last 7 years have seen over seven times as many publications indexed by Medline dealing with pomegranate and *Punica granatum* than in all the years preceding them. The use of juice, peel and oil have also been shown to possess anticancer activities, including interference with tumor cell proliferation, cell cycle, invasion and angiogenesis. The phytochemistry and pharmacological actions of all *Punica granatum* components suggest a wide range of clinical applications for the treatment and prevention of cancer, as well as other diseases where chronic inflammation is believed to play an essential etiologic role.[15]

Carica papaya

Aim of the study various parts of *Carica papaya* Linn. CP have been traditionally used as ethnomedicine for a number of disorders, including cancer. There have been anecdotes of patients with advanced cancers achieving remission following consumption of tea extract made from CP leaves. However, the precise cellular mechanism of action of CP tea extracts remains unclear.[16]

Capsicum annum

We have demonstrated a synergy between a decaffeinated green tea concentrate and a vanilloid-containing *Capsicum* preparation obtained commercially. At a ratio of 25 parts green tea concentrate to 1 part *Capsicum* preparation, the resultant product exhibited efficacy in the killing of cancer cells in culture 100-times that of green tea on a weight basis. The activity of the protein target was inhibited by the tea catechins and the *Capsicum vanilloids*. As with growth, the tea and *Capsicum* preparations evaluated were synergistic in their inhibition of the target enzymatic activity.[17]

Vernonia amygdalina

Evidence suggests that most chemotherapeutic agents are less effective as treatment in patients with estrogen receptor-negative (ER-) breast carcinomas compared to those with estrogen receptor-positive (ER+) breast carcinomas. Moreover, African American Women (AAW) is disproportionately diagnosed with ER- breast cancer compared to their white counterparts. Novel therapies effective against ER- breast carcinomas are urgently needed to ameliorate the health disparity. Previous reports show that low concentrations (microgram/ml) of water-soluble leaf extracts of a Nigerian edible plant, *V. amygdalina* (VA), potently retards the proliferative activities of ER+ human breast cancerous cells (MCF-7) *in vitro* in a concentration-dependent fashion.[18]

Vitis vinifera

Grapes and grape extracts were compared for inhibition of a growth-related and cancer-specific form of cell surface NADH oxidase with protein disulfide-thiol interchange activity designated tNOX from human cervical carcinoma (HeLa) cells and growth of HeLa and mouse mammary 4T1 cells in culture and transplanted tumors in mice. Grapes and grape extracts of several varieties had activity. The grape extracts interacted, often synergistically, with decaffeinated green tea extracts both in the inhibition of tNOX activity and in the inhibition of cancer cell growth.[19]

Ziziphus jujuba

Herbs have always been the natural form of medicine in India. Medicinal plants have curative properties due to presence of various complex chemical substances of different composition which contain secondary metabolites such as alkaloids, flavonoids, terpenoids, saponin and phenolic compounds distributed in different parts of the plants. *Ziziphus jujuba* Mill, a member of the family Rhamnaceae, commonly known as Bor, is used traditionally as tonic and aphrodisiac and sometimes as Hypnotic-sedative and Anxiolytic, anticancer (Melanoma cells), Antifungal, Antibacterial, Antiulcer, Anti-inflammatory, Cognitive, Antispastic, Antifertility/contracepti on, Hypotensive and Antinephritic, Cardiotonic, Antioxidant, Immunostimulant, and Wound healing properties.[20]

Passiflora incarnata

In our country, more than 2000 medicinal plants have been recognized. *Passiflora incarnata* (Passifloraceae; passion flower Family) is an important medicinal plant of tropical and subtropical India. Its medicinal usage has been reported in the traditional systems of medicine such as Ayurveda, Siddha and Unani. P. incarnata has been described as a passion flower and has been used extensively for treatment of some diseases like as anxiety, insomnia, convulsion, sexual dysfunction, cough and cancer. The present article including the detailed exploration of phyto-pharmacological properties of *P. incarnata* is an attempt to provide a direction for further research.[21]

Emblica officinalis

Aqueous extract of *Emblica officinalis* (E.O) was found to be cytotoxic to L 929 cells in culture in a dose dependent manner. Concentration needed for 50% inhibition was found to be 16.5 µg/ml. E.O and chyavanaprash (a non-toxic herbal preparation containing 50% E.O) extracts were found to reduce ascites and solid tumours in mice induced by DLA cells. Animals treated with 1.25 g/kg b.wt. of E.O extract increased life span of tumour bearing animals (20%) while animals treated with 2.5 g/kg b.wt. of chyavanaprash produced 60.9% increased in the life span. Both E.O and chyavanaprash significantly reduced the solid tumours.[22]

Poncirus trifoliata

Recently several plant derived natural compounds have been screened for their anticancer activity in order to identify putative compounds with novel structures or mechanism of action. In the present study, fruits of *Poncirus trifoliata* were extracted with acetone and loaded onto silica gel column chromatography. The column was eluted with different solvents to obtain two bioactive compounds. The purity of compounds was analyzed by HPLC and their structures were identified by ^{1}H and ^{13}C NMR experiments as β-sitosterol and 2-hydroxy-1,2,3-propanetricarboxylic acid 2-methyl ester (HPCME). β-Sitosterol, HPCME, and trolox were tested for their antioxidant capacity by oxygen radical absorbance capacity (ORAC) measurement. Growth inhibition assay suggested the potential use of bioactive compounds as cancer chemopreventive and therapeutic agents. This is the first report on HPCME isolation and identification from Rutaceae family and β-sitosterol from *P. trifoliata*.[23]

Hedychium spicatum

Phytochemical investigation of $CHCl_3$ extract of the rhizomes of *Hedychium spicatum* led to the isolation of two new labdane-type diterpenes, compounds **1** and **2** along with five known compounds (**3-7**). Their structures were established on the basis of NMR (1D and 2D) and mass spectroscopic analysis. In addition, all the isolates were tested for their cytotoxicity against the Colo-205 (Colo-cancer), A-431 (skin cancer), MCF-7 (breast cancer), A-549 (lung cancer) and Chinese hamster ovary cells (CHO). Two new compounds **1** and **2** were shown good cytotoxic activity.[24]

Cynodon dactylon

The present study was aimed at evaluating the chemopreventive property of*Cynodon dactylon*. The antioxidant, antiproliferative and apoptotic potentials of the plant were investigated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, nitric oxide radical scavenging activity (NO^{-}) and MTT assay on four cancer cell lines (COLO 320 DM, MCH-7, AGS, A549) and a normal cell line (VERO). *In vivo*chemopreventive property of the plant extract was studied in DMH-induced colon carcinogenesis. The methanolic extract of *C. dactylon* was found to be antiproliferative and antioxidative at lower concentrations and induced apoptotic cell death in COLO 320 DM cells. The present investigation revealed the anticancer potential of methanolic extract of *C. dactylon* in COLO 320 DM cells and experimentally induced colon carcinogenesis in rats.[25]

Tabernaemontana divaricata

In the present investigation, the cytotoxic, hydroxyl radical scavenging and topoisomerase inhibition activities of *Tabernaemontana divaricata* (Apocynaceae) were evaluated.

The extracts from leaves of the plant were prepared with different solvents viz. chloroform, methanol, ethyl acetate and hexane. In, *in vitro* cytotoxicity assay, with cell lines viz HCT-15 (Colon), HT-29 (Colon), 502713 (Colon), MCF-7 (Breast), PC- 3 (Prostrate), it was observed that the ethyl acetate extract was effective against only one colon cell line (502713) at the lowest dose i.e. 10 µg/ml, whereas the chloroform extract was effective against all the three colon cancer cell lines, at 30 µg/ml. In order to evaluate the mechanism of cytotoxicity of these extracts, they were assessed for their ability to scavenge hydroxyl radicals in plasmid nicking assay with pBR322.[26]

Nigella sativa

Aim of the study *Nigella sativa*, also known as blackseed, has long been used in traditional medicine for treating various conditions related to the respiratory and gastrointestinal systems as well as different types of cancers. In this study, the potential immunomodulatory effects ofNigella sativa are investigated in light of splenocyte proliferation, macrophage function, and NK anti-tumor activity using BLAB/c and C57/BL6 primary cells.[27]

Zingiber officinalis

Ginger, the rhizome of *Zingiber officinalis*, one of the most widely used species of the ginger family, is a common condiment for various foods and beverages. Ginger has a long history of medicinal use dating back 2500 years. Ginger has been traditionally used from time immemorial for varied human ailments in different parts of the globe, to aid digestion and treat stomach upset, diarrhoea, and nausea. Some pungent constituents present in ginger and other zingiberaceous plants have potent antioxidant and anti-inflammatoryactivities, and some of them exhibit cancer preventive activity in experimental carcinogenesis. The anticancer properties of ginger are attributed to the presence of certain pungent vallinoids, viz. [6]-gingerol and [6]-paradol, as well as some other constituents like shogaols, zingerone etc.[28]

Berberis vulgaris

Berberine, an isoquinoline plant alkaloid, is widely distributed in plant used in the traditional Chinese medicine. It displays a wide range of biological activities and the mechanism of action. Our previous studies of the anticancer activity of berberine against the cancer cell lines HeLa and L1210 were extended to the human tumour U937 cell line and the murine melanoma B16 cell line growing *in vitro*. Berberine acted cytotoxically on both tumour cell lines. The melanoma B16 cells were much more sensitive to berberine treatment than the U937 cells. The value of IC_{100} was below 100 µg/ml for the U937 cells and below 1 µg/ml for the B16 cells. As for both cell lines under the long-term influence the values of IC_{50} were found to be less than 4 µg/ml. No effect of berberine on the cell cycle profile of the U937 and B16 cells was detected.[29]

Sauromatum venosum

A new lectin with the potent mitogenic and *in vitro* anti-proliferative activity was isolated from the tubers of a wild monocotyledonous plant *Sauromatum venosum*(Schott), from the family Araceae. The apparent native molecular mass of *S. venosum* lectin (SVL), as determined by gel filtration chromatography, was 54 kDa. In HPLC, size exclusion and cation exchange chromatography, SVL gave a single peak and also a single band of 13.5 kDa in SDS-PAGE, pH 8.3, under reducing and non-reducing conditions, indicating that the lectin is composed of four identical subunits. The amino acid composition showed that lectin contained a high amount of aspartic acid and glycine but totally devoid of cysteine. However, trace amounts of methionine was present. The lectin showed a potent mitogenic response towards BALB/c splenocytes and human lymphocytes. This lectin is endowed with proliferation of T cells as revealed by IL-2 bioassay but showed no production of immunoglobulins thus indicating the non-stimulation of B cells. SVL significantly inhibited the proliferation of murine cancer cell-lines. Thus the anti-proliferative ability of SVL may be helpful in identification of new lectin probes that can lead to better understanding in the detection and study of certain types of cancer.[30]

Eulophianuda nuda

Ethnopharmacological relevance *Eulophia nuda* L. (Orchidaceae) is a medicinally important terrestrial orchid used for the treatment of tumours and various health problems by the local healers throughout the Western Ghats region in Maharashtra (India).[31]

Ipomoea bahiensis

Four new antimicrobial glycosides have been isolated from *Ipomoea bahiensis*. Spectroscopic properties and basic and acidic hydrolysis characterized them as derivatives of 11 -hydroxy hexadecanoic and 11-hydroxy tetradecanoic acid, glycosidically linked in the 11-position to a trisaccharide unit composed of glucose, rhamnose and fucose which is esterified by tiglic and 3-hydroxy-2-methylbutyric acid. One of these compounds revealed significant activity against Sarcoma 180 in mice.[32]

Mangifera indica

The antioxidant and antiproliferative properties of flesh and peel of mango (*Mangifera indica* L.) were investigated. The cytoprotective effect of mango flesh and peel extracts on oxidative damage induced by H_2O_2 in a human hepatoma cell line, HepG2, were determined, and the underlying mechanism was examined by a single-cell electrophoresis assay (comet assay). Treatment of HepG2 cell with mango peel extract prior to oxidative stress was found to inhibit

DNA damage. Thus, mango peel, a major by-product obtained during the processing of mango product, exhibited good antioxidant activity and may serve as a potential source of phenolics with anticancer activity.[33]

Podophyllum emodi

This review deals with the historical discovery of particularly important lignan derivatives used in cancer chemotherapy. From isolation of the naturally occurring podophyllotoxin, an inhibitor of microtubule assembly, to hemisynthesis of the clinically import antanticancer drugs etoposide and tenoposide, it will be demonstrated how the activities and the ability of this class of compounds to inhibit topoisomerase II were discovered by different research teams. By virtue of these discoveries, new hemisynthetic derivatives, with different mechanisms of action, are bringing improvements in the ability to treat cancer.[34]

Polyalthia longifolia

Polyalthia longifolia is a lofty evergreen tree found in India and Sri Lanka. We are reporting first time the anticancer potential of *P. longifolia* leaves extract (A001) and its chloroform fraction (F002). Both inhibited cell proliferation of various human cancer cell lines in which colon cancer cells SW-620 showed maximum inhibition with IC_{50} value 6.1 µg/ml. Furthermore, F002 induce apoptosis in human leukemia HL-60 cells as measured by several biological end points. F002 induce apoptotic bodies formation, DNA ladder, enhanced annexin-V-FITC binding of the cells, increased sub-G_0 DNA fraction, loss of mitochondrial membrane potential, release of cytochrome *c*, activation of caspase-9, caspase-3, and cleavage of poly ADP ribose polymerase (PARP) in HL-60 cells. All the above parameters revealed that F002-induced apoptosis through the mitochondrial-dependent pathway in HL-60 cells.[35]

Tinospra cordifolia

Exposure of HeLa cells to 0, 5, 10, 25, 50 and 100 µg/ml of guduchi extracts (methanol, aqueous and methylene chloride) resulted in a dose-dependent but significant increase in cell killing, when compared to non-drug-treated controls. The effects of methanol and aqueous extracts were almost identical. However, methylene chloride extract enhanced the cell killing effect by 2.8- and 6.8-fold when compared either to methanol or aqueous extract at 50 and 100 µg/ml, respectively. Our results demonstrate that guduchi killed the cells very effectively *in vitro* and deserves attention as an antineoplastic agent.[36]

Bauhinia variegata

The antitumour activity of the ethanol extract of *Bauhinia variegata* (EBV) has been evaluated against Dalton's ascitic lymphoma (DAL) in Swiss albino mice. A significant enhancement of mean survival time of EBV-treated tumour bearing mice was found with respect to control group. EBV treatment was found to enhance peritoneal cell counts. After 14 days of inoculation, EBV is able to reverse the changes in the haemotological parameters, protein and PCV consequent to tumour inoculation.[37]

Thea sinensis

We investigated the anticancer effects of green and black tea polyphenols alone and in combination with bovine milk lactoferrin (bLF) on human tongue squamous carcinoma (CAL-27) and normal human gingival fibroblast (HGF) cells. Both green (Polyphenon-E;P-E) and black tea polyphenols (Polyphenon-B;P-B) pre- ferentially inhibit the growth of CAL-27 cells in a dose-dependent manner. Based on the IC_{50} values, P-E was found to be more effective than P-B and the combination of P-E and bLF (1:2 ratio) exhibited synergistic inhibition of CAL-27 cells.[38]

Phyllanthus amarus

This study deals with establishment of hairy root cultures of *Phyllanthus amarus* using *Agro- bacterium rhizogenes* and cytotoxic effects of methanolic extract of hairy roots on human adenocarcinoma cell line, MCF-7. The hairy root extract displayed a linear concentration- and time-dependent cytotoxicity. Further, increased concentration of the root extract showed an increase in the percent apoptotic cells from 26% to 36% as determined by annexin V-FITC and propidium iodide.[39]

Bidens pilosa

Aim of the study *Bidens pilosa* (L.) (Asteraceae) is a medicinal plant traditionally used in Brazil for treating conditions that can be related to cancer. Therefore the present study was carried out to evaluate the antitumor activity of extracts obtained from the aerial parts of this plant species.[40]

Solanum nigrum: Plants are used worldwide for the treatment of diseases, and novel drugs continue to be developed through research from plants. It possesses antiviral, anti-inflammatory, and analgesic effects. Also, *Solanum nigrum* has been used as a diuretic and an antipyretic agent and it has also been used to cure inflammation, edema, mastitis and hepatic cancer. In this investigation, cytotoxicity of specific concentrations of hydro-alcoholic extracts of *C. pepo* and *S. nigrum* was studied on normal [Chinese hamster ovarian cells (CHO) and rat fibroblast] and cancer (HepG2 and CT26) cell lines.[41]

Vinca rosea

The novel third-generation bifluorinated semisynthetic vinca alkaloid, vinflunine, is a microtubule inhibitor that shows superior antitumor activity and a favorable safety profile compared with other vinca alkaloids. The main antineoplastic effects of vinflunine arise from its interaction with tubulin,

the major component of microtubules in mitotic spindles. Vinflunine is known to have low affinity for tubulin, high intracellular accumulation, and important effects on microtubule dynamics. It has been shown to have activity against transitional cell carcinoma of the urothelial tract.[42]

Eurycoma longifolia

Twenty-four quassinoids isolated from Eurycoma longifolia Jack were investigated for their cytotoxicity against a panel of four different cancer cell lines, which includes three murine cell lines [colon 26-L5 carcinoma (colon 26-L5), B16-BL6 melanoma (B16-BL6), Lewis lung carcinoma (LLC)] and a human lung A549 adenocarcinoma (A549) cell line. Among the tested compounds, eurycomalactone (9) displayed the most potent activity against all the tested cell lines; colon 26-L5 (IC50 = 0.70 microM), B16-BL6 (IC50 = 0.59 microM), LLC (IC50 = 0.78 microM), and A549 (IC50 = 0.73 microM). These activities were comparable to clinically used anticancer agent doxorubicin (colon 26-L5, IC50 = 0.76 microM; B16-BL6, IC50 = 0.86 microM; LLC, IC50 = 0.80 microM; A549, IC50 = 0.66 microM).[43]

Coptidis rhizoma

Coptidis Rhizoma (*Huanglian*) and its major component, berberine, have drawn extensive attention toward their antineoplastic effects in the recent years. The antineoplastic effects are related to the Chinese Medicine (CM) properties of Huangliang in treating diseases by removing *damp-heat* and purging *fire* and counteracting toxicity.[44]

Ximenia americana

The antineoplastic activity of a plant powder used in African traditional medicine for treating cancer was investigated by analyzing the activity of various extracts *in vitro*. The most active, aqueous extract was subsequently subjected to a detailed investigation in a panel of 17 tumor cell lines, showing an average IC_{50} of 49 mg raw powder/ml medium. The sensitivity of the cell lines varied by two orders of magnitude, from 1.7 mg/ml in MCF7 breast cancer cells to 170 mg/ml in AR230 chronic-myeloid leukemia cells.[45]

Eruca sativa

Erucin (ER) is a dietary isothiocyanate present in cruciferous vegetables, such as rocket salads (*Eruca sativa* Mill., *Diplotaxis* sp.), that has been recently considered a promising cancer chemopreventive phytochemical. Biological activity of ER was investigated on human lung adenocarcinoma A549 cells, analyzing its effects on molecular pathways involved in apoptosis and cell cycle arrest, such as PARP-1 cleavage, p53 and p21 protein expression.[46]

Podophyllum hexandrum

Polymer-linked (podophyllotoxin) conjugates have been designed to improve the therapeutic efficacy of PT. A new PT-conjugate, 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalimido-acetamidoglycinylglycine podophyllotoxin ester (ETPA-gly-gly-PT), was synthesized by covalently coupling its hydroxyl group onto the phthalimido monomer through a glycine-glycine-glycine spacer. Its homo- and copolymer with acrylic acid (AA) were prepared by photopolymerization using 2,2-dimethoxy-2-phenylacetophenone (DMP) as a photoinitiator. The *in vitro* antitumor activity of these conjugates and polymers were determined and used to evaluate the potential applications in antitumor drugs. The IC_{50} values indicated that the synthesized ETPA-gly-gly-PT and its polymers against cancer cells were much better inhibitors than PT.[47]

Taxus bacata

The taxanes, paclitaxel and docetaxel are microtubule-stabilizing agents that function primarily by interfering with spindle microtubule dynamics causing cell cycle arrest and apoptosis. However, the mechanisms underlying their action have yet to be fully elucidated. These agents have become widely recognized as active chemotherapeutic agents in the treatment of metastatic breast cancer and early-stage breast cancer with benefits gained in terms of overall survival (OS) and disease-free survival (DFS). overexpression of the drug efflux pump MDR-1/P-gp, altered expression of microtubule-associated proteins (MAPs) including tau, stathmin and MAP4 may help to identify those patients who are most at risk of recurrence and those patients most likely to benefit from taxane treatment.[48]

Cedrus deodara

AP9-cd, a standardized lignan composition from *Cedrus deodara* consisting of (−)-wikstromal, (−)-matairesinol, and dibenzyl butyrolactol, showed cytotoxicity in several human cancer cell lines reported earlier. An attempt was made in this study to investigate the mechanism of cell death in human leukemia Molt-4 and HL-60 cells. It inhibited Molt-4 cell proliferation with 48-h IC_{50} of ~15 μg/ml, increased sub-G0 cell fraction with no mitotic block, produced apoptotic bodies and induced DNA ladder formation.[49]

Podophyllum hexandrum

It has been hypothesized that cancer stem cell is responsible for the refractoriness of glioblastoma therapy. This study is to observe the influence of Etoposide on anti-apoptotic and multidrug resistance-associated protein genes in glioblastoma stem-like cells. U251 glioblastoma cells were cultured and CD133 positive cancer stem-like cells were isolated and identified. After Etoposide intervention glioblastoma stem-like cells showed a stronger resistance to apoptosis and death, and the anti-apoptotic gene livinβ was more related with the high survival rate and MRP1 appeared to be more related with transporting chemotherapeutics out of glioblastoma stem-like cells.[50]

Scutellaria baicalensis

Traditional Chinese medicines have been recently recognized as a new source of anticancer drugs and new chemotherapy adjuvant to enhance the efficacy of chemotherapy and to ameliorate the side effects of cancer chemotherapies however their healing mechanisms are still largely unknown. *Scutellaria baicalensis* is one of the most popular and multi-purpose herb used in China traditionally for treatment of inflammation, hypertension, cardiovascular diseases, and bacterial and viral infections. Accumulating evidence demonstrate that *Scutellaria* also possesses potent anticancer activities. The bioactive components of *Scutellaria* have been confirmed to be flavones. The major constituents of *Scutellaria baicalensis* are Wogonin, Baicalein and Baicalin. These phytochemicals are not only cytostatic but also cytotoxic to various human tumor cell lines *in vitro* and inhibit tumor growth *in vivo*. The antitumor functions of these flavones are largely due to their abilities to scavenge oxidative radicals, to attenuate NF-κB activity, to inhibit several genes important for regulation of the cell cycle, to suppress COX-2 gene expression and to prevent viral infections.[51]

Salybum marianum

Natural compounds play a key role in the cancer prevention and treatment. Among them stilbene-based compounds are widely distributed in nature and show a wide range of biological activities. Certain poly-hydroxylated *cis*-stilbenes and their analogues bind in the colchicin site of tubulin and inhibit cancer cell proliferation. Up-to-now the most promising of these compounds is combretastatin A-4 (*cis*-3,4,4',5tetramethoxy-3'-hydroxy stilbene) which has been shown to cause mitotic arrest in a variety of cancer cell lines, including multi-drug-resistant ones, and has also demonstrated cancer antiangiogenetic properties.[52]

Panax ginseng

Our previous studies demonstrated that KG-135, a quality-controlled red ginseng-specific formulation containing approximately equal amounts of three major ginsenosides (Rk1, Rg3 and Rg5), down-regulated G1 cyclin-dependent kinase in HeLa cells. In the present work, we have found that KG-135 potentates cytotoxicity of etoposide by modulating apoptotic signaling. Co-treatment of etoposide and KG-135 markedly elevated the expression and phosphorylation at the serine 15 residue of p53 as well as the cellular levels of Bax and $p21^{Waf1/Cip1}$. The increased accumulation and phosphorylation of p53 (Ser15) were attenuated by treatment of cells with wortmannin, a pan-phosphatidylinositol-3 kinase inhibitor.[53]

Cynara cardunculus

Sixteen edible plants from Southern Italy were evaluated for their *in vitro* antiproliferative properties, using the sulforodamine B (SRB) assay, on four human cancer cell lines: breast cancer MCF-7, prostate cancer LNCaP, amelanotic melanoma C32 and renal adenocarcinoma ACHN. After 48 h of incubation the most antiproliferative plant extract was *Cynara cardunculus* ssp. *cardunculus* on C32 and ACHN cell lines with IC_{50} of 21 and 18 µg/ml, respectively. *Mentha aquatica* showed a selective antiproliferative activity on breast cancer while significant activity was exerted by *Cichorium intybus* on melanoma. These species contained the highest amount of phenolics.[54]

Radix sophorae

The Chinese herbal medicine *Radix Sophorae* is widely applied as an anti-carcinogenic/anti-metastatic agent against liver cancer. In this study, Leachianone A, isolated from *Radix Sophorae*, possessed a profound cytotoxic activity against human hepatoma cell line HepG2 *in vitro*, with an IC_{50} value of 3.4 µg/ml post-48-h treatment. Its action mechanism via induction of apoptosis involved both extrinsic and intrinsic pathways. Its anti-tumor effect was further demonstrated *in vivo* by 17-54% reduction of tumor size in HepG2-bearing nude mice, in which no toxicity to the heart and liver tissues was observed. In conclusion, this is the first report describing the isolation of Leachianone A from *Radix Sophorae* and the molecular mechanism of its anti-proliferative effect on HepG2 cells.[55]

Sophora flavescens

The objective of this study was to investigate the anti-tumor activity of a lectin from *Sophora flavescens* and explore its potential apoptotic induction mechanism. Here, an elegant series of biochemical and cell biology methods were carried out in a sequential procedure (e.g., MTT, cell morphologic changes and LDH assays, DNA ladder as well as flow cytometric assay). As a result, we found that this lectin shows a strong cytotoxicity against HeLa cells and induces apoptosis in a time- and dose-dependent manner. Subsequently, according to caspase inhibition and Western blot analysis, we further demonstrated that it is a typical caspase-dependent apoptotic mechanism.[56]

Allium sativum

The reputation of garlic (*Allium sativum*) as an effective remedy for tumours extends back to the Egyptian Codex Ebers of 1550 B.C. Several garlic compounds including allicin and its corresponding sulfide inhibit the proliferation and induce apoptosis of several human non-leukaemia malignant cells including breast, bladder, colorectal, hepatic, prostate cancer, lymphoma and skin tumour cell lines. Ajoene (4,5,9-trithiadodeca-1,6,11-triene-9-oxide) is a garlic-derived compound produced most efficiently from pure allicin and has the advantage of a greater chemical stability than allicin. Ajoene was shown to inhibit proliferation and induce apoptosis of several human leukaemia CD34-negative cells

including HL-60, U937, HEL and OCIM-1. Also, ajoene induces 30% apoptosis in myeloblasts from chronic myeloid leukaemia patient in blast crisis. More significantly, ajoene profoundly enhanced the apoptotic effect of the two chemotherapeutic drugs: cytarabine and fludarabine in human CD34-positive resistant myeloid leukaemia cells through enhancing their bcl-2 inhibitory and caspase-3 activation activities.[57]

Solanum lycopersicum

Epidemiologic studies suggested a protective effect of tomatoes against prostate cancer brought by lycopene, a carotenoid conferring the red colour of tomatoes. However, intervention studies on patients have shown that the preventive effect of tomato was more potent than that of lycopene. The aim of this study was to compare the effects of red tomato, yellow tomato (devoid of lycopene) and lycopene on Connexin43 (Cx43) expression, a protein regulating cell growth, on a prostate cancer cell line expressing the androgen receptor.[58]

Leucaena leucocephala: This work aimed to prove that simple chemical modification could provide new cancer chemopreventive and/or anticancer properties to the inactive extracted polysaccharide derived from Leucaena leucocephala. Unmodified crude extract was neither active as cancer chemopreventive nor as anti-proliferative. In conclusion, chemical modification of Leucaena gum induced its cancer chemopreventive and anti-proliferative activities.[59]

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Cedrus deodara (Roxb.) Loud.: A Review on its Ethnobotany, Phytochemical and Pharmacological Profile

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ABSTRACT

Cedrus deodara (Roxb.) Loud. commonly called as deodar, is a species of cedar native to the Western Himalayas in Eastern Afghanistan, Northern Pakistan, North-Central India, South Western Tibet and Western Nepal. The chemical constituents obtained from different parts of plant include wikstromal, matairesinol, dibenzylbutyrolactol, berating, isopimpillin, lignans 1, 4 diaryl butane, benzofuranoid neo lingam, isohemacholone, sesquiterpenes LIII: deodarone, atlantone, deodarin, deodardione, limonenecarboxylic acid, α-himacholone, β-himacholone, α-pinene, β-pinene, myrcene, cedrin (6-methyldihydromyricetin), taxifolin, cedeodarin (6-methyltaxifolin), dihydromyricetin and cedrinoside. Various parts of this plant are used in traditional system of medicine for the treatment of different ailments such as fever, inflammation, pain, ulcer, apoptosis, spasmodic, hyperglycemia, infections, insomnia, disorder of mind, disease of skin and blood. Recent *in-vivo* and *in-vitro* studies have indicated its anti-inflammatory, analgesic, anti-hyperglycemia, anti-spasmodic, insecticidal, anti-apoptotic, anti-cancer, immmunomodulatory, molluscidal, anxiolytic and anticonvulsant properties. Exhaustive literature survey reveals that there are some activities which are still not validated scientifically. The current review compiles and presents an up-to-date comprehensive review of the traditional and folklore medicinal uses, phytochemistry and biological activities of *Cedrus deodara* plant.

Keywords: *Cedrus deodara*, Ethnomedicinal, Phytochemical, Traditional uses.

INTRODUCTION

Various drugs have entered into the international market through exploration of ethnopharmacology and traditional medicine. Although scientific studies are carried out on a large number of plants but smaller numbers of phytochemical entities have entered the evidence-based therapeutics. Efforts are therefore needed to establish and validate evidence regarding practice of Ayurvedic medicines. *Cedrus deodara* (Roxb.) Loud. commonly called as deodar, is a species of cedar native to the Western Himalayas in Eastern Afghanistan, Northern Pakistan, North-Central India, South Western Tibet and Western Nepal. It is a majestic and handsome tree, growing to a great height and wide girth and living to a great age. It is a pyramidal shape tree having soft grayish-green (or blue) needles and drooping branches, growing rapidly to 40-50 feet tall and 20-30 feet wide. It is an oldest tree of 745 years with 900 rings as described by Bhattacharyya *et al* in 1988.[1,2]

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Classification

Kingdom – Plantae, Subkingdom – Trachibionta, Division – Conoferophyta, Class – Dinopsida, Order – Pinales, Family – Pinaceae, Genus – Cedrus, Species – Deodara.[3]

Morphological characteristics

Cedrus deodara is a large evergreen tree often reaching 60 meter in height (Figure 1A). Branches and branchlets are horizontal, tips slender and nodding. The leaves are 2.5 to 5 cm. long, acicular glaucous green, mostly in dense fasciles with few solitary scattered between fassicles, leaf is niddle like as shown in Figure 1B.[3] Bark is grayish or radish brown with vertical and diagonal fissures. It is monocious plant, although male and female cones appear

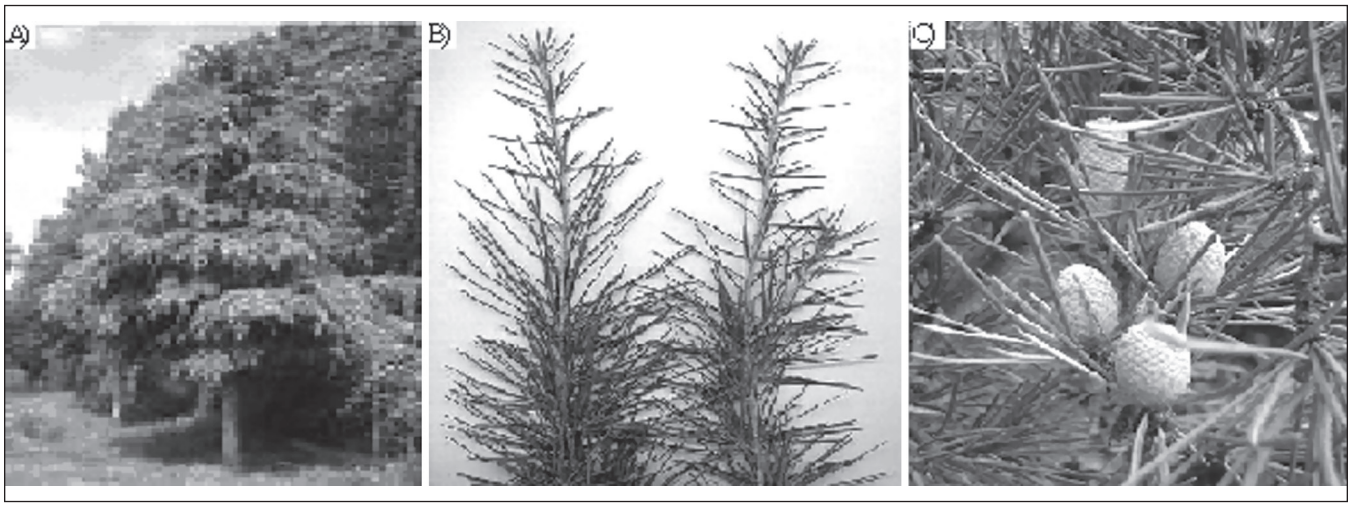

Figure 1: *Cedrus deodara*: (A) Tree (B) Needles (C) Cone

on separate branches. Female cones are barrel-shaped and borne singly at the tip of the dwarf shoots (Figure 1C). They are cylindrical and 2.5 to 4.5 cm. in length. Fruit shape is oval, 3 to 6 inches in length, brown in color and covering is dry or hard. Flowers are bisexual and appear in the month of the autumn.[4]

PHYTOCHEMICAL CONSTITUENTS

Cedrus deodara has been explored phytochemically by various researchers and found to possess number of chemical constituents of wide range of structures as illustrated in figure 2. The main chemicals in wood of *Cedrus deodara* include: wikstromal, matairesinol, dibenzylbutyrolactol,[5,6] 1, 4 diaryl butane, benzofuranoid neo lingam,[7] cedrin (6-methyldihydromyricetin), taxifolin, cedeodarin (6-methyltaxifolin), dihydromyricetin, cedrinoside,[8] deodardione, diosphenol, limonenecarboxylic acid,[9] (–)-matairesinol, (–)-nortrachelogenin, and a dibenzylbutyrolactollignan (4, 4', 9-trihydroxy-3, 3'-dimethoxy-9, 9'-epoxylignan).[10] A new dihydroflavonol named deodarin (3, 4, 5, 6-tetrahydroxy-8-methyl dihydroflavonol) has been isolated from the stem bark.[11] The ethanolic extract of pine needles of *Cedrus deodara* showed presence of many compounds viz. 10-nonacosanol, dibutyl phthalate, protocatechuic acid, phthalic acid bis-(2-ethylhexyl) ester, (E)-1-O-p-coumaroyl-beta-D-glucopyranoside and 5-p-trans-coumaroylguinic acid, 9-hydroxy-dodecanoic acid, ethyl laurate, ethyl stearate, 3-beta-hydroxy-oleanolic acid methyl ester, beta-sitosterol, shikimic acid, methylconiferin and ferulic acid beta-glucoside.[12,13] The essential oil from wood was reported to contain a sesquiterpenes-L II: isohemacholone and sesquiterpenes L III: deodarone, atlantone,[14] α-himacholone, β-himacholone,[15,16] ά-pinene, β-pinene, myrcene,[17] himachalene, cis-atlantone, ά-atlantone.[18]

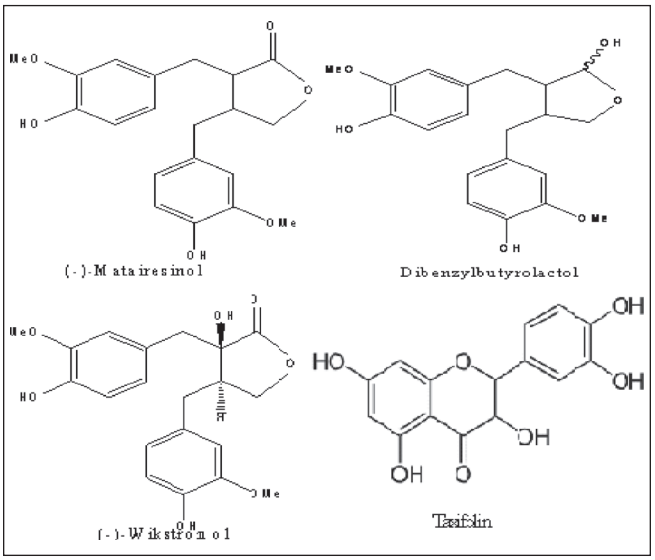

Figure 2: Chemical structure of some phytoconstituents isolated from *Cedrus deodara*

ETHNOPHARMACOLOGICAL USES

Cedrus deodara oil and gum have medicinal value and are used in treatment of inflammations, dyspepsia, insomnia, hiccough, fever, urinary discharge, ozoena, bronchitis, itching, elephantitiasis, tuberculous glands, leucoderma, ophthalmia, piles, disorder of mind, disease of skin and blood. Leaves are used in treatment of inflammation and applied to tuberculous glands. Wood is bitter and is used as diuretic, diaphoretic, carminative. It finds wild application in treatment of fever, rheumstism, piles, palsy, epilepsy, prolapsus recti, skin disease, pulmonary and urinary disorder. All parts of *Cedrus deodara* are useful in Ayurveda system of medicine for the treatment of insomnia, disorder of

mind, disease of skin and blood.[19] Oil is used as analgesic, alexipharmic, diaphoretic and is used in treatment of bruises, injuries to joint, boils, tuberculous glands, skin diseases and for ulcers. Bark is astringent and is useful for fever, diarrhea and dysentery. Turpentine oil is used for treatment of ulcer, skin diseases and leprosy.[4] *Cedrus deodara* used in preparation of V-gel, which is commonly used as antiseptic.[20]

PHARMACOLOGICAL ACTIVITY

Several workers have reported various biochemical activities of *Cedrus deodara* in various *in-vivo* and *in-vitro* test models. Different parts of this plant have been found to exhibit anti-inflammatory, analgesic, immunomodulatory, anti-spasmodic, anti-hyperglycemic, anti-cancer, molluscidal, insecticidal, anti-apoptotic, anti-bacterial, antisarcoptic, anxiolytic and anticonvulsant activities.

Anti-inflammatory activity

The volatile oil extract (50 and 100 mg/kg body weight) of wood of *Cedrus deodara* was evaluated for its oral anti-inflammatory activity by carragenan induced rat paw edema method. Diclofenac sodium (10 mg/kg) served as reference control for the study. The extract showed significant inhibition of carrageenan induced rat paw edema.[21] In another study, volatile oil extract (50 and 100 mg/kg body weight) was examined for its anti-inflammatory activity by adjuvant induced arthritis method. The extract showed significant inhibition of both exudative-proliferative and chronic phase of inflammation.[22]

Analgesic activity

The wood oil (50 and 100 mg/kg body weight) of *Cedrus deodara* was evaluated for its analgesic potentiality by acetic acid induced writhing response and hot plate reaction time model in mice. Aspirin (25 mg/kg p.o.) and morphine (1 mg/kg, s.c.) served as reference control for the study, respectively. *Cedrus deodara* wood oil showed significant analgesic activity at both the dose levels, relative to the control group in both the pain models.[23]

Immunomodulatory activity

Immunomodulatory potential of volatile oil of wood of *Cedrus deodara* was evaluated by using several pharmacological models like neutrophill adhesion test in rats,[24] Athrus reaction in mice,[25] SRBC- induced delay type hypersentivity (DTH) and hemagglutination antibody titer in mice[26,27] and oxazolone-induced contact hypersensitivity in mice.[28] *Cedrus deodara* wood oil at doses of 50 and 100 mg/kg significantly inhibited the adhesion of neutrophils to nylon fibers which simulate the process of margination of cells in blood vessels. This indicates that the *Cedrus deodara* wood oil reduces the number of neutrophils, thus decreasing their phagocytosis action and the release of various enzymes and mediators that make inflammation worse.[27] At the same doses, *Cedrus deodara* wood oil significantly inhibited the Arthrus rection due to its inhibitory effects on any of the following steps which characterize the following reaction: formation of antibodies against antigenic MBSA, formation and precipitation of an immune complex at the site of injection, activation of complement system, neutrophil aggregation, and release of lysosomal enzymes.[29] Mast cell degranulation has been reported to be an early event in contact hypersensitivity reaction to oxazolone.[30] Inhibition of edema due to oxazolone-induced contact hypersensitivity might also be due to mast cell stabilization. These result shows that *Cedrus deodara* wood oil produces an inhibitory effect on humoral and cell-mediated immune response in experimental animals justified its therapeutic usefulness in inflammatory disorder.

Anti-spasmodic activity

The wood of *Cedrus deodara* has himachalol identified as the major antispasmodic constituent. The pharmacological studies of himachalol on various isolated smooth muscles (guinea pig ileum, rabbit jejunam, rat uterus and guinea pig seminal vesicles) and against different agonist (acetyl choline, histamine, serotonin, nicotine and barium chloride) indicated spasmolytic activity similar to that of papavarine. In the conscious immobilize cat, iritragastric administration of himachalol or papavarine (100 mg/kg) produced equal inhibition of carbachol and induced spasm of intestine, lasting about 2 h, but himachalol had a faster on set of action. Himachalol devoid of spasmolytic on the bronchial musculature of guinea pig but was 3.3 times more potent than papaverine in antagonizing epinephrine-induced contraction of the guinea pig seminal vesicles. Intravenous injection of himachalol (3-10 mg/kg) in the cat produced dose dependent fall in blood pressure and an increased femoral blood flow.[16]

Anti-hyperglycemic activity

The ethanolic extract of wood of *Cedrus deodara* exhibited antihyperglycemic activity on streptozotocin-induced diabetic rats from 1 to 7 h. The maximum lowering in blood pressure was found to be at 7 h. post treatment.[31] *Cedrus deodara* shows 6% significant fall in blood glucose profile in a single dose experiment on streptozotocin-induced diabetic rats.[32,33,34] Shivanand *et al* in 2009 formulated and evaluated an antihyperglycemic preparation obtained from the ethanolic extract of *Cedrus deodara*.[35]

Anti-cancer activity

"CD lignan mixture" viz. (–)-wikstromal (75-79%), (–)-matairesinol (9-13%) and benzy1butyrolactol (7-11%) isolated from stem wood of *Cedrus deodara* were evaluated for their in-vitro cytotoxcity against human cancer cell lines

and *in-vivo* anti-cancer activity using Ehrlich ascites carcinoma and colon carcinoma (CA-51) models in mice. *In-vitro* studies showed significant dose dependent effects against several cancer cell lines from different tissues such as breast, cervix, neuroblastoma, colon, liver and prostrate at 10, 30 and 100 μg/ml. The results obtained also revealed IC_{50} values ranging from 16.4 ng/ml to 116.0 μg/ml depending on the cell lines. Comparative data of IC_{50} values of CD lignan mixture showed a synergistic effect in comparison to the individual molecules, i.e., (–)-matairesinol, (–)-wikstromol present in CD lignan mixture. The tumor regression observed with Ehrlich ascites carcinoma and CA-51 was 53% and similar to 54%, respectively, when CD lignan mixture was given at 300 mg/kg, i.p. for nine days in the Ehrlich ascites carcinoma model and 400 mg/kg, i.p. for the same period in the CA-51 model. It was comparable with 5-fluorouracil at 22 mg/kg and 20 mg/kg, respectively. CD lignan mixture at 10, 30 and 100 pg/mL increased the percentage of annexin V positive HL-60 cell is to 1.9 17.18% as compared to control (1.04%). In K562 cells CD lignan mixture at 10, 30 or 100 pg/ml and staurosporine (1 μM) showed 9.13%, 11.38%, 17.22% and 28.07% intracellular caspases activation, respectively. It indicates that CD lignan mixture has cytotoxic potential against human cancer cell lines. It has the ability to induce tumor regression *in-vivo*.[36]

Molluscidal activity

Cedrus deodara wood essential oil was tested for its ovicidal and molluscicidal activities against *L. a. rufescens*. In the laboratory, the 8% suspension of wood oil showed 100% ovicidal and molluscicidal at 15 and 20 ppm, respectively, at 24 h exposure time. In field trials, the solution sprayed to a final concentration of 30 ppm in small pools of water with naturally occurring fauna and flora and large numbers of *L. a. rufescens* and other species of aquatic snails (*Indoplanorbis exustus, Gyraulus convexiusculus* and *Vivipara* sp.) showed 100% mortality of snails and their eggs within 24 h whereas freshwater fishes (*Channa* sp. and *Heteropneustes* sp.), dipterous naiads, leeches and some microfauna was found to be insignificant.[37] In a further study the mixture of cedar and neem oils was most toxic to *Lymnaea acuminata* of the combinations tested.[38,39] It was also observed that *L. inermis* seed powder in combination with *Cedrus deodara* oil and *Azadirachta indica* oil was more toxic than their individual components and other combinations.[40]

Insecticidal activity

Chromatographic fractions of Himalayan cedar wood oil were bioassayed against the pulse beetle (*Callosobruchus analis* F.) and the housefly (*Musca domestica* L.). Almost all fractions showed insecticidal activity against both test species. Fractions I and V led to the highest mortality and also produced a quick knockdown effect. Fractions I and V, after rechromatography and purification, yielded himachalol (3%) and β-himachalene (31%), based on essential oil weight, respectively. Further evaluation of these two naturally occurring sesquiterpenes indicated 97.5% mortality at 0.56 μ mol/insect against the pulse beetle. These biologically active natural products of plant origin may serve as suitable prototypes for development of commercial insecticides.[41]

Anti-apoptotic activity

AP9-CD, a standardized lignan composition from consisting of (–)-wikstromal (75-79%), (–)-matairesinol (9-13%) and dibenzylbutyrolactol (7-11%) was obtained from wood powder of *Cedrus deodara*. The individual constituents isolated from chloroform extract showed lesser cytotoxicity than AP9-CD when tested against many cancer cell lines.[5,6] Wikstromal is the major ingredient that occurs both in (–) and (+) forms; the (−) form is active against leukemia and HIV while the (+) form is biologically inactive.[42] The AP9-CD has been reported to be cytotoxic to numerous human cancer cell lines at concentration between 10 to 30 μg/ml *in-vitro*.[43] The apoptotic assays using light electron microscopy revealed that this agent induced Molt-4 cell process at varied concentration. The morphological change of intracellular organelles in Molt-4 cell treated with 30 μg/ml of AP9- CD revealed the disruption of mitochondrial cristae. Incubation of Molt-4 cells with different concentration of AP9-CD for 6-24 h resulted in time and dose-dependent toxicity. After 6, 12, and 24 h incubation, the cell viability at concentrations of 10, 30 and 50 μg/ml was reported to be 93.78, 84.36, 76.93, 87.50, 79.86, 68.95 and 73.59, 53.16, 35.93 respectively. The decrease in cell viability was significant at all the concentrations from 6-24 h.[44]

Antibacterial activity

The antibacterial activity of the alcoholic and aqueous extracts of the roots, stems and leaves of *Cedrus deodara* was evaluated against *E. coli* only. The aqueous extract of the leaf was observed to be a better inhibitory agent as compared to the stem and root as revealed by larger zone of inhibition. However the alcoholic extract of leaf showed much stronger inhibitory potentiality as compared to the stem extract. It may be noted that the alcoholic extract of root was devoid of any action against *E. coli*. However one needs to study against other microorganisms before claming it to be an anti-bacterial agent.[45]

Antisarcoptic mange activity

Two commonly used acaricidal drugs in India containing oil *Cedrus deoduru* (OCD) and benzyl benzoate (BB), respectively, were used in 24 lambs (3-6 months) naturally infected with Sarcoptes mites. Drugs were applied locally on affected parts on alternate days and recovery changes in skin lesions were observed regularly at the time of application. Blood samples from each group were

collected and analyzed for total erythrocytes, leukocytes, and haemoglobin concentration every 10 day of Post treatment (PI). The two treated groups responded to the treatment but recovery in the *Cedrus deodara* group (CDG) was faster and lesions were free from mites after 5 applications (tenth day) as compared to the seventh application (fourteenth day) in the benzyl benzoate group (BBG). Erythrocyte and leukocyte counts were significantly different in treated groups as compared to control. Animals treated with OCD had significantly more erythrocyte and leukocyte counts compared to control; however, haemoglobin did not show significant difference. Oil of *Cedrus deodara* was found more efficacious in controlling sarcoptic mange in sheep.[46]

Anxiolytic activity

The alcoholic extract of heart wood (50, 100 and 200 mg/kg body weight) of *Cedrus deodara* was evaluated for its anxiolytic activity by elevated plus maze model, light-dark model and actophotometer. The results suggested that alcoholic extract reduced the aversion fear and produced anxiolytic activity in a dose dependent manner. Estimation of GABA in rat brain after administration of extract showed significant modulation of GABA levels. These findings suggested that the alcoholic extract of heartwood of *Cedrus deodara* possess significant anxiolytic through modulation of GABA levels in brain.[47,48]

Anticonvulsant activity

The alcoholic extract of heart wood (50, 100 and 200 mg/kg body weight) of *Cedrus deodara* was evaluated for its anticonvulsant activity by Pentylenetetrazole (PTZ) induced convulsions and maximal electro shock (MES) induced convulsions models in mice. The results showed that 100 and 200 mg/kg of alcoholic extract increased the onset of clonus and tonic seizures in PTZ induced convulsions model and decreased the duration of tonic extensor phase in MES induced convulsions model and also increased the percentage protection in PTZ and MES induced convulsions. Estimation of GABA in rat brain after administration of extract showed significant modulation of GABA levels. These findings suggest that the alcoholic extract of heartwood of *Cedrus deodara* possess significant anticonvulsant activity through modulation of GABA levels in brain.[47,48]

TOXICOLOGICAL STUDIES

In many study, safety profile of *Cedrus deodara* showed non-toxic, non- irritant to the skin of rabbit and sheep and did not alter blood urea nitrogen and blood glucose levels.[49,50] Perveen *et al*., in 2008 reported the mammalian toxicity of *Cedrus deodara* root oil against Albino rats. They reported 34.4 gm/kg as LD50, which was quite safe as compared to neem oil LD50, 5 gm/kg.[51]

CONCLUSION

Cedrus deodara (Roxb.) Loud. is a well known plant used in the Indian system of medicine, besides which folklore medicine also claims its use in cancer, fever, inflammation, pain, ulcer, apoptosis, spasmodic, hyperglycemia and neurological disorder. It is used in the manufacture of various Ayurvedic preparations for a wide range of ailments. Research carried out using different *in-vivo* and *in-vitro* techniques of biological evaluation support most of these claims. Recent study have focused mainly on its anti-inflammatory, analgesic, immunomodulatory, anti-spasmodic, anti-hyperglycemic, anti-cancer, molluscidal, insecticidal, anti-apoptotic, antisarcoptic, anxiolytic and anticonvulsant activities. Some of the compound present in *C. deodara* (himachalol, wikstromal, matairesinol, dibenzylbutyrolactol) are pharmacologically well known and provide additional supporting evidence for possible mechanism of action. This study is an attempt to compile an up-to-date and comprehensive review of *Cedrus deodara* that covers its distribution, description, traditional and folk medicinal uses, phytochemistry and pharmacology.

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Pharmacognostic and Phytochemical Investigations of *Pseuderanthemum palatiferum* (Nees) Radlk. ex Lindau Leaves

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ABSTRACT

Introduction: Fresh leaves of *Pseuderanthemum palatiferum* (Nees) Radlk. ex Lindau are claimed to cure various diseases and have become a popular medicinal plant among Thai people. Products made from *P. palatiferum* leaves – including powder decoctions, herbal tea bags and capsules – have been developed and are available in markets in Thailand, but without quality control. **Methods:** Microscopic characteristics were observed under a light microscope. Physicochemical properties – including loss on drying, total ash, acid-insoluble ash, and water extractive values – were determined. Phytochemical screening for major groups of compounds was performed, and a thin-layer chromatographic (TLC) fingerprint of this plant was developed. **Results:** The microscopic characteristics showed the arrangement of palisade cells into two layers, and the presence of multicellular trichomes with a warty wall in the upper epidermis. Phytochemical screening revealed that *P. palatiferum* leaf extract contains flavonoids, phenolic compounds, unsaturated lactone rings, and steroid nuclei. Flavonoids, phenol carboxylic acid and terpenoids were detected in TLC fingerprints of *P. palatiferum* extract developed using blends of toluene: ethyl acetate: formic acid (5:4.5:0.5) and ethyl acetate: formic acid: water (90:2:2) as solvent systems. **Conclusion:** The results are useful for the establishment of a monograph on this plant which could be used for quality control of plant materials.

Key words: *Hoan-ngoc*; Pharmacognosy; Phytochemistry; *Pseuderanthemum palatiferum*

INTRODUCTION

Pseuderanthemum palatiferum (Nees) Radlk. ex Lindau belongs to the Acanthaceae family; its vernacular names are *Hoan-ngoc* and *Xuan-hoa* in Vietnam, and *Payawanorn* in Thailand. In recent years, *P. palatiferum* has become popular among Thai people. According to folkloric medicine, its fresh leaves are claimed to cure various diseases including diarrhea, peptic ulcer, hepatitis, nephritis, hypertension and diabetes.[1] It has been reported that the hypoglycemic effects of 80% ethanolic leaf extract of *P. palatiferum* have been observed in streptozotocin-induced diabetic rats. The extract also has shown beneficial effects on lipid profile, renal and liver functions of streptozotocin-induced diabetic rats.[2] Recently, leaf aqueous extracts of *P. palatiferum* have been reported as showing acetylcholinesterase inhibitory activity in rats.[3]

The leaves of *P. palatiferum* have been found to contain phytosterols, flavonoids and saponins. Compounds including *n*-pentacosan-1-ol, β-sitosterol, stigmasterol and their corresponding 3-O-β-glucosides, as well as kaempferol 3-methyl ether 7-O-β-glucoside and apigenin 7-O-β-glucoside, have been isolated from *P. palatiferum* leaves.[4]

In Thailand, *P. palatiferum* products from leaves – including dried powder for decoctions, herbal tea bags, and capsules – have been developed and commercialized without quality control. A monograph on *P. palatiferum* leaves has not yet been completed. Thus, observations regarding the pharmacognostic aspects of this plant – including macroscopic and microscopic characteristics, physicochemical

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properties, and thin-layer chromatographic fingerprints – are necessary to provide important information for controlling the raw material quality of these products.

MATERIALS AND METHODS

Plant materials

Fresh leaves of *Pseuderanthemum palatiferum* (Nees) Radlk. were collected from Hoan Ngoc Garden, Khlong Luang district, Pathum Thani province, Thailand, and were identified by Prof. Dr. Wongsatit Chuakul of the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. Voucher specimens (PBM 04831) were deposited at the department's herbarium.

Pharmacognostic study

Anatomical and histological examinations

The transverse sections and paradermal sections of fresh leaves were cleared by chloral hydrate solution, and then mounted in glycerine-water. The sections were examined under a light microscope.[5] Paradermal sections of the lower epidermis were observed under a camera lucida to obtain the number of stomata and ordinary epidermal cells in the same area. The stomatal index was calculated by the following equation:[6]

$$\text{Stomatal index} = \left[\frac{S}{(E+S)}\right] \times 100$$

where S = number of stomata per unit area, and E = number of epidermal cells per unit area.

Powdered drug examination

The dried materials were ground and passed through a #40 sieve to obtain powder. The leaf powders were prepared and examined by the same procedure as described by the World Health Organization.[5] Palisade ratio, the average number of palisade cells beneath each upper epidermal cell, was observed using a camera lucida. The palisade cells lying beneath a group of four epidermal cells were counted and divided by four to obtain the palisade ratio of the group.[6]

Physicochemical properties

The physicochemical properties – including loss on drying, total ash, acid-insoluble ash, and water extractive values – were evaluated by the standard methods as described in The British Pharmacopoeia (BP).[7] The examination was done in triplicate.

Phytochemical study

The dried powder (70 g) was macerated with 350 ml of 80% ethanol for 1 week. The filtered ethanol extract was used in phytochemical screening and thin-layer chromatography fingerprinting.

Phytochemical screening

The dried powder was observed for the presence of alkaloids, cardiac glycosides, saponins, tannins, flavonoids, anthraquinone glycosides, and coumarins by color or precipitation tests as described by Farnsworth (1966).[8]

Thin-layer chromatographic (TLC) fingerprinting

The ethanol extract was streaked as a 3 mm band on four precoated silica gel aluminum plates 60_{F254}. Two solvent systems were used as mobile phases. Solvent system I consisted of toluene: ethyl acetate: formic acid (5:4.5:0.5), while solvent system II was composed of ethyl acetate: formic acid: water (90:2:2). Two plates were developed using solvent system I, while the others were developed by solvent system II. All plates were examined under short (254 nm) and long (365 nm) wavelength UV light, the chromatograms were recorded. Then two plates from different solvent systems were sprayed with natural product/polyethylene glycol (NP/PEG) spray reagent and observed under long-wave UV light after spraying; whereas the other plates were sprayed with anisaldehyde-sulfuric acid (AS) spray reagent, heated at 105 °C for 5-10 min, and observed under visible light and long-wave UV light.

RESULTS

Pharmacognostic study

Microscopic characteristics

Anatomical and histological characteristics: Transverse sections of *P. palatiferum* leaves show the characteristics of bifacial leaves. Upper epidermis cells are rectangular in shape and covered with a cuticle layer (Figure 1). The mesophyll layers are composed of palisade cells and spongy cells. Palisade cells are arranged in two layers: cells in the upper layer are cylindrical in shape, while cells in the lower layer are almost spherical in shape. The palisade ratio is about 3. Spongy cells are irregular in shape, with intercellular spaces between them. Vascular bundles are scattered in the mesophyll. Stomata and glandular trichomes are found in abundance in the lower epidermis. The average stomatal index is about 17.

Powdered drug: The dried powders of *P. palatiferum* leaves are dark green in color, odorless and tasteless. The diagnostic characteristics are as follows:

- The abundant leaf fragments in surface view are the upper and lower epidermises. Epidermal cells are polygonal in shape (Figure 2A, 2D). There are many lithocyst cells and glandular trichomes scattered on both surfaces (Figure 3A, 3B). Multicellular trichomes with warty walls are found scattered only on the upper epidermis (Figure 3C). The lower epidermis is comprised

of diacytic stomata (Figure 2D, 8F). In paradermal view, the palisade cells are found to be circular in shape and tightly arranged, while spongy mesophyll cells are irregular in shape with intercellular spaces between them (Figure 2B, 2C).

- Some leaf fragments in sectional view show the upper epidermis with glandular and multicellular trichomes (Figure 3D).

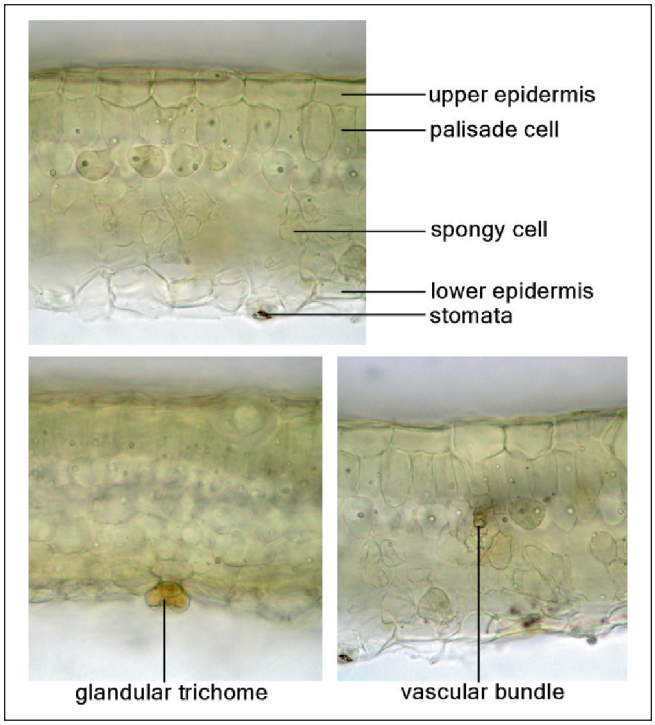

Figure 1: Transverse section of *P. palatiferum* leaf

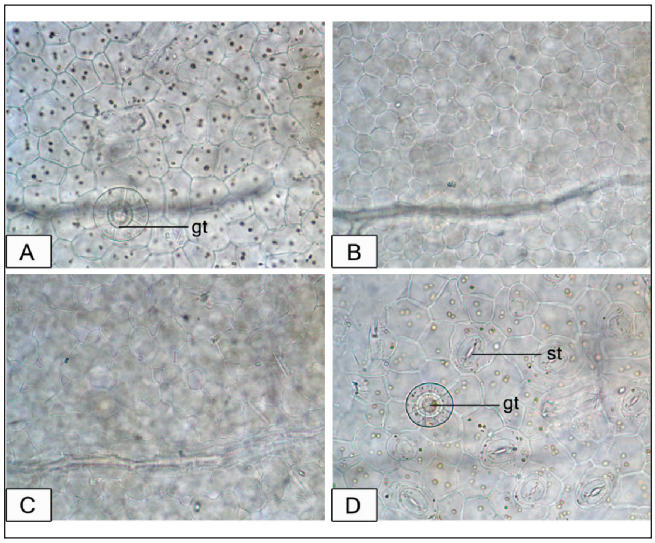

Figure 2: Surface view and paradermal view of leaf fragment: **(A)** surface view of upper epidermis with glandular trichome (gt); **(B)** paradermal view of palisade mesophyll; **(C)** paradermal view of spongy mesophyll; **(D)** surface view of lower epidermis with glandular trichome (gt) and stomata (st)

- Some fragments of the petiole in surface view show rectangular epidermal cells. Lithocyst cells are also found in petiole fragments (Figure 4).

Physicochemical properties

The percentages of loss on drying, total ash, acid-insoluble ash, and water-soluble extractive of *P. palatiferum* leaf powder were as follows. The values are expressed as mean ± S.D.

Loss on drying : 9.45 ± 0.10%
Total ash : 18.14 ± 0.08%

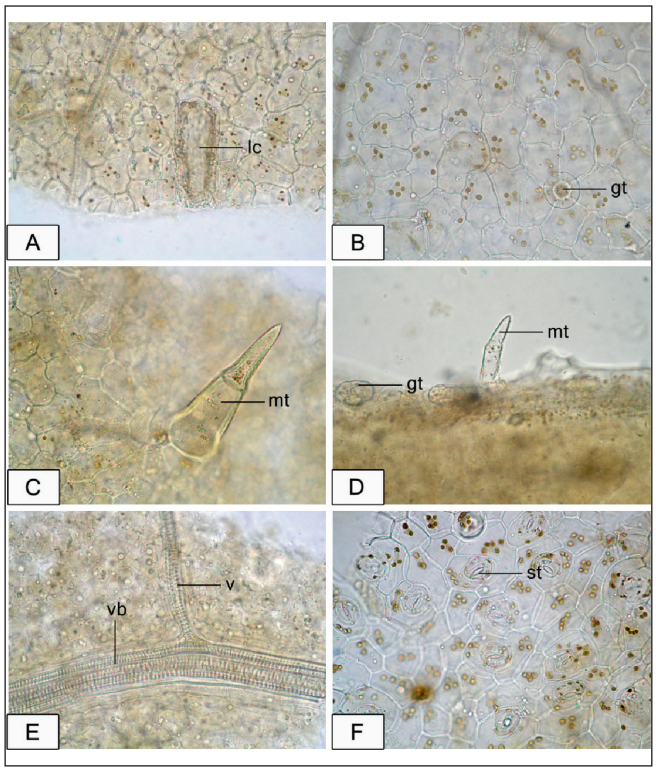

Figure 3: Microscopic characteristics of powdered *P. palatiferum* leaf: **(A)** surface view of upper epidermis with lithocyst cell (lc), and **(B)** with glandular trichome (gt); **(C)** upper epidermis with multicellular trichome (mt) and glandular trichome (gt) in surface view, and **(D)** in sectional view; **(E)** paradermal view of mesophyll with vascular bundle (vb) and veinlet (v); **(F)** surface view of lower epidermis with diacytic stomata (st)

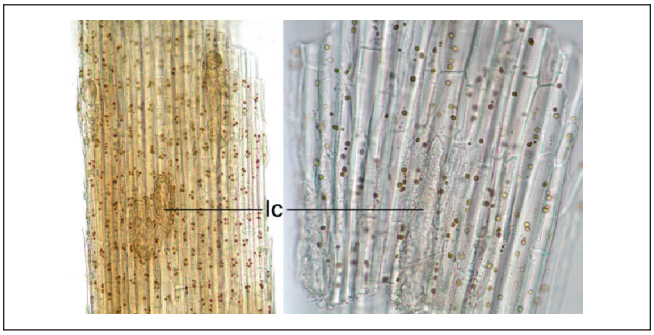

Figure 4: Fragments of epidermis from the petiole in surface view showing lithocyst cells (lc)

Acid-insoluble ash : 6.11 ± 0.25%
Water-soluble extractive : 25.96 ± 1.63%

Phytochemical study

Phytochemical screening

The 80% ethanol extract of *P. palatiferum* leaves was found to contain flavonoids, phenolic compounds, unsaturated lactone rings, and steroid nuclei; while alkaloids, tannins, anthraquinones, coumarins, saponins, and deoxy sugars were not found, as shown in Table 1.

Thin-layer chromatography (TLC) fingerprints

TLC chromatograms, developed using toluene: ethyl acetate: formic acid (5:4.5:0.5) as a solvent system, are shown in Figure 5. After NP/PEG spraying, four blue fluorescence bands (bands 3, 4, 6 and 8; hR_f = 16, 26, 32 and 40) observed under 365 nm were defined as phenol carboxylic compounds. After AS spraying, band 5 (hR_f = 29) gave off a pink color, while bands 2, 8 and 11 (hR_f = 13, 40, and 55) gave off a violet color under visible light. These bands were defined as terpenoids.

TLC chromatograms developed using a solvent system of ethyl acetate: formic acid: water (90:2:2) are shown in Figure 6. After NP/PEG spraying, bands 5, 7 and 8 (hR_f = 58, 67 and 78), which gave of blue fluorescence at 365 nm, were defined as phenol carboxylic compounds. Bands 1, 2 and 3 (hR_f = 17, 22 and 29) gave off yellow, orange and blue-green fluorescence, respectively, under 365 nm UV light. These bands were defined as flavonoids. AS spraying resulted in two visible bands, bands 4 and 6 (hR_f = 37 and 62), which showed violet and pink color, respectively, and were defined as terpenoids.

DISCUSSION

Microscopic examination of fresh leaves and dried powder of *P. palatiferum* revealed some special microscopic characteristics. Palisade cells are arranged into two layers: cells in the upper layer are cylindrical in shape, and those in the lower layer are spherical in shape. Multicellular trichomes with warty walls are present in the upper epidermis. Some characteristics

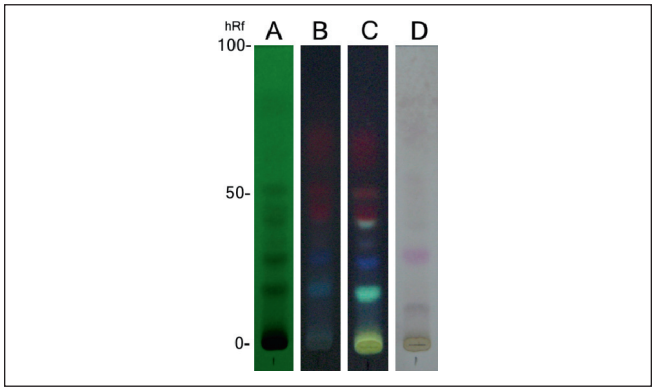

Figure 5: TLC chromatograms of *P. palatiferum* using toluene: ethyl acetate: formic acid (5:4.5:0.5) as a solvent system.
Absorbent: silica gel 60 GF_{254}
Detection: **(A)** 254 nm; **(B)** 365 nm; **(C)** NP/PEG; **(D)** AS

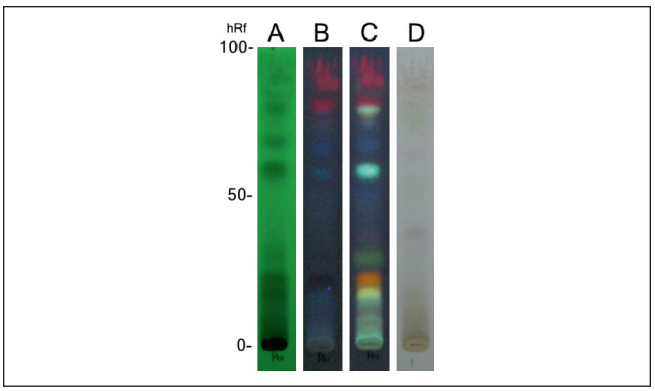

Figure 6: TLC fingerprint of *P. palatiferum* using ethyl acetate: formic acid: water (90:2:2) as a solvent system
Absorbent: silica gel 60 GF_{254}
Detection: **(A)** 254 nm; **(B)** 365 nm; **(C)** NP/PEG; **(D)** AS

Table 1: Groups of compound detected in *P. palatiferum* leaf extract

Screening tests	Results	+/–
Alkaloids	Precipitated with Dragendorff's only	(–)
Flavonoids	Color changed from brown to red-brown	(+)
Tannins & phenolic compounds		
– Ferric chloride	Dark green color present	(+)
– Gelatin solution	Precipitate not present	(–)
– Gelatin-salt solution	Precipitate not present	(–)
Anthraquinones	Pink color not present	(–)
Lactones glycosides		
– Coumarins	Fluorescence was not observed	(–)
– Large molecule lactone	Fluorescence was not observed	(–)
Saponins		
– Froth test	Foam not present	(–)
Cardiac glycosides		
– Steroids nucleus	Green color present	(+)
– Unsaturated lactone ring	Violet color present	(+)
– Deoxy sugar	Violet ring not present	(–)

Table 2: TLC chromatogram of *P. palatiferum* in the solvent system of toluene: ethyl acetate: formic acid (5:4.5:0.5)

No.	hR_f value	UV (nm)		NP/PEG (365 nm UV)	AS (Visible light)
		254	365		
1	10	–	–	–	–
2	13	–	–	–	✓ (violet)
3	16	✓	✓ (blue)	✓ (light blue)	–
4	26	✓	✓ (blue)	✓ (blue)	–
5	29	–	–	–	✓ (pink)
6	32	✓	✓ (blue)	✓ (blue)	–
7	36	–	–	–	–
8	40	✓	✓ (blue)	✓ (light blue)	✓ (violet)
9	44	✓	✓ (red)	✓ (red)	–
10	50	✓	✓ (red)	✓ (red)	–
11	55	–	✓ (red)	✓ (red)	✓ (violet)

✓ = band detected in TLC chromatogram

Table 3: TLC chromatogram of *Pseuderanthemum palatiferum* (Nees) Radlk. ex Lindau in the solvent system of ethyl acetate: formic acid: water (90:2:2)

No.	hR_f value	UV (nm)		NP/PEG (365 nm UV)	AS (Visible light)
		254	365		
1	17	✓	✓ (blue)	✓ (yellow)	–
2	22	✓	✓ (dark)	✓ (orange)	–
3	29	✓	–	✓ (blue green)	–
4	37	–	–	–	✓ (violet)
5	58	✓	✓ (blue)	✓ (light blue)	–
6	62	–	–	–	✓ (pink)
7	67	✓	✓ (blue)	✓ (blue)	–
8	78	✓	✓ (blue)	✓ (light blue)	–
9	81	–	✓ (red)	✓ (red)	–

✓ = band detected in TLC chromatogram

are similar to other species in the Acanthaceae family, such as *Andrographis paniculata* and *Clinacanthus nutans*,[6] including diacytic-type stomata, the presence of glandular trichomes and lithocyst cells in both the upper and lower epidermis, as well as the presence of lithocyst cells in leaf and petiole fragments.

Examination of the physicochemical properties of *P. palatiferum* dried leaf powder revealed that the loss on drying value, which represents the amount of water and volatile matter in the sample, was 9.45% of leaf powder. The total ash value represents the amount of chemical elements remaining after ignition. This includes only physiological ash derived from plant tissue itself, because all of the foreign matter was definitely removed from the sample. Therefore the total ash value could be set as the maximum limitation of total ash value, which is useful for examination of foreign matter such as sand and soil. The 18.14% of total ash value of *P. palatiferum* leaf powder was near the maximum limitation of total ash values of *Andrographis paniculata* and *Clinacanthus nutans* (15% and 21%, respectively).[6] These high values might be due to calcium carbonate residue contained in lithocyst cells which were found in these three species but rarely observed in others. The amount of water-soluble compounds in the leaf powder was expressed by water-soluble extractive value, which was 25.96% of leaf powder.

The phytochemical screenings for the group of major compounds were examined by color and precipitation tests. The results indicated that *P. palatiferum* leaves contained flavonoids, phenolic compounds, unsaturated lactone rings and steroid nuclei. These results correspond with the study by Giang et al. (2003), in which flavonoids were found in ethyl acetate, chloroform and the *n*-butanol fraction, and phytosterols were found in the *n*-hexane fraction of *P. palatiferum* leaves.[4] However, a froth test indicated that saponin was not present in the leaves; but in contrast, Giang et al. (2003) reported the presence of saponins in the *n*-butanol soluble fraction.[4] It might be suggested that the leaves may contain no saponins, or only a small amount which cannot be detected by a froth test.

TLC fingerprints of *P. palatiferum* were developed using two solvent systems – toluene: ethyl acetate: formic acid (5:4.5:0.5), and ethyl acetate: formic acid: water (90:2:2). The detection was performed by spraying with NP/PEG or anisaldehyde-sulfuric acid. With NP/PEG, flavonoids gave off yellow, orange, or blue-green fluorescence, and phenol carboxylic compounds demonstrated blue fluorescence under 365 nm UV light. With anisaldehyde/sulfuric acid spray reagent, terpenoids gave off a pink or violet color under visible light.[9] Phenol carboxylic compounds and terpenoids were detected in TLC chromatograms developed with both solvent systems, while flavonoids were detected only in the solvent system of ethyl acetate: formic acid: water (90:2:2). Therefore, this solvent system was more appropriate for controlling the chemical content of the plant materials.

CONCLUSION

The microscopic characteristics and physicochemical properties of *P. palatiferum* leaves were recorded. The special anatomical and histological characteristics are the arrangement of palisade cells into two layers, cylindrical-shaped cells in the upper layer and almost spherical-shaped cells in the lower layer. In addition, multicellular trichomes with warty walls are present in the upper epidermis. Phytochemical screenings revealed that this plant contains flavonoids, phenolic compounds, unsaturated lactone rings, and steroids nuclei. TLC chromatograms of ethanol extract of *P. palatiferum* leaves, which were developed using two solvent systems – toluene: ethyl acetate: formic acid (5:4.5:0.5), and ethyl acetate: formic acid: water (90:2:2) – revealed that the extract may contain

phenolic compounds, flavonoids and terpenoids, which corresponded with the results of phytochemical screenings.

These results are useful for the establishment of a monograph on this plant which could be used for quality control of plant materials. Nevertheless, these preliminary data were obtained from one sample. To develop a monograph, other samples of *P. palatiferum* should be studied. The determination of contamination by microorganisms, pesticides and heavy metals in plant materials and plant extract must be performed for safety control.

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Pharamcognostic Evaluation of *Acorus calamus* L.

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ABSTRACT

Introduction: *Acorus calamus* L. is a semi aquatic medicinal plant, commonly used in traditional medicinal systems of Asian and European countries. Rhizomes are used as therapeutic agent for various diseases. The present study focuses on pharmacognostic evaluation of the rhizomes. **Methods:** Pharmacognostic evaluation was carried out by organoleptic evaluation, anatomical studies, powder microscopic analysis and ash valve studies. **Results and Conclusions:** Organoleptic studies showed presence of characteristic triangular leaf scars and the anatomical studies showed the presence of aerenchyma and amphivasal vascular bundles. Presence of tannins was also observed

Key words: *Acorus calamus*, Anatomy, Powder Microscopy, Ash value, Phytochemistry, terpenoids,

INTRODUCTION

Acorus calamus L. (commonly called as 'Sweet flag') of family Araceae, is a semi-aquatic, perennial, aromatic herb with creeping rhizomes, sword shaped leaves and spadix inflorescence (Figure 1 a, b). *A. calamus* grows either as wild or cultivated crop throughout India.[1] The rhizomes are used in almost all civilisations of world; Asians have been using the plant for at least 2000 years for many medicinal purposes. Sumerians and Egyptians used calamus as sacred incenses. Aromatic leaves of *A. calamus* were placed on floors of medieval churches and houses as effective air-fresheners and insecticides.

Pharmacological applications of *A. calamus*

A. calamus is used in traditional medical systems of India, Thailand, China and native America for various pharmacological applications such as insomnia, melancholia, neurosis, epilepsy, hysteria, loss of memory, antispasmodic, carminative, antihelmintic, anti-inflammatory, therapeutic against diseases like eczema, rheumatism, diarrhoea, bronchial catarrh, intermittent fevers, abdominal tumours, liver disorders and for diseases of kidney.[2-9] In addition, insecticidal,[10] hypolipidemic,[11] antimicrobial[12] and antidiabetic[13] properties of *A. calamus* rhizomes are reported. Essential oils of *A. calamus* are used in cosmetic and brewing industries.[11]

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Pharamcognostic tools are essential in identifying the medicinal plants. This study covers the entire pharmacognostic study on the rhizomes of *Acorus calamus*.

MATERIALS AND METHODS

Chemicals

Chemicals required for pharmacognostical, phytochemical analysis were purchased from SRL Pvt. Ltd., India. Solvents and acids of Analytical grade were purchased from Rankem Ltd, India. Standards like α asarone, β asarone and eugenol were purchased from Sigma (USA). Filter papers were purchased from Whatman Ltd., UK.

Collection of plant

Rhizomes were collected from *A. calamus* plants grown in loamy red soil (pH 5.5-8) at moderate climate and optimum temperature (25-30 °C) for a period of 6 months at M/s JK Herbal Farm, Veerakanur, Perambalur District, Tamil Nadu, India (11°14' 0" North, 78°53' 0" East). Collected rhizomes were thoroughly washed with tap water once, subsequently with distilled water (4 times) and shade dried (Room temperature 37 °C).

Identification and Pharmacognostical studies of *A. calamus*

Plant was identified on the basis of organoleptic, macroscopic and microscopic (anatomy) observations as summarised below:

Organoleptic analysis

Surface appearance, texture, colour, size and odour of rhizomes were evaluated by physical examinations.

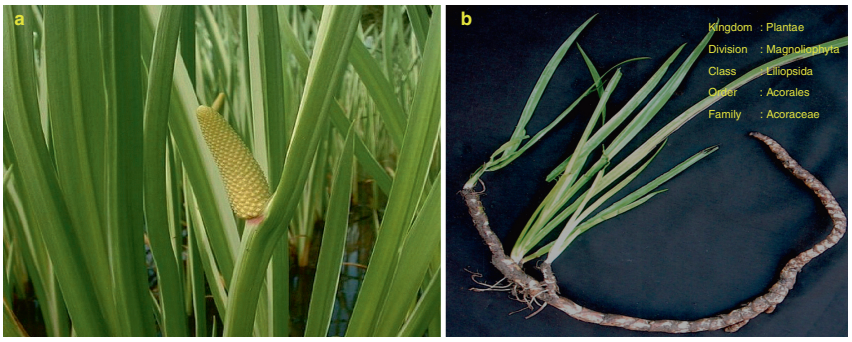

Figure 1: *Acorus calamus* L. a) Habit b) Morphology of rhizome

Anatomy

For anatomical studies, fresh rhizome bits were fixed in FAA (5% Formalin, 5% Acetic acid and 90% of 70% Ethanol) for 24 hours and subsequently dehydrated using series of t-Butyl Alcohol.[14] Fixed samples were embedded in paraffin wax blocks, sectioned using rotary microtome (10 µm thickness), stained with toluidine blue, safranin, fast-green, IKI as per protocols of O'Brien et al,[15] viewed under Nikon Labphoto2 polarized microscope (Nikon Inc, USA). For powder microscopic observations, coarse powders of rhizome were spreaded in a glass slide, viewed in bright field and polarised light microscope (Nikon Labphoto2 polarized microscope, USA).

Deposition of voucher samples

A voucher sample of identified plant was deposited in National Institute of Herbal science-Plant Anatomy Research Centre, Chennai for further authentification.

Physico-Chemical analysis

All physico-chemical analyses were carried as triplicates on three different days.

Moisture content

Moisture content of *A. calamus* rhizomes was estimated by drying one gram of rhizome powder in hot air oven at 105 °C for 1 hour, subsequently cooled in desiccators and the loss in weight was recorded.

Ash valve

Ash value was calculated as per the protocols of Kokate et al[16] and WHO report (1998).[17] To estimate total ash value, four grams of rhizome powder was evenly spreaded in a pre-ignited silica crucible, heated to 600°C in a muffle furnace for a period of 6 hours, eventually the residue was cooled and weighed.

Water-soluble ash content was estimated by adding 25 ml of water to crucible containing total ash, boiled for 5 minutes and filtered through Whatman no.1 filter paper. Remains in the filter paper was transferred to crucible and ignited for 15 minutes. Water-soluble ash content was calculated as: difference in weight between total ash and the residue obtained after treatment.

Acid-insoluble ash was quantified by adding 25 ml of dilute hydrochloric acid to crucible containing total ash, boiled for 5 minutes, subsequently filtered through whatman no.1 filter paper, washed with hot water until solution becomes neutral and the residue was transferred to a crucible, ignited for 30 minutes and weighted.

Energy Dispersive X-ray analysis (EDAX)

Energy Dispersive X-ray analysis was carried out by spreading the ash evenly on aluminium stub using double sided adhesive tape, sputter coated with gold, analysed in a Hitachi VP-SEM S-3400N scanning electron microscope with EDS detector system. Data was analysed qualitatively as well as quantitatively using EDS software.

RESULTS

Humankind knows usage of medicinal plants and their preparations from time immemorial. Herbal medicines are still mainstay for about 75-80% of world population for primary health care, due to better cultural acceptability, compatibility and lesser side effects. Complimentary medicinal systems are now in lime light in developed countries like Germany, France, European Unions, and United States America.[18] Medicinal plants are source of raw materials for both traditional and modern medicine. Rationalisation of new multidrug and multitarget concept of therapy in classical medicine will have great implication on future basic research in phytomedicine and on evidence based phytotherapy.[19]

Quality control in phytomedicine

In general, there are three main factors in quality control of phytomedicine namely: a) identity of the plant, b) purity of the compounds and c) content and processing.

Pharmacognosy

Organoleptic evaluation

Pharmacognostical studies are pivotal in herbal sciences, as it ensures plant identity and prevents adulteration. *Acorus calamus*, a perennial herb grows up to 2 meters with sword shaped leaves and spadix inflorescence (Figure 1a). Figure 1b illustrated the morphology of rhizomes. Rhizomes were brown in colour, tortuous, cylindrical, curved, and shortly nodded. Adventitious fibrous roots and erect aerial shoots were produced along nodes. Upper surface consists of triangular leaf scars, while under surface bears tubercled, irregular, circular root-scars. Rhizomes were highly aromatic with sweet odour and bitter taste.

Anatomy of rhizome

Anatomical studies of *A. calamus* rhizomes illustrate the following characteristic features: Ground plan view (5X) showed the presence of central stele surrounded by narrow cortex and thin epidermis. Epidermal cells were circular or squarish, thin walled and thickly cuticularised (300 µm width) (Figure 2a), followed by aerenchymatous cortex (600 µm width) with shapeless air chambers; Stele wide and circular (Figure 2a). Scattered in the cortical zone there were diffused fibre–strands as well as vascular strands. The vascular strands were 200µm wide with wide, thick walled angular xylem elements, surrounded by phloem and ensheathed by sclerenchymatous cells. The sclerenchyma strands were about 50µm thick with wider, thick walled lignified fibres. The vascular system consists of a peripheral ring of vascular bundles abutting the endodermis and central scattered vascular bundles (Figure 2a1). Vascular strands were amphivasal in nature i.e. Phloem surrounded by a layer of xylem elements (Figure 2c). The central bundles were large, amphivasal with massive phloem tissue surrounded by two or more layers of xylem elements. While the peripheral vascular bundles attached to the endodermis has a central mass of phloem surrounded by a layer of xylem elements. Figure 2b illustrated, polarised light picture of lignified fibre sheath of vascular bundles. Central ground tissue was parenchymatous; the cells were wide, thin walled and compact; no air chambers were evidenced. Scattered in the cortical and central ground parenchyma, the dark masses of cell contents were may be of tannins (Figure 2a1).

Powder microscopy

The images of powder microscopy analysis display the following components; a) Tracheids- were quite long with blunt end and the lateral wall thickenings are scalariform or close helicals; b) Fibres were abundant in the powder and are short, wide in the middle and tapering towards the centre, the walls were thick and lignified and the lumen was wide, the lateral wall pits were not evidenced. With regard to the size, the fibres were 300 -250 µm in length and 20 µm in wide in the middle (Figure 2 d,e,f,g).

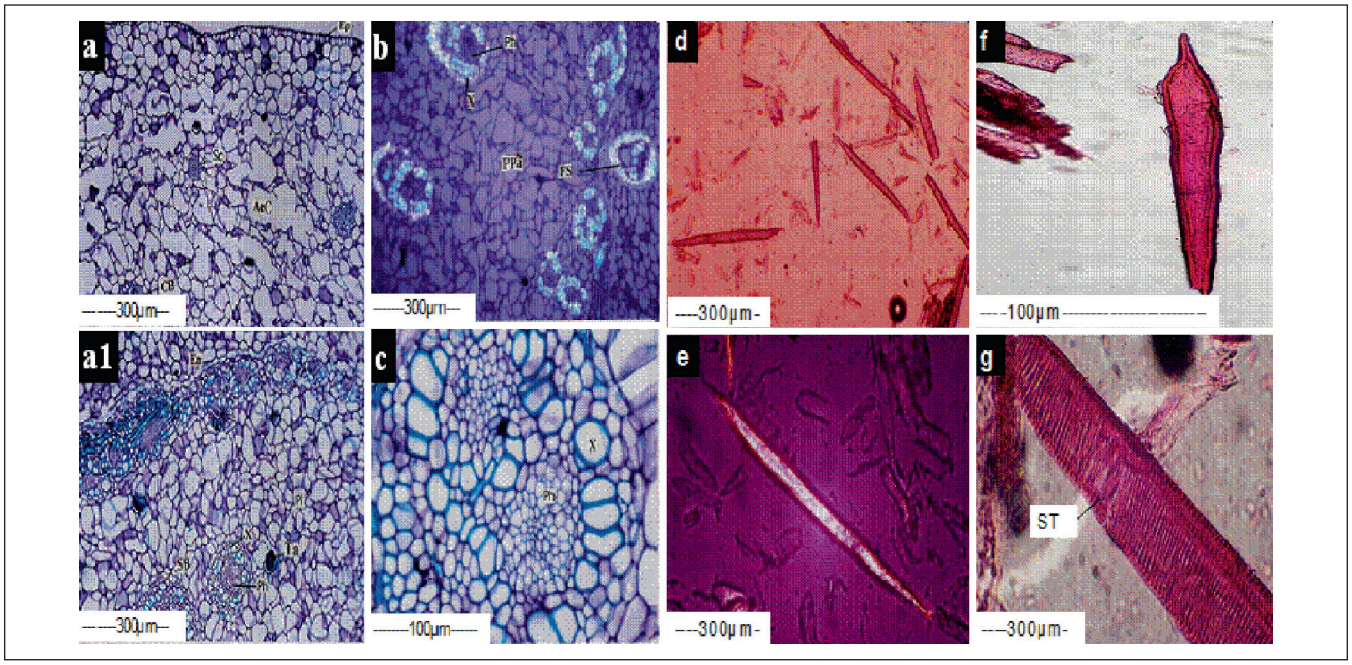

Figure 2: Anatomical studies of *A. calamus* rhizome
a & a1) Transverse section (T.S) of Acorus calamus rhizome (5X magnification);
b) T.S. of Acorus calamus rhizome under polarised light (20X magnification)
c) Entire view of vascular bundles of A. calamus rhizome (40X magnification)
d & e) Powder microscopy of A. calamus rhizome;
f & g) Powder microscopy of A. calamus rhizome- tracheids

Ash value

The total ash valve was 6.12% of which 99% was acid soluble and 66% was water soluble (Table 1). EDAx study revealed the presence of sodium, Magnesium, silicon, phosphorous, sulphur, chlorine, potassium and calcium. Of which, potassium content was more followed by chlorine, calcium, sulphur, phosphorous, sodium, magnesium, and silicon. (Figure3; Table 1)

Results on anatomical studies of rhizomes were well coincided with the descriptions summarized in manuals of *Acorus calamus* L..[20] Based on organoleptic and anatomic features, the plant was identified as *Acorus calamus* and the same was authenticated by Plant Anatomy Research Centre with registration number PARC/2008/203.

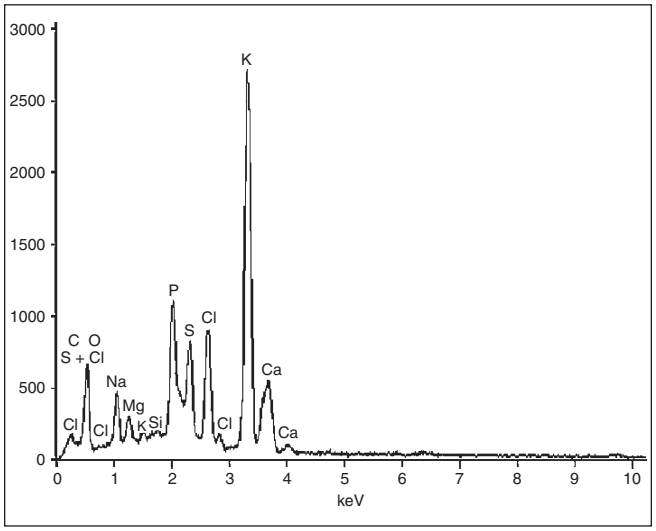

Figure 3: EDAX analysis of *A. calamus* rhizome ash

Table 1: Ash value of rhizomes of *A. calamus* L. and Elemental analysis of Ash

S No		Weight (%)
	Ash Analysis	
1	Total ash value	6.12
2	Water soluble ash	66%
3	Acid soluble ash	99. 09%
4	Acid insoluble ash	0.01%
	Elemental Analysis	
5	Carbon	15.07
6	Oxygen	26.88
7	Sodium	3.36
8	Magnesium	1
9	Silicon	0.27
10	Phosphorous	5.59
11	Sulphur	4.45
12	Chlorine	8
13	Potassium	29.01
14	Calcium	6.25

CONCLUSION

A golden triangle of traditional medicine, modern medicine and science may result in discovery of newer, safer and cost-effective therapies. Globally there is a positive trend towards holistic health, integrative sciences, systems biology approaches in drug discovery and therapeutics. Quality control is pivotal in phytomedicine and quality assurance begins with the identity of the raw material i.e. the plants and plant parts. Thus, pharmacognostical studies were carried out to ensure the identity of the plant. Upon identifying the plant, the purity was monitored for contaminations of heavy metals and pesticides.

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Phytochemical and Pharmacognostic Evaluation of Leaves of *Psidium guajava* L. (Myrtaceae)

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ABSTRACT

Psidium guajava L. known as Guava, is a medicinal plant belonging to the family Myrtaceae. *P. guajava* is a well known traditional medicinal plant used in various indigenous systems of medicine. It is widely distributed throughout India. The present study provides pharmacognostical, physicochemical and phytochemical details of the leaves of the *P. guajava* which are useful in laying down standardization and pharmacopoeial parameters.

Key words: *Psidium guajava* L., pharmacognostic study, phytochemical analysis, physicochemical analysis, solubility, leaves

INTRODUCTION

Psidium guajava L., commonly known as Guava, of the family Myrtaceae, is a native plant of tropical America. Different parts of the plant are used in the indigenous system of medicine for the treatment of various human ailments such as wounds, ulcers, bowels and cholera.[1] *P. guajava* leaves are astringent, anodyne, febrifuge, antispasmodic and tonic. The roots are astringent, haemostatic, constipating, antiemetic, and used in haemorrhages, diarrhea, dysentery, ulcers, gingivitis, proctoptisis, vomiting, nephritis, cachexia, vata, epilepsy, odontalgia. The flowers are cooling, laxative, tonic, and used in bronchitis, opthalmodynia, colic and ulemorrhagia. The fruits are sweet, astringent, sour, cooling, aphrodisiac, laxative, tonic, and used in pitta, dipsia, burning sensation, colic, ulemorrhagia, diarrhea, dysentery and debility.[2]

P. guajava is reported to posses antibacterial,[3,4] antistress,[5] antigenotoxic,[6] anti-inflammatory,[7] antihyperglycemic,[8] trypanocidal,[9] anti-rotavirus,[10] cytotoxicity,[7] hepatoprotective,[11] anticestodal,[12] and antioxidant activities.[13,14]

In spite of the numerous medicinal uses attributed to *P. guajava*, there is no pharmacognostical report on the leaf of the plant to determine the anatomical and other physicochemical standards required for quality control of the crude drug. Hence, the present investigation is an attempt to evaluate the morphological and anatomical features, physicochemical constants and qualitative phytochemical analysis of *P. guajava* leaves.

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MATERIAL AND METHODS

Collection and extraction of plant material

The fresh leaves of *Psidium guajava* L. were collected from Jamjodhpur, Gujarat, India in August 2008. The plant was compared with voucher specimen (voucher specimen No. SU/BIO/510/Thakrar) deposited at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The leaves were washed under tap water, air dried, homogenized to fine powder and stored in airtight bottles. For physicochemical investigation, 10 g of dried powder was extracted by sequential method using different solvents with different polarities in Soxhlet apparatus. The solvent was evaporated to dryness and the dried crude extracts were stored in air tight bottle at 4 °C. The Acetone extract was used for the solubility study.

Pharmacognostic studies

Macroscopic characteristics

For morphological observations, fresh leaves (approx. 10-13 cm in length) were used. The macromorphological features of the leaf were observed under magnifying lens.[15]

Microscopic characteristics

Free hand section of leaf was taken and stained by safranine reagent to confirm its lignification. Powder microscopy

was also carried out and their specific diagnostic characters were recorded.[16]

Physicochemical parameters

The physicochemical parameters were like total ash value, loss on drying, water soluble ash, acid insoluble ash, petroleum ether, methanol, acetone and water soluble extractive value, pH value, heavy metal analysis, solubility, etc were determined as per WHO guidelines.[17]

Phytochemical analysis

The qualitative phytochemical tests of crude powder and acetone extract were carried out to identify different phytoconstituents.[18,19]

RESULTS AND DISCUSSION

Macroscopic characteristics

Macroscopically, the leaf was simple in composition, opposite, decussate, oblong or elliptic-oblong, entire, softy hair beneath, prominent below, densely tomentose below, apex acute. The average leaf size was 10 to 13 cm (length) and 2 to 6 cm (width). The fresh leaf was green in color (Figure 1).

Microscopic characteristics

The transverse section of *P. guajava* leaf showed presence of upper and lower epidermis. The epidermis was covered with a single layer of cuticle. The vascular bundle was surrounded by 4-6 layers of cortex. Xylem was lignified, phloem was non-lignified, vascular bundles were arc shaped. The pith was made up of large cells. The anomocytic stomata were present in epidermis. Oil glands were present below the epidermis. Plenty of blunt and pointed unicellular trichomes were present. Prismatic and cluster type of crystals of calcium oxalate were found. Xylem vessels were also found in transverse section of leaf (Figures 2-4).

The salient diagnostic characteristics of leaf were arc shaped vascular bundle, animocytic stomata, xylem vessels, prism and cluster type of calcium oxalate crystals**.** These characters can be used for standardization of drugs and also used for preparation of plant monographs. Similar study is reported in other plants like *Actinodaphne hookeri* Meissn,[20] *Oxystelma esculentum* (L.f.) R.br. Ex Schltes,[21] *Datura fastuosa* Linn,[22] *Manilkara hexandra* (Roxb.) Dubard,[23] *Polyalthia longifolia* var. pendula,[24] *Vitex trifolia* Linn.,[25] and *Citrus paradisi* Var. *Duncan*.[26]

Powder study

The crude powder of *P. guajava* leaf was green in color with characteristic odour and astringent taste. The diagnostic features of powder were prism and cluster types of crystals of calcium oxalate present on surface of epithelial cells. In surface view, fragments of epidermis were embedded with anomocytic stomata. Xylem vessels with spiral thickening were observed (Figure 5).

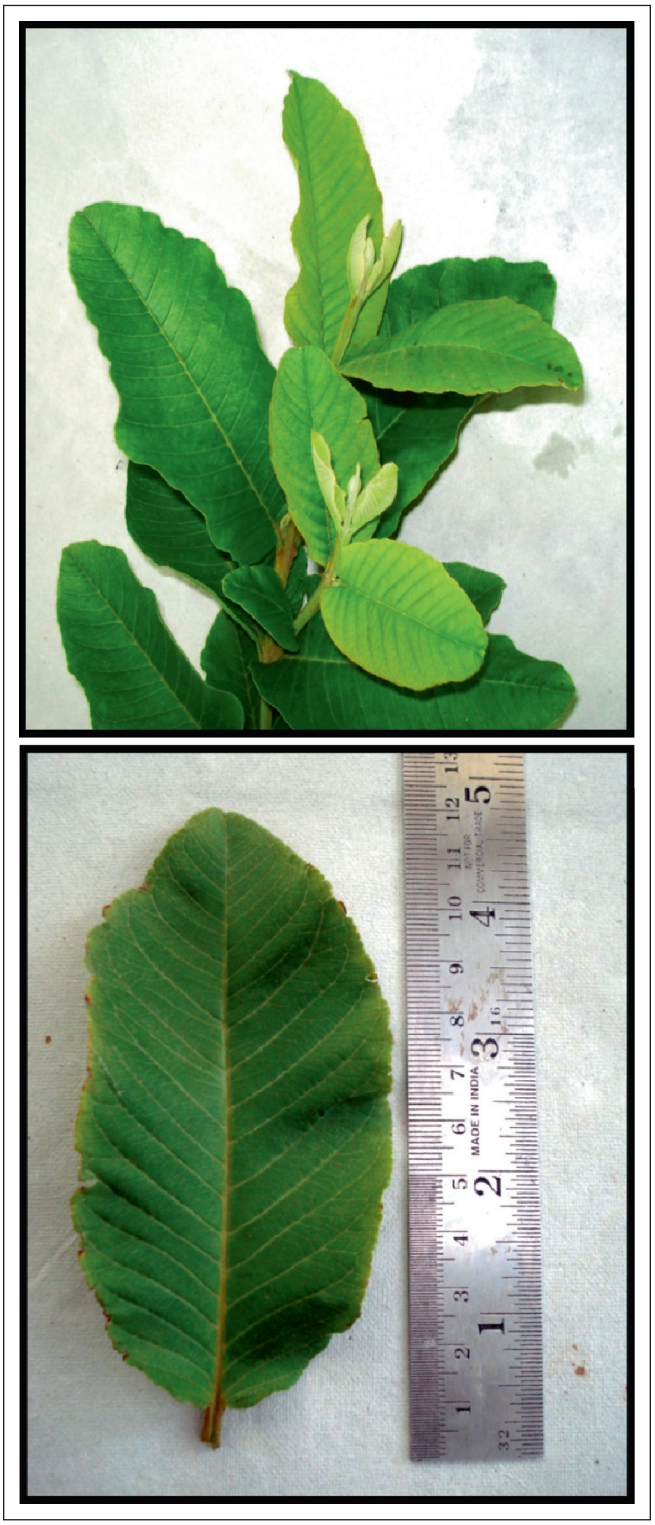

Figure 1: Macroscopic characteristics of *P. guajava* leaf

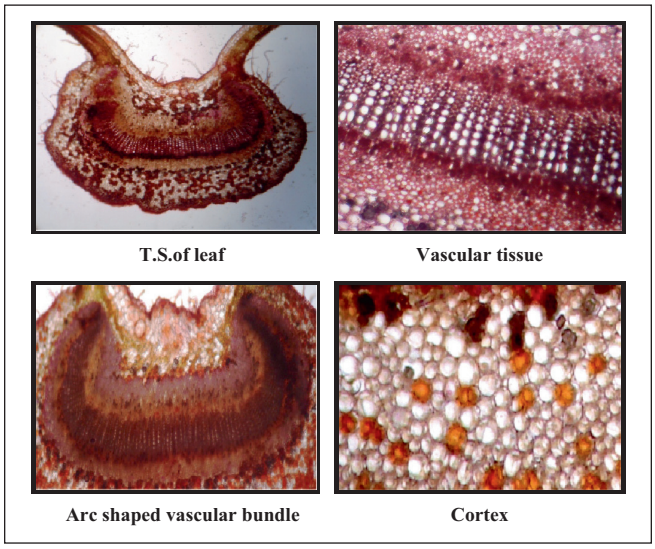

Figure 2: Photomicrographs of microscopic characteristic of *P. guajava* leaf

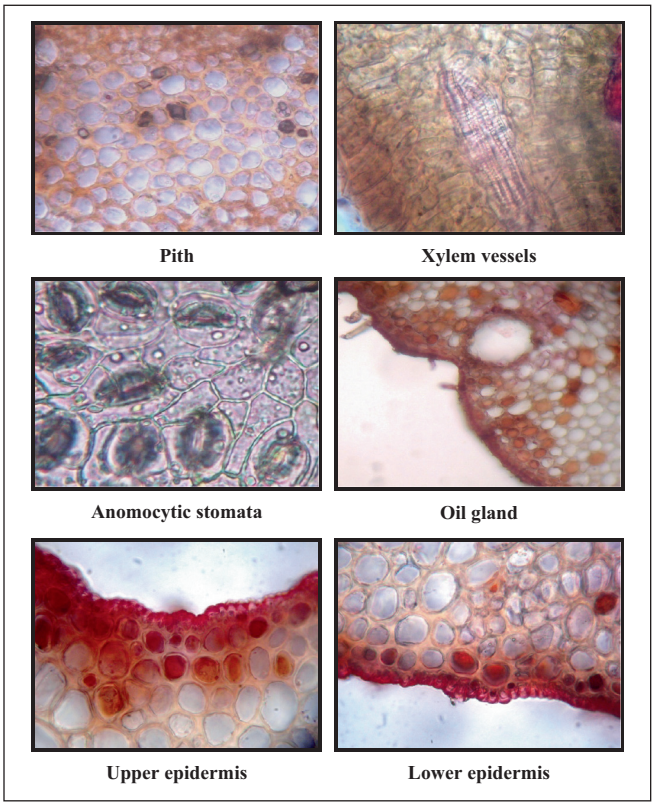

Figure 3: Photomicrographs of microscopic characteristic of *P. guajava* leaf

Physiochemical investigations

The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. The moisture content of dry powder of *P. guajava* leaves was 8.5% which is not very high, hence it would discourage bacteria, fungi or yeast growth. The ash value was determined by three different forms viz., total ash,

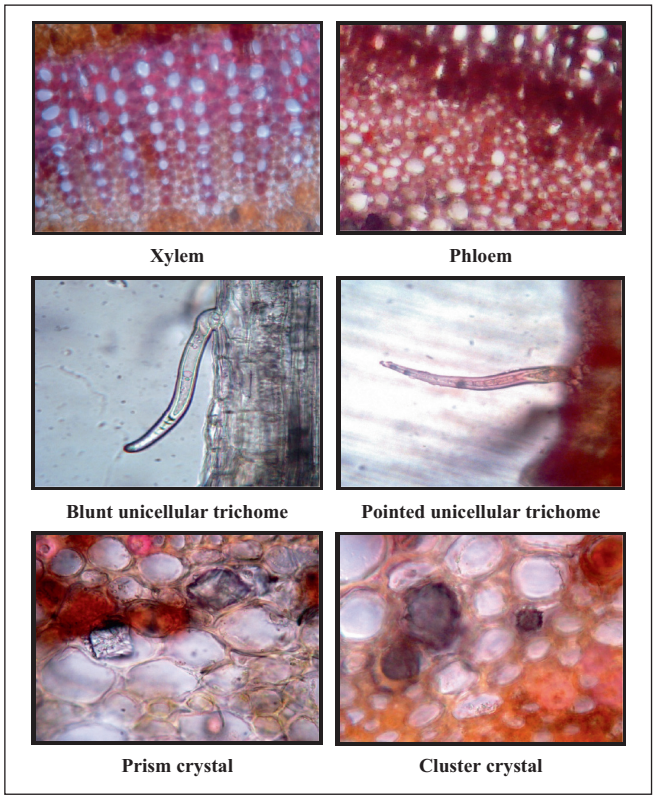

Figure 4: Photomicrographs of microscopic characteristic of *P. guajava* leaf

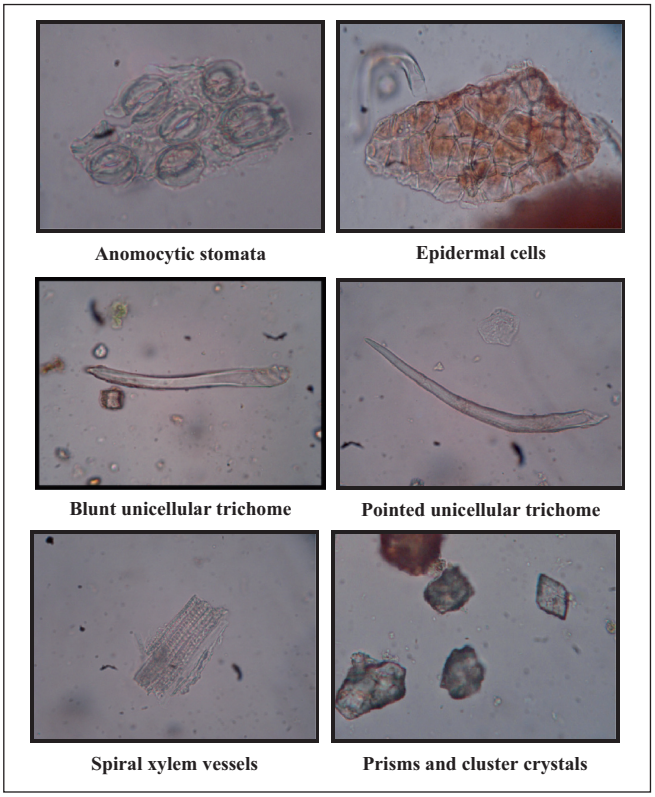

Figure 5: Photomicrographs of microscopic characteristic of powder of *P. guajava* leaf

water soluble ash and acid insoluble ash. The total ash is particularly important in the evaluation of purity of drugs; i.e. the presence or absence of foreign inorganic matter such as metallic salts or silica. Acid insoluble ash measures the amount of silica present, especially sand. Water soluble ash is the water soluble portion of the total ash.[24] The total ash of crude powder of *P. guajava* leaves was 7.65%, water soluble ash was 1.50% and acid insoluble ash was 7.5%. Less amount of these three parameters indicate that the inorganic matter and silica was less in *P. guajava* leaves. The extractive value of crude powder was maximum in methanol (24%) followed by water (16.92%) and minimum was in petroleum ether (3.32%). pH of acetone extract was 3.57 (Table 1). Mercury, lead, chromium, arsenic and cadmium were absent in the acetone extract as well as in crude powder of *P. guajava* leaves (Table 2).

Solubility test

The acetone extract of *P. guajava* leaves was evaluated for its solubility in 11 solvents with varied polarities. The extract was highly soluble in dimethylformamide and methanol, but insoluble in all non polar solvents (Table 3).

Phytochemical analysis

The results of qualitative phytochemical analysis of the crude powder and the acetone extract of *P. guajava* leaves are shown in Table 4. The acetone extract contained maximum amount of alkaloids and saponins while triterpenes was present in moderate amount. In the crude powder tannins and cardiac glycosides were present in high amount. Phlobatanins, steroida and cardiac glycosides were absent in the acetone extract while phlobatanins was absent in crude powder.

Table 1: Physiochemical parameters of *P. guajava* leaves

Parameters	Value
Loss on drying	8.50% (w/w)
Total ash	7.65% (w/w)
Water soluble ash	1.50% (w/w)
Acid insoluble ash	7.50% (w/w)
Petroleum ether soluble extractive value	3.32% (w/w)
Methanol soluble extractive value	24.00% (w/w)
Acetone soluble extractive value	9.00% (w/w)
Aqueous soluble extractive value	16.92% (w/w)
3.57	

Table 2: Heavy metal analysis of *P. guajava* crude powder and acetone extract

Heavy metal	Crude powder	Acetone extract
Mercury	Not detected	Not detected
Lead	Not detected	Not detected
Chromium	Not detected	Not detected
Arsenic	Not detected	Not detected
Cadmium	Not detected	Not detected

CONCLUSIONS

As there is no pharmacognostical work on record of this traditionally much valued drug, the present work was taken up with a view to lay down standards, which could be useful to establish the authenticity of this medicinally useful plant. Macro and micro morphological standards discussed here can be considered as identifying parameters to authenticate the drug. In the present study, we have found that most of the biologically active phytochemicals were present in the acetone extract and crude powder of *P. guajava* leaves. The medicinal properties of *Psidium guajava* leaves may be due to the presence of above mentioned phytochemicals. Further studies are in progress in our laboratory to isolate the active components.

ACKNOWLEDGMENT

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Table 3: Solubility of *P. guajava* acetone extract in different solvents

Solvents		Solubility (mg/ml)
Non polar solvents	Petroleum ether	0.0
	Hexane	0.0
	Chloroform	0.0
	Ethyl acetate	0.0
	Toluene	0.0
Polar solvents	1,4 Dioxan	0.0
	Acetone	4.2
	Methanol	207.1
	Dimethyl sulfoxide (DMSO)	155.0
	Dimethyl formamide (DMF)	389.3
	Water	120.3

Table 4: Qualitative phytochemical analysis of *P. guajava* leaves

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

–: No presence; +: Less presence; ++: Moderate presence; +++: High presence

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Physicochemical and Preliminary Phytochemical Studies on Petals of *Crocus sativus* 'Cashmerianus'

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ABSTRACT

Crocus sativus 'Cashmerianus' is a plant cultivated in various parts of the world. The present communication attempts to evaluate the physicochemical and preliminary phytochemical studies on the petals of most expensive *Crocus* species found in Kashmir is *Crocus sativus* 'Cashmerianus' (Iridaceae). These observations would be of immense value in the botanical identification and standardization of the drug in crude form and would help to distinguish the drug from its other species. The percentage of total ash value, acid insoluble ash value, water soluble ash value and sulphated ash value were found as 18.36, 3.48 11.36 and 9.01% respectively. Fluorescence characters of different extracts with various reagents were noted under UV (254 nm and 365 nm) and under normal ordinary light. Loss of weight on drying was found as 6.68% w/w. The percent yield of successive extract of petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts were found as 6.18%, 7.79%, 6.28%, 30.63%, and 35.16% w/w respectively. The preliminary phytochemical screening was carried out for the presence of alkaloids, flavonoids, carbohydrate glycosides, tannins, terpeniods, phenol, steroids and saponins for different petal extracts of *Crocus sativus* 'Cashmerianus'. These studies provide referential information for correct identification and standardization of this plant material.

Key words: *Crocus sativus,* physicochemical, florescent behaviors. Pharmacognostical analysis, phytochemicals.

INTRODUCTION

Crocus sativus var. cashmerian is a perennial stem less herb of the Iridaceae family commonly known as Kesar (Hindi), Saffron (English) and Zafran (Kashmiri). The word "saffron" is derived from the Arabic word za'faran, which translates to "yellow." Commercial saffron is made from the dried stigmas of the saffron flower, a triploid sterile plant and has a unique and distinctively pungent, honey-like flavor and aroma. Saffron is produced worldwide at an annual rate of 50 tons with a commercial cost of about 50 million dollars.[1] It comprises of the dried red stigma, purple petal with a small portion of the yellowish style attached. It is cultivated in Azerbaijan, France, Greece, Iran, Italy, Spain, China, Israel, Morocco, Turkey, Egypt, Mexico and Kashmir in India.[2] It is most expensive spice and used for cooking, staining, medicine, cosmetics[3] and is also used in folk medicine as antispasmodic, carminative, stomachic, expectorant, aphrodisiac, hypolipaemic effects[4] petals possesses antidepressant activity[5] cardio-tonic and stimulant.[6] In traditional medicine this plant is utilized as an exhilarant and curative of anxiety.[7] The chemical composition of saffron has attracted the interest of several research groups during the last decades, and among the estimated more than 150 volatile and several nonvolatile compounds of saffron, approximately 40-50 constituents have been isolated.[1] Saffron contains three main pharmacologically active metabolites: Saffron-colored compounds are crocins, which are unusual water-soluble carotenoids (mono and diglycosyl esters of a polyene dicarboxylic acid, named crocetin). The digentiobiosyl ester of crocetin - α-crocin is the major component of saffron. Picrocrocin is the main substance responsible of the bitter taste in saffron. Safranal in the volatile oil is responsible for the characteristic saffron odor and aroma. Furthermore, saffron contains proteins, sugars, vitamins, flavonoids, amino acids, mineral matter, gums, and other chemical compounds.[1]

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Plant Material and Method

The petals of *Crocus sativus* 'Cashmerianus' were collected from Pampore area of Kashmir (J&K, India). *Crocus sativus* 'Cashmerianus' was properly identified (Voucher specimen-KUCS03) by Dr. A. R. Naqshi, Taxonomist, Department of Pharmaceutical Sciences, University of Kashmir, Srinagar. The collected petals were shade dried and coarsely powdered. The coarse powder was subjected to continuous extraction in a soxhlet apparatus separately using petroleum ether, chloroform, ethyl acetate, methanol and water as solvents.

Preparation of the extract

The petals of *Crocus sativus* 'Cashmerianus' were dried under shade, separated and crushed to form dry powder. 150 g of weighed petals were first defatted using petroleum ether (60-80 °C) in soxhlet apparatus. The marc was removed and dried after the completion of defatting process. Defatted petals were subjected to successively continuous extraction with 500 mL each of chloroform, ethyl acetate, methanol and distilled water to give petroleum ether extract (PE), chloroform extract (CE), ethyl acetate extract (EAE) methanol extract (ME) and aqueous extract(AE). After complete extraction the solvent was evaporated and concentrated to dry residue.[8] The percentage yield of successive extracts of petroleum ether, chloroform, ethyl acetate, methanol and aqueous extract of petals of *Crocus sativus* 'Cashmerianus' were obtained as 6.18%, 7.79%, 6.28%, 30.63%, and 35.16% respectively. All the extractive values of the plant *Crocus sativus* 'Cashmerianus' with different solvents were calculated and are shown in the table 1.

Preliminary phytochemical analyses

Successively obtained materials were filtered and concentrated using vacuum distillation. The different extracts were subjected to qualitative tests for the identification of various phytochemical constituents as per the standard procedure[9] and shown in the table no: 4.

Physico-chemical parameters

Physicochemical constants were evaluated for the purpose of standardization such as the percentage of total ash, water soluble ash; acid insoluble ash, sulphated ash, loss on drying and total extract values were calculated according to standard procedures.[10] The values of physicochemical constants were calculated and are shown in table no: 2.

Fluorescent behavior of powder

In fluorescence analysis[11] the powdered sample were treated with various chemical reagents like aqueous 10% Sodium hydroxide, alcoholic 1N sodium hydroxide, 1N hydrochloric acid, 50% sulphuric acid and concentrated nitric acid, picric acid, acetic acid, ferric chloride, conc. HNO3 + NH3 etc. and their extracts were subjected to fluorescence analysis in day light and UV light (254 nm and 365 nm). The observed values of fluorescence analysis are shown in Table 3.

Table 1: Successive Extraction values of the petal extracts of *Crocus sativus* 'Cashmerianus' in different solvents

Nature of extracts	%Yield (w/w)	Color of the extract
Petroleum ether (60-80°)	6.18	Golden yellow
Chloroform	7.79	Dark brown
Ethyl acetate	6.28	Dark brown
Methanol	30.63	Dark brown
Aqueous	35.16	Black

Table 2: Physicochemical parameters of petal of *Crocus sativus* 'Cashmerianus'

Parameters	% Content
Foreign organic matter	0.28
Methanol soluble extractive	39.63%
Water soluble extractive	46.10%
Total ash	18.76%
Acid-insoluble ash	03.48%
Water soluble ash	11.36%
Sulphated ash	09.01%
Loss on drying	06.68%

Table 3: Fluorescence characteristics of the powdered sample of Petal of *Crocus sativus* 'Cashmerianus'

Treatment	Visible/Day light	Short UV light (254 nm)	Long UV light (365 nm)
Powder as such	Violet	Purple	Black
Powder + 50% H_2So4+ water	Reddish brown	Brown	Dark brown
Powder + Con. Hcl	Golden brown	Light brown	Green
Powder + Con. Hcl+ water	Pink	Purple	Light purple
Powder + Con. nitric acid	Golden brown	Green	Purple
Powder + Con. nitric acid+ water	Light green	Light green	Yellow
Powder + Acetic acid	Pink	Purple	Light pink
Powder + Methanol	Colorless	Intense light green	Light green
Powder + Chloroform	Purple	Colorless	Light green
Powder + Petroleum ether	Colorless	Colorless	Light green
Powder + Distilled water	Purple	Colorless	Light green
Powder + 10% NaOH (aqua.)	Yellow	Light green	Light yellow
Powder + 5% iodine	Light yellow	Light green	Light green
Powder + Picric acid	Light yellow	Intense green	Light green
Powder + Ferric chloride (5%w)	Muddy yellow	Intense green	Yellow green
Drug Powder + NH_3 solution	Light blue	Colorless	Light green
Powder + Ethyl acetate	Colorless	Colorless	Purple
Powder + 1N NaOH(alcoholic)	Light Yellow	Colorless	Light yellow
Powder + Conc. H_2SO_4	Greenish yellow	Green	Light green

Table 4: Preliminary Phytochemicals Screening of Petal extracts of *Crocus sativus* 'Cashmerianus'

Test	P.E.	C.E.	E.A.E.	M.E.	A.E.
Carbohydrates-					
Molish test	+	+	+	++	++
Fehling's test	+	+	+	+	+
Seliwnoffs test	–	–	–	+	+
Alkaloids					
Mayer's test	++	++	–	+	+
Hager's test	+	++	–	++	++
Wagner's test	+	+	–	+	–
Saponins					
Foam test	–	–	–	–	++
Steroids					
Salkovaski test	++	–	–	++	++
Fats and oils					
Filter paper test	–	–	–	–	–
Proteins					
Millon's test	–	–	–	–	++
Biuret test	++	–	++	–	++
Amino acid test					
Ninhydrine test	+	–	–	–	+
Glycosides					
Anthraquinone test	++	–	–	++	++
Keller kiliani test	–	–	–	+	+
Starch					
Iodine test	–	–	–	–	+
Tannins and Phenolic					
Ferric chloride test	–	–	–	++	++
Resins					
Ferric chloride test	+	–	–	++	++
Flavonoids					
Shinoda test	+	–	–	++	++
Lead acetate test	–	+	–	+	+

Symbol denoted: +: Positive, ++: Strong positive –: Negative (Symbol Based on color intensity)

RESULT

The preliminary qualitative chemical tests performed shows that the petals are credited with different secondary metabolites such as alkaloids, glycosides, tannins, phenolics compounds, flavonoids, steroids, saponins, proteins, amino acids and carbohydrates. The percentage of total ash value, acid insoluble ash value, water soluble ash value and sulphated ash value were found to be 18.36, 3.48 11.36 and 9.01% respectively. Foreign organic matter in the petals was 0.28%. Loss of weight on drying was found as 6.68% w/w, which is not too high, hence could discourage bacterial, fungal, or yeast growth.[13] The percent yield of successive extract of petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts were found to be 6.18%, 7.79%, 6.28%, 30.63%, and 35.16% w/w respectively. Extractive value was highest in water and alcohol indicating the possibility of considerable amount of polar compounds in crocus petals. Preliminary phytochemical screening of different petals extracts of *Crocus sativus* 'Cashmerianus'. Indicated the presence of alkaloids, flavonoids, carbohydrate, glycosides, tannins, terpeniods, phenol, steroids and saponins.

CONCLUSION

Physicochemical constant values are useful in determining authenticity and purity of drug and also for quantitative standards as reference.[12] In this study total ash value is considerably low, which may be due to low content of carbonates, phosphates, silicates and silica. Study of crude fiber content could be useful in distinguishing between similar drugs or in the detection of adulteration. It will also help to remove the more resistant parts of plant organs which can be used for microscopic examination. Fluorescence behavior of drugs sample is an important character exhibited by various chemical constituents present in plant material. Some plant constituents have unique tendency to show fluorescence in the visible range in day light. The ultra violet light produces fluorescence in many natural products (e.g. alkaloids like berberine), which do not visibly fluoresce in day light. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives by applying different reagents hence some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostical evaluation.[13] Preliminary phytochemical screening of different petals extracts have shown that the petals of *Crocus sativus* 'Cashmerianus' posses various secondary metabolites. These secondary metabolites may be responsible for various pharmacological effects of the drug and justify their use by herbal practitioners.

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Antioxidant, Antimicrobial and Cytotoxicity Activities of *Acacia farnesiana* (L.) Willd. Leaves Ethanolic Extract

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ABSTRACT

The antioxidant activity was evaluated from the ability to chelate iron, reducing property, scavenging DPPH radical and nitric oxide. The *A. farnesiana* ethanolic extract responded to all the antioxidant assays in concentrations dependent manner. Evaluated by broth microdilution assay, the extract exhibited MIC value at 0.8 mg/ml against *Bacillus subtilis* and MIC value at 2.5 mg/ml against *Saccharomyces* cerevisiae. The absence of cytoxicity was observed from the brine shrimp lethality test. The quantification of total phenolic content by Folin-Ciocalteu reagent resulting 209.78 ± 3.21 mg gallic acid/g ethanolic extract of *A. farnesiana* leaves. Analysis of the extract by both HPLC-PDA and LC/MS have tentatively identified flavonoid galloylglycoside and flavonoid glycosides from the extract. It was plausible that the preparation method of the extract influenced the consituents of the extract, and that later contributed to the exhibited antioxidant and antibacterial activities. Quercetin deoxyhexoside was tentatively identified as the major consituent of the extract. Bioassay guided isolation is suggested as future research to study the biological activities and elucidate the chemical structure of flavonoids involved.

Key words: Electrospray negative ionization, Mimosoideae, Soxhlet extraction

INTRODUCTION

Biological studies of plant extracts have been carried out to verify the pharmacological properties of the plants. The radical scavenging, reducing capacity and metal chelating properties of antioxidants are known to eliminate and prevent the generation of free radical. The properties have been contributing directly or indirectly in the prevention of pathogenesis and deterioration of food,[1] whereas the ability of plant extract to kill or inhibit the growth of microorganisms is at interest for the development of antimicrobial agent. Thus, such studies add value and provide scientific information to continually validate the potential of the plant known as ethnomedicine.[2]

Along with the determination of the pharmacological properties of plant extract, study on the toxicity property of plant extract is indispensable to assure the safety of the extract.[3] Solvents and extraction procedures,[4] together with different methods available to evaluate antioxidant[5] and antibacterial activity[6] have been contributed to the variation of observed plant extract effects. In frequently reported studies, polyphenols have been suggested as responsible for the potent biological activities of extracts prepared using methanol and ethanol.[7]

Acacia farnesiana is a plant from the subfamily Mimosoideae of Leguminosae (Fabacea). It is well known cultivated for its flower to be utilized in the production of cassie perfume.[8] The plant is also known for its ethnopharmacological properties and a number of scientific studies have been carried out previously. For instance, the bronchodilator and anti-inflammatory effect of glycosidal fraction of *A. farnesiana* was reported.[9] The root is reputed active against snake bite venom[10] and compounds of diterpenes and flavonoids were previously isolated from the root extract.[11] The methanolic extract of *Acacia farnesiana* bark exhibited antidiarrhoeal activity against castor oil and magnesium sulphate induced diarrhea along with antimicrobial activity against common pathogens responsible for diarrhoea *in vitro*.[12] Previously, the anti-inflammatory property of the

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leaves ethanolic extract evaluated by carrageenan induced paw oedema for acute inflammation and cotton pellet induced granulation for chronic inflammatory model has been reported.[13] In addition to the previous works, the objective of this study was to study the antioxidant, antimicrobial and cytotoxicity activities of *Acacia farnesiana* leaves ethanolic extract. The quantification of phenolic content by Folin ciocalteu reagent, and characterization of the extract by high performance liquid chromatography coupled with photo diode array (HPLC-PDA) and mass spectrometry (LC/MS) were also carried out.

MATERIALS AND METHODS

Plant material

Leaves of *Acacia farnesiana* were collected from botanical garden in Bangkok. The identification and authentication of species was carried out by Associate Prof. Dr. Nijsiri Ruangrungsi. Samples were deposited at the Faculty of Pharmaceutical Sciences Herbarium, Chulalongkorn University. All samples were air dried before grounded and were stored at room temperature.

Preparation of ethanolic extracts

The dried grounded powder of sample was successively extracted with petroleum ether, dichloromethane and ethanol in soxhlet apparatus. The ethanol in the extract was removed with rotary evaporator and water bath, respectively.

Metal chelating assay

The assay was carried out according to[14] with modifications. In the 96 well microplate, 15 µl of iron (II) chloride ($FeCl_2$) in ultrapure water (2 mM) was added into 110 µl of extract in ethanol (1-10 mg/ml). The reaction started by addition of 75 µl of aqueous Ferrozine (5 mM). The mixture was left for 10 minutes, before the absorbance of reaction was measured at 562 nm. Control was ethanol reacted with $FeCl_2$ and Ferrozine. The Fe^{2+} chelating activity of test compound was calculated as: Chelating activity (%) = (Absorbance $_{control}$ – Absorbance $_{sample}$)/Absorbance $_{control}$ × 100. EDTA was used as positive control (0.01-0.1 mg/ml). The effective concentration of EDTA that chelate 50% of ferrozine (EC_{50}) was obtained from a graph of chelating activity (%) against the EDTA concentration.

Reducing power assay

Extract in DMSO (20 mg/ml) was diluted with 0.1 M potassium phosphate buffer (pH 6.6) to obtain different concentrations in a range of 0.02 to 1.0 mg/ml. The reducing assay was carried out according to[15] with modifications. A 148 µl of each concentration was pipette into a 96-well microplate, followed by 50 µl of 1% potassium ferricyanide (w/v). The mixture was incubated at 50 °C for 20 min. Next, 50 µl of 10% trichloroacetic acid (w/v) and 2 µl of 1% of ferric chloride (w/v) was added into each well. The addition of $FeCl_3$ leads to the formation of colored complex which can be measured at 700 nm. The mixture was mixed until homogenized before being measured spectrophotometrically. Increase in absorbance of the reaction mixture indicates good reducing capacity of extract. The assay was carried out in triplicate. The graph of absorbance at 700 nm against the correspondent extract concentration was plotted. The EC_{50} was arbitrarily defined as the concentration of extract that exhibited absorbance of 0.5 at 700 nm obtained from a line of best fit from the plotted graph.

DPPH radical scavenging assay

The activity was determined according to.[16] Aliquot of 100 µl of DPPH in methanol (126µM) was pipette into 96 well microplate, followed by 100µl of extract prepared in ethanol. The reaction was allowed to incubate in 30 minutes at room temperature. Next, the absorbance was measured at 517 nm using microplate reader. All extract were analysed in triplicate. The control was reaction mixture with ethanol substituting the extract. The DPPH radical scavenging activity was calculated by the following equation: Radical scavenging activity (%) = (Absorbance $_{control}$ – Absorbance $_{sample}$)/Absorbance $_{control}$ × 100. The scavenging activity of extract was expressed as the concentration necessary to scavenge free radical by 50% (EC_{50}).

Nitric oxide scavenging assay

Extract in DMSO (20 mg/ml) was diluted with ultrapure water to obtain different concentrations in a range of 0.5-5 mg/ml. The assay was carried out according to[17] with modifications. Into a 96 microwell plate, 50 µl of extract or control (ultrapure water) was added into 50 µl of 5 mM sodium nitroperusside (prepared in a phosphate buffer (pH 7.4)). The mixture was incubated for 2 hours. Next 100 µl of Griess reagent (0.5% sulphanilamide in ultrapure water, 0.16% naphtylethylenediamine dihydrochloride in 20% acetic acid, 1:1) was added and was immediately read at 570 nm. Different concentrations of aqueous sodium nitrite (1-120 µM) were treated in the same way with Griess reagent. A graph of sodium nitrite concentration against the absorbance of chromophore formed at 570 nm was used to calculate the nitrite produced in the reactions. The nitric oxide scavenging activity was calculated as, scavenging activity (%) = (Absorbance $_{control}$ – Absorbance $_{sample}$)/ Absorbance $_{control}$ × 100. The concentration of extracts necessary to scavenge 50% of the produced nitric oxide (EC_{50}) was obtained from a graph of scavenging activity (%) against a plant extract concentrations.

Broth microdilution assay for antimicrobial evaluation

Bacterial strains studied were: *Staphylococcus aureus* (ATCC 6538P), *Bacilus subtillis* (ATCC 6633) and *Escherichia coli*

(ATCC 25922), whereas fungal strains were: *Saccharomyces cerevisiae* (ATCC 9763) and *Candida albicans* (ATCC 10230). They were obtained from the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Both bacterial and fungal strains were maintained on Muller-Hinton and Sabouraud agar, respectively. The subcultures were prepared 24 hours before use. Two to three colonies of the microbial culture from 24 hour old plate were suspended in normal saline and the turbidity of the culture was adjusted to match the 0.5 Mc Farland standard. Next, a microbial suspension in broth was prepared by adding 10 µl of normal saline microbial suspensions to 1 ml Muller-Hinton or Sabouraud broth. The assay was carried out according to[18] with modifications. Into a sterile 96-well microplate, 50 µl of microbial suspended in broth was added to the wells containing 50 µl of plant extract (final concentration: 0.2-1.2 mg/ml for bacteria and 1.0 to 5.0 mg/ml for fungi) or control. Control was prepared by diluting 50 µl DMSO with broth to obtain final volume of 1 ml. After 20 hours incubation at 37 °C, 20 µl of *P*-Iodonitrotetrazolium (INT) reagent dissolved in water (1 mg/ml) was added into each well. The lowest concentration of extract inhibiting the growth of test microorganisms indicated by INT was defined as the MIC of an extract. Gentamicin was used as a reference antibiotic.

Brine shrimp lethality assay

Fresh eggs of *A. salina* were purchased from the local pet shop at Chatuchak market, Bangkok. Artificial sea water was prepared by dissolving 3.8 g sea salt per litre of distilled water and was aerated for 24 hours. Into a two portioned aquarium, the eggs were hatched in the darkened area of the aquarium covered by aluminium foil whereas the other part of the aquarium was put under a light. With the help from a light source, the larvae (nauplii) were attracted to the side of the aquarium and were easily collected from the non hatched eggs. One day old nauplius was transferred into a vial containing 5 ml of artificial sea water (30 per vial) and was allowed to stand for another 24 hours under illumination. Extract prepared in DMSO was added into each vial to obtain final concentration of extract at 10, 100 and 1000 µg/ml. Five vials were prepared for each concentration. The controls were prepared in the same manner except that DMSO was used instead of the extract. Three replicates were prepared for each concentration. After 24 hours, the numbers of survivors were counted. Next, the percentage of death and LD_{50} was calculated by probit analysis.[19]

Determination of total phenolic content

Quantification of total phenolic content of extract was determined using Folin-Ciocalteu's phenol reagent according from[20] with modifications. Phenolic compounds in the extract will form a blue color complex with Folin Ciocalteu reagent after adjusted with alkali. Briefly, 80µl of extract in methanol was pipette into 96 well microplate, followed by 100µl of 15% Folin Ciocalteau. Distilled water was added to adjust the volume to 200 µl. The mixture was left for 5 minutes before addition of 100 µl Na_2CO_3 aqueous (0.105 g/ml). The absorbance of extract was measured at 756 nm after incubation at 30 °C for 60 minutes. All determinations were performed in triplicate. Different concentrations of gallic acid (0.03, 0.06, 0.12, 0.25, 0.5 and 1 mg/ml) were used to prepare a standard graph. The concentration of total phenolic compounds in all extract was expressed as mg of gallic acid equivalents per g dry weight of extract using a linear equation.

Detection and characterization of extract constituents by HPLC-PDA and LC/MS

One mg/ml sample was filtered through the Ultrafree-MC membrane centrifuge-filtration unit (hydrophilic PTFE, 0.20 µm, Milipore), and 5µl of the filtrate was loaded into a TSK-gel Super ODS column (2.0 µm, 2.0 × 100 mm, TOSOH, Tokyo, Japan) at 40 °C. The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% formic acid with the flow rate of 200 µl/min. The separation and detection was performed by an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) with gradient condition of 5%-60%B for 35 minutes. The HPLC system was consisted of a pair of LC 10AD VP pumps, a DGU 12A degasser, a CTO 6A column oven, an SPD 10A VP photodiode array detector (PDA), and a SCL 10A VP system controller (Shimadzu, Kyoto, Japan). For the analysis by LC/MS, the HPLC system coupled with mass spectrometer (MS) was consisted of Finnigan LCQ Deca XP plus Ion Trap MS instrument (Thermo Fischer Scientific, San Jose, CA) equipped with electrospray ionization (ESI) interface. The ESI conditions of negative mode were as the following: capillary temperature 300 °C; sheath gas flow rate, 35 (arbitrary units); ESI source voltage, 5000 V; capillary voltage, 43 V; and tube lens offset 15 V. Scan range 150 to 1000 was applied. Tandem mass spectrometry (MS/MS) was applied to ions that acquired further structural analysis. Standards employed for both HPLC-PDA and LC/MS analyses were gallic acid, epicatechin, quercetin, ellagic acid, genistein, rutin (Nacalai Tesque, Kyoto, Japan) and tannic acid (Wako Pure Chemicals, Osaka, Japan).

RESULTS AND DISCUSSIONS

Antioxidant activities

Various mechanisms have been attributed to the antioxidant activity of an extract. Metal chelating capacity of antioxidant was significant in reducing the concentration of transition metal participating in lipid peroxidation.[21] Transition metal as iron (Fe^{2+}) has been described contributing in free radical generation, stimulating lipid peroxidation that eventually disturbing the function of membrane protein.[22] The metal

chelating property of an extract has been evaluated from the ability of extract to bind with metal in a presence of Ferrozine.[23] Ferrozine is a chromophoric complexing agent that able to bind with free iron (Fe^{2+}). The ability of extract to interfere with the formation of Fe^{2+}-Ferrozine complex or simultaneously bind to the iron is regarded as metal chelating activity.[24] The ethanolic extract of *A. farnesiana* exhibited the ability to chelate metal, but it was observed that the chelating activity was saturated at high concentration of extract, which higher concentration of extract was found ineffective to increase the metal chelating activity of the extract. The activity of extract was compared with the activity of EDTA; a known metal iron chelator. Extract of *A. farnesiana* exhibited the highest metal chelating activity at the concentration of 5 mg/ml, whereas the chelating activity of EDTA was increasing linearly with its concentrations (Figure1). At the concentration of 5 mg/ml the extract was able to chelate 15.55 ± 0.38% of available iron, whereas EDTA exhibited EC_{50} = 0.052 mg/ml which was defined as the concentration of EDTA required to chelate 50% of available iron.[25]

Both reducing power and free radical scavenging activity indicate the antioxidant activity of extract through the ability to donate electron or hydrogen that is crucial in the inhibition of deleterious effects caused by oxidation process and free radicals.[26] The reducing capacity of *A. farnesiana* ethanolic extract was evaluated from the ability of extract to reduce potassium ferricyanide, whereas DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was employed to evaluate the free radical scavenging activity. The reducing ability of *A. farnesiana* was observed from the formation of colour complex which was measured at 700 nm. Increase in absorbance of the reaction mixture indicates good reducing capacity of extract.[27] As an indication to the potency of the extract and standard quercetin, EC_{50} was arbitrarily obtained as concentration of extract that exhibited 0.5 absorbance value calculated from the linear plotted graph[15] (Figure 2). Results showed that the extract exhibited EC_{50} = 40.4 μg/ml compared to quercetin with EC_{50} = 3.75 μg/ml.

From the reaction of the extract with DPPH radical, it was observed that the scavenging activity of *A. farnesiana* ethanolic extract was also concentration dependent (Figure 3). The concentration of extract required to scavenge 50% of DPPH radical present in the reaction mixture (EC_{50}) was higher compared to quercetin. Ethanolic extract of *A. farnesiana* scavenge 50% of DPPH radical at the concentration of 56 μg/ml, whereas quercetin exhibited EC_{50} = 3.25 μg/ml.

Other than free radical, the non radical species as nitric oxide (NO) has been associated with chronic inflammation, cell death, and onset of atherosclerosis.[28] Nitric oxide scavenging activity has been studied using sodium nitroprusside (SNP) and Griess reagent. SNP decomposes in an aqueous solution at physiological pH to produce NO. Under aerobic conditions, NO reacts with oxygen to produce stable nitrite product which can be quantify from the reaction with Griess reagent. Antioxidants were reported able to prevent the cytotoxity and lipid peroxidation activities of SNP derived by NO.[29] When solution of 5 mM SNP in PBS was incubated at 25 °C for 2 hours, a time-dependent nitrite was generated, which was decreased by the presence of extracts in a dose-dependent manner.[30] In this study, the mean of nitrite produced during the 2 hours incubation with SNP was 59.56 ± 2.26 μM. As in Table 1, *A. farnesiana* ethanolic extract inhibited NO production in a concentration dependent.

The evaluation of antioxidant activity was carried out by several assays. The concentration of extract required to obtain EC_{50} in the in DPPH experiment compared to NO assay and metal chelating was observed. Other than

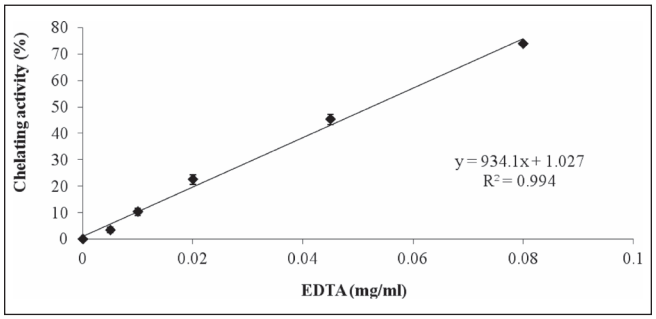

Figure 1: Iron chelating activity of EDTA. Values are mean ± SD (*n* = 3).

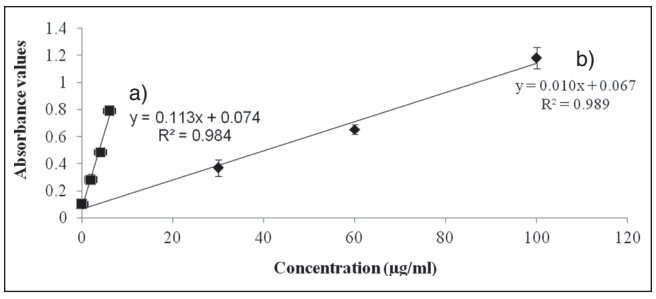

Figure 2: Reducing power of a) Quercetin and b) *A. farnesiana* ethanolic extract. Values are mean ± SD (*n* = 3).

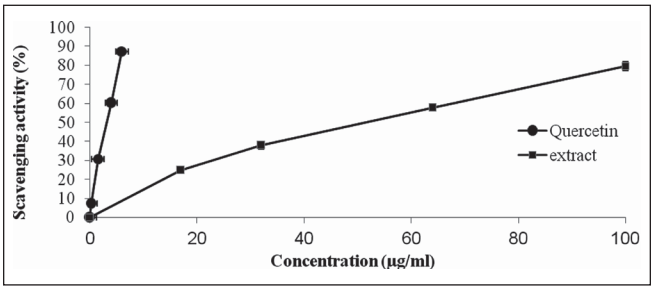

Figure 3: DPPH radical scavenging activity of extract and quercetin. Values are mean ± SD (*n* = 3).

differences in the basis of each assay, it was reported that solvent influences the kinetics of reaction with DPPH.[31] DPPH is a synthetic free radical which readily to undergo reduction by antioxidant substances in a polar organic solvent such as methanol, while the NO radical has to be generated from SNP in a buffer.[32] Application of Tris buffer was suggested able to reduce the interference of solvent with the complexing capacities of phenolic compound in metal chelating assay.[33] In comparison with the quercetin, the activities of *A. farnesiana* ethanolic extract were found low. The structure activity relationships of quercetin[34,35] as a metal chelator, DPPH radical scavenger and reducing power have been widely published, resulting quercetin as a favourable positive control over synthetic antioxidants as BHT (Butylated hydroxytoluene), and BHA (Butylated hydroxyanisole), especially in the study of antioxidant substances. The OH group at the position C_3, presence of ortho-dihydroxy group and absence of glycosylation group particularly at position C_3 have been reported as contributing to the pronounced antioxidant activity of quercetin (Figure 4).[36]

Antimicrobial activity

The ability of *A. farnesiana* extract to inhibit the growth of selected bacterial and fungal strains was evaluated by broth microdilution method. In the broth microdilution method, the microbial growth was indicated by a presence of reddish-pink colour after the addition of tetrazolium salt (INT). The lowest concentration of extract inhibiting growth of test microorganisms indicated by INT was defined as the minimum inhibition concentration (MIC). MIC value indicates the antimicrobial property of an extract. Extract with MIC < 1 mg/ml was regarded as exhibiting activity.[37] It was found that ethanolic extract of *A. farnesiana* leaves exhibited MIC = 0.8 mg/ml against bacteria *Bacillus subtilis* (ATCC 6633), whereas the MIC to the growth of *Staphylococcus aureus* (ATCC 6538P) and *Escherichia coli* (ATCC 25922), were more than 1.0 mg/ml, thus the extract was regarded as not active against those bacteria. *E. coli* has been found resistant to plant extracts and frequently the gram positive bacteria are more susceptible to plant extract than gram negative.[38] Nevertheless, the antibacterial activity of extract against *B. subtilis* was low compared to Gentamicin (MIC = 0.05 mg/ml). Fungi unlike bacteria are eukaryotic cells which are more complex to be inhibited; therefore the MIC for bacteria is usually lower compared to fungal. Towards the fungal, the final concentration of extract was increased because the extract showed no inhibitory effect at final concentration of 1.0 mg/ml. The extract exhibited MIC = 2.5 mg/ml against *Saccharomyces cerevisiae* (ATCC 9763) whereas no activity was observed against fungal *Candida albicans* (ATCC 10230) at final concentration of 5 mg/ml.

Table 1: Nitric oxide scavenging activity. Values are mean ± SD

	Concentration (mg/ml)	Inhibition (%)
Extract	5	48.64 ± 3.08
	3	36.12 ± 1.46
	1	23.59 ± 4.98
Quercetin	0.5	76.41 ± 4.61
	0.3	46.13 ± 3.23
	0.1	16.16 ± 1.41
	0.05	9.44 ± 0.99

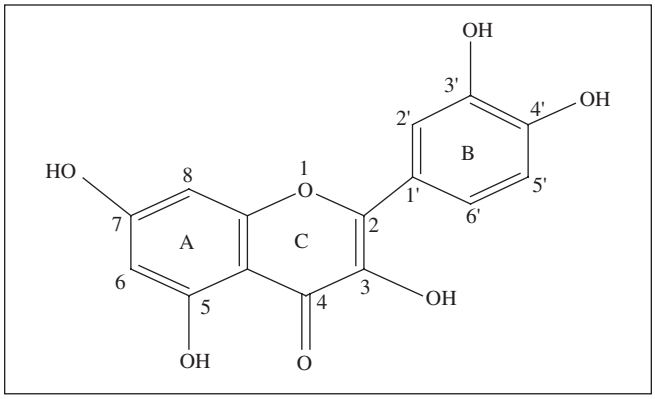

Figure 4: Quercetin[36]

Cytotoxic activity

Substituting the use of laboratory animals in toxicological tests due to the high cost for chemicals and animal suffering have been proposed.[39] The utilization of brine shrimp or *Artemia salina* (Artemiidae) for predicting the cytoxicity of plant extracts has been employed as an alternative. In this test, the tiny crustacean *Artemia salina* was used. After exposure of 24 hours to the extract, the surviving *A. salina* were counted. Increases of lethality which linear to the increase of extract concentration allow the determination of the dose or concentration of extract that causing 50% of lethality (LD_{50}) to the *A. salina*. The results of brine shrimp lethality test has been expressed as the extract was not toxic (LD_{50} >1000), weak toxicity (LD_{50} 500-1000 µg/ml), toxic (LD_{50} 100-500 µg/ml) and very toxic (LD_{50} <100 µg/ml).[40] The brine shrimp lethality assay not only indicates the safety of the extract but this bioassay has a good correlation with cytotoxic activity in human solid tumors. The compound toxic to *A. salina* was reported exhibiting the possible antineoplastic activity.[41] From the result, the *A. farnesiana* extract was not lethal to the *A. salina* at the final concentration of 1000 µg/ml. Thus, the extract was considered as not toxic.

Analyses of extract

In this study, the ethanolic extracts were prepared by successively extracting the plant materials with petroleum ether, dichloromethane and ethanol in soxhlet apparatus. The use of sequential solvents with different polarity allows a separation of compounds according to their solubility in the solvents, thus simplify and reduce the chemical complexity

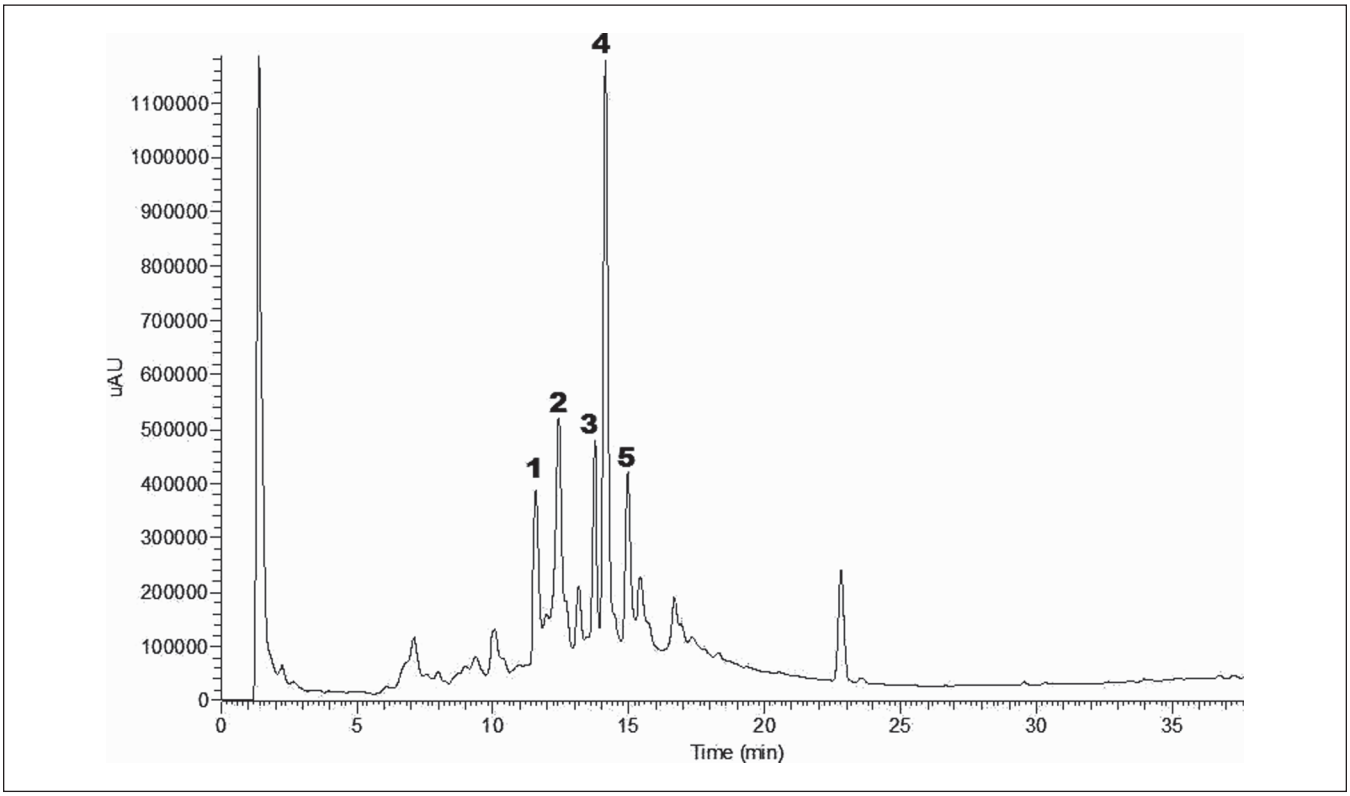

Figure 5: HPLC profile of *A. farnesiana* leaves ethanolic extract. Compounds were identified as follow: peak 1. Quercetin-galloylglucoside; 2. Rutin 3. Quercetin-pentoside; 4. Quercetin-deoxyhexoside; 5. Diosmetin diglycoside

of the extract, whereas it was suggested that the heat disrupted the cell wall and liberate the polyphenol compounds which in turn increased the pool of accessible compounds.[42] In addition, such preparation of extract also facilitated the LC/MS analysis since requiring no procedures of extract clean up. The quantification of total phenolic content by Folin-Ciocalteu reagent resulting 209.78 ± 3.21 mg gallic acid/g ethanolic extract of *A. farnesiana* leaves. Nevertheless, the values given by extract reaction with Folin-Ciocalteu reagent was only an approximate estimation of the total phenolic content.[43] Analysis by HPLC-PDA and LC/MS in the negative ionization with a scanning range between *m/z* 150-1000 tentatively characterized *A. farnesiana* leaves ethanolic extract. Tandem mass spectrometry (MS/MS) was applied to ions that acquired further structural analysis. The HPLC profile of *A. farnesiana* extract recorded at 280 nm is depicted in Figure 5. Previously, high tannin content was reported from *A. farnesiana* leaves;[44] however the UV spectra for the numbered peaks exhibited two major absorption bands: around λ_{max} 260 nm and 350 nm, thus were putative flavonoids.[45] Results obtained through the mass analysis in the negative ion mode are shown in Table 2. The characterizations were aided by comparison with reference standard where available and by correlation with previous literature reports.

Table 2: Results by HPLC-PDA and LC/MS of *A. farnesiana* leaves ethanolic extract

Peak	R_t (min)	λ_{max}	Molecular weight	Product ions (*m/z*)
1	11.61	269, 334	616	615, 463, 301
2	12.43	268, 340	610	463, 301
3	13.20	264, 346	434	433, 301
4	14.16	264, 348	448	447, 301
5	14.97	269, 342	608	607, 299

Ion at *m/z* 615 was identified as the deprotonated ion $[M-H]^-$ of peak 1. Product ions at *m/z* 463 and 300 indicated neutral loss of galloyl group and hexoside, respectively. The aglycone was identified as quercetin which was in corresponded with the standard. The gallic acid group was most likely bounded to the sugar component because compounds of this type are quite sporadic in the flora.[46] Therefore, peak 1 was tentatively identified as quercetin-galloylglucoside. Flavonoids galloyl glucosides have been previously reported from *Acacia farnesiana*. For instance, naringenin 7-*O*- galloylglucoside was described from the pod of *Acacia farnesiana*,[47] along with other flavonoids galloyl glucosides; consisted of kaempferol, quercetin and myricetin 7-*O*- galloylglucoside were also identified from the pod.[48]

Peak 2 was initially suspected as rutin according to the matched retention time, however the product ions of the peak was not corresponded to standard rutin, therefore peak 2 was suggested to be quercetin diglycoside. Ion at m/z 433 and 447 fragmented in MS/MS to produce ion that corresponded to standard quercetin, neural loss of 132 and 146 from each ion, respectively demonstrating presence of pentoside and deoyhexoside.[49] Thus, peaks 3 and 4 were tentatively identified as quercetin-pentoside and quercetin-deoxyhexoside. Peak 5 was tentatively identified as diosmetin-diglycoside based on the deprotonated ion $[M-H]^-$ at m/z 607.[50] Diosmetin was expected in the extract in corresponding to the report of diosmetin from the seed of *A. farnesiana*.[51] Previously, a number of compounds have been previously isolated and identified from *A. farnesiana*. For instance, diosmetin, a new flavone described as farnisin and sitosterol glucoside were reported from the seed.[51] A novel diterpene β-D-glucoside, designated as farnesiaside was also isolated of the seed.[52] This study reported the presence of flavonoids galloyl glucosides and flavonoid glycosides from the leaves of *A. farnesiana* ethanolic extract.

Influences of phenolics compound such as phenolic acid, flavonoids and tannin with the pharmacological properties of an extract have been frequently reported. From the HPLC results, it could be deduced that the biological activity of *A. farnesiana* extract observed in this study was contributed by the presence of quercetin-deoxyhexoside. Rhamnose is an example of deoxyhexose sugar found associated with flavonoids, commonly found in the form of disaccharide as rutinose (6-*O*-α-L-rhamnosyl-D-glucose) and neohesperidose (2-*O*-α-L-rhamnosyl-D-glucose). The glycosylated flavonoids which are ubiquitously found in fruits and vegetables have been frequently studied due to their health effects. For instance, although the metal chelating activity of flavonoid *in vitro* was lower than EDTA,[53] quercetin and rutin were effective chelators of transition metals.[54] Excessive intakes of food rich in metal chelators reduce the absorption of iron in the gut and eventually developing iron deficiency particularly to infant, children and pregnant woman.[55]

Evaluation of crude extract frequently demonstrated the synergistic and antagonistic effects of constituents in the extract. For example, the green tea extract exhibited high NO scavenging activity compared to the extract components; caffeine and theanine at concentration 5 μg/ml, but the activity was lower compared to tannin which indicated the contribution of tannin to the NO scavenging activity of the green tea extract.[56] The structure activity relationship of compound such as glycosylation at position 3 exhibited by kaempferol-3-*O*-glucoside and quercetin 3-*O*-glucoside reduced the antioxidant activity of an extract.[57] Previously, the essential oil from *A. farnesiana* was responsible for the antifungal activity against rice pathogenic fungi.[58] However, the method applied to prepare the ethanol extract in this study had eliminated any volatile compound thus the antimicrobial and antioxidant observed was presented by the flavonoid compounds in the extract. It was reported that quercetin and its isomer; morin were active antimicrobial agent, nevertheless, compared with the glycosidic form of quercetin; rutin which was also found in the extract of *A. farnesiana* leaves extract was inactive against microbial growth.[59] Previously, plant extracts with MIC values 1.25 to 5.0 mg/ml determined by broth microdilution against *B. subtilis* was regarded as showing antibacterial activity.[60] Therefore, from the MIC values exhibited by *A. farnesiana* extract (0.8 mg/ml) against *B. subtilis* in this study, the extract could be regarded as effective antimicrobial agent in comparison with those medicinal plant extracts.

In this study, the assays selected have been carried out frequently as preliminary or complementary assays in evaluating the antioxidant, antimicrobial and cytotoxicity activities of plant extract. Nevertheless, the assays may not be able to reflect the effect of the extract *in vivo*. For instance, the ability of *A. farnesiana* ethanolic extract to chelate a metal in this study only reflected the nature of the extract which consisted of a number of compounds with different affinities for iron compared with EDTA in competing with ferrozine to bind the available iron.[15] Result on cytotoxicity was also in accordance with the absence of toxicity or mortality exhibited by ethanolic extract of lotus which consisted of catechin glycosides and flavonoid glycosides including quercetin-3-*O*-rhamnoside.[61] The *A. farnesiana* ethanolic extract may not suitable to be developed as an anticancer agent but the cytotoxicity test suggested the safety of extract rich in flavonoid compounds.

This study is a part of a continuous effort to appreciate and provide scientific study to the plants with relevance to food and health. The method used to prepare extract has contributed to the extraction of constituents detected in the extract and the structures of flavonoid were responsible for the observed activities. The presence of quercetin-deoxyhexoside in the extract influenced the biological activity of *A. farnesiana* extract in agreement with the reports from other studies. Nevertheless, bioassay guided isolation is suggested to study the phytochemicals involved.

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Comparative Study of *In vitro* Antioxidant, Antibacterial and Cytotoxic Activity of Two Bangladeshi Medicinal Plants- *Luffa cylindrica* L. and *Luffa acutangula*

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ABSTRACT

Luffa cylindrica L. (LUCY) and *Luffa acutangula* (LUAC) are popular & consumed as vegetables and foods and in folklore medicine to treat various ailments in Bangladesh. The purpose of the present study was to investigate the antioxidant, antibacterial and cytotoxic activities of the n-hexane, chloroform and ethyl acetate extracts of leaves of LUCY & LUAC. Antioxidant and antibacterial activities were evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and disc diffusion method, respectively. The plants were also assessed for cytotoxic activity by brine shrimp lethality assay. Antioxidant activity of different concentrations of plant extracts were found to increase in a concentration dependent manner. The most prominent activity was found with ethyl acetate extract of LUCY and n-hexane extract of LUAC with an IC_{50} value of 50.32 & 50.95 µg/mL, respectively. All extracts/fractions showed low to moderate levels of anti-bacterial activity against most of the tested strains (zone of inhibition = 5-13 mm). All extracts/fractions displayed considerable general toxicity towards brine shrimps. The LC_{50} values of the extracts/fractions were of 15.92 to 33.69 µg/mL compared to vincristine sulphate (LC_{50} = 0.91 µg/mL). This is the first report for cytotoxic activity of LUCY and antibacterial activity of LUAC leaves extracts. On the basis of results obtained, it is suggested that both LUCY and LUAC leaves extracts may be a potential source of natural antioxidants, antimicrobial compounds and anticancer agents to be used in the treatment of various oxidative disorders, infectious diseases caused by resistant microorganisms and cancer, respectively.

Key words: *Luffa cylindrica* L., *Luffa acutangula*, DPPH, disc diffusion, brine shrimp lethality bioassay

INTRODUCTION

The study of diseases and their treatment have been existing since the dawn of human civilization. Also for the treatment of a range of diseases, herbal drugs have been used since ancient times as medicines. Medicinal plants have played a key role in world health. The World Health Organisation (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care.

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A large proportion of the Bangladeshi population for their physical and psychological health needs depend upon traditional systems of medicine. Medicinal plants have become the focus of intense study in terms of conservation and as to whether their traditional uses are supported by actual pharmacological effects or merely based on folklore.[1-2]

The luffa, loofah, or lufah are tropical and subtropical vines comprising the genus *Luffa*, the only genus of the subtribe Luffinae. The fruit of at least two species, *Luffa acutangula* and *Luffa aegyptiaca (Luffa cylindrica)*, is grown, harvested before maturity, and eaten as a vegetable, popular in Asia and Africa. The plant *Luffa cylindrica* L. belonging to family Cucurbitaceae is commonly called as Rajakoshaataki or Sponge gourd[3] cultivated throughout the world and is distributed mainly in tropical to warm-temperate areas.[4] The plant is reputed to have antitubercular and antiseptic properties.[5-7] Antioxidant activity of the seed oil[8] & antiinflammatory activity of the seed extract were reported.[3] Extracts of fruits showed antioxidant,[9] antibacterial and antifungal activity.[10] On the other hand *Luffa acutangula* also known as ridge gourd is an important member of the

family Cucurbitaceae grown in Bangladesh as a year-round vegetable. It is nutritionally rich in vitamin A, C and Fe[11] and was found to contain carbohydrate, protein, fat and rich in Cu, Ni, Zn, Pb, Co, Cd, Fe, Cr, Ca and Na.[12] LUAC has a considerable medicinal importance. The fresh fruits of it showed a certain antioxidant activity.[13] Alcohol and chloroform extracts of fruits were shown to have antidiabetic activity.[14] The methanolic fruit extract was reported for anticancer activity.[15] Its abortifacient, antitumor, ribosome inactivating and immunomodulatory activities were reported earlier.[16-18] Recently it has gained attention from the nutritionists due to the presence of antioxidant activity.[13]

Literature reviews pointed out that no studies combining the antioxidant, antibacterial and cytotoxic activities of the leaves of LUCY & LUAC have so far been undertaken. Coupled with our continuous interest of pharmacological screening of Bangladeshi medicinal plants, in this study we aimed to investigate the antioxidant, antibacteiral and cytotoxic activities of the n-hexane, chloroform and ethyl acetate extracts of leaves of LUCY & LUAC.

MATERIALS AND METHODS

Drugs and chemicals

DPPH (1, 1-diphenyl, 2-picryl hydrazyl), was obtained from Sigma chemical co. USA. Ascorbic acid was obtained from SD Fine chem. Ltd., Biosar, India. DMSO (dimethyl sulfooxide) was purchased from Merck, Germany. Kanamycin was collected from Square Pharmaceuticals Ltd., Bangladesh.

Collection and Identification of the plant

The fresh leaves of LUCY and LUAC were collected during the month of January 2010 from the area of Barguna, Bangladesh and identified by DR. M.A. Razzaque Shah, Tissue Culture Specialist, BRAC Plant Biotechnology Laboratory, Bangladesh. The fresh leaves of the plants were first washed with water to remove adhering dirt and then cut into small pieces, sun dried for 4 days. After complete drying, the entire portions were pulverized into a coarse powder with the help of a grinding machine and were stored in an airtight container for further use.

Extraction and solvent-solvent partitioning of plant material

The dried leaves were coarsely powdered from which about 50 gm powders of each plant were extracted with 3 times ethanol of their weight in a flat bottom glass container, through occasional shaking and stirring for 7 days. The extracts were then filtered through filter paper (Double Rings filter paper 102, 11.0 cm). The filtrates were concentrated at 50 ºC under reduced pressure using vacuum pump rotary evaporator (STUART RF3022C, UK) to afford a greenish mass. The concentrated ethanol extract was made slurry with water. The slurry was taken in a separating funnel and few ml of n-hexane (40 ml) was added. The funnel was shaken vigorously and allowed to stand for few minutes. The n-hexane layer (upper layer) was collected. The process was repeated two times. The combined n-hexane extract was concentrated. After n-hexane extraction, chloroform (40 ml) was added to the aqueous solution and the mass was shaken vigorously in a separating funnel. Then the funnel was allowed to stand for few minutes for the complete separation of the layers. The organic (lower layer) layer was collected. The process was repeated two times. The aqueous layer left after chloroform extraction was again extracted two times with ethyl acetate.

ANTIOXIDANT ACTIVITY TEST

DPPH radical scavenging activity

i) Qualitative analysis

A suitably diluted stock solutions were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extracts. The plates were dried at room temperature and were sprayed with 0.02% DPPH in methanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted.[19]

ii) Quantitative analysis

The free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Brand-Williams *et al*..[20] During this experiment the test samples n-hexane, chloroform and ethyl acetate extracts of both plants at different concentrations were mixed with 3.0 ml of DPPH methanol solution. The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extracts as compared to that of ascorbic acid by UV spectrophotometer (UV–1501PC SHIMADZU, Japan) at 517 nm. Ascorbic acid was used as a positive control. Percent scavenging of the DPPH free radical was measured using the following equation-

$$\% \text{ DPPH radical scavenging} = \left[1 - \left(\frac{As}{Ac}\right)\right] \times 100$$

Here, Ac = absorbance of control, As = absorbance of sample solution.

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated. The lower IC_{50} indicates higher radical scavenging activity and vice versa.

IN VITRO ANTIBACTERIAL SCREENING

Antibacterial activities of n-hexane, chloroform and ethyl acetate extracts of both plants were carried out by disc diffusion method.[21-22] In this method, measured amount of the test samples were dissolved in definite volumes of solvent to give solutions of known concentration. Then sterile filter paper discs (5 mm diameters) were impregnated with known test substances and dried. The dried discs were placed on plates (Petri dishes, 120 mm diameters) containing a suitable medium (nutrient agar) seeded with the test organisms. These plates are kept at low temperature (4 ºC) for 24 hours to allow maximum diffusion. The dried discs absorb water from the agar medium and the material under test is dissolved. The test material diffuses from the discs to the surrounding medium according to the physical law that controls the diffusion of molecules through agar gel. There is a gradual change of the tested material concentration on the agar surrounding each disc. The plates are then kept in an incubator (37 ºC) for 24 hours to allow the growth of microorganism. If the test material has antimicrobical activity, it will inhibit the growth of the microorganism, giving a clear, distinct zone called "Zone of Inhibition". The antimicrobial activity of the test agent is determined in terms of millimeter by measuring the diameter of the zone of inhibition. The greater zone of inhibition indicates the greater activity of the test material against the test organism.

In our present study, the antibacterial activity of n-hexane, chloroform, and ethyl acetate fraction of both plants were investigated in comparison with standard kanamycin (30 µg/disc) against a number of pathogenic Gram- positive (*Bacillus megaterium, B. subtilis, Staphylococcus aureus* and *Sarcina lutea)* and eight Gram- negative *(Salmonella paratyphi, S. typhi, Vibrio parahemolyticus, V. mimicus, Escherichia coli, Shigella dysenteriae, S. boydii* and *Pseudomonas aeruginosa*) bacteria. The microorganisms were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. The sample solution of the material to be tested was prepared by dissolving a definite amount of material in appropriate solvent to attain a concentration of 50 mg/ml. 10 µl of such solution was applied on sterile disc (5 mm diameter, filter paper) and allowed to dry off the solvent in an aseptic hood. Thus, such discs contain 500 µg of crude extracts. To compare the activity with standard antibiotics, Kanamycin (30 µg/disc) was used.

CYTOTOXICITY TEST

Brine shrimp Lethality Bioassay

Brine shrimp lethality bioassay was used for probable cytotoxic activity.[23-25] The eggs of Brine Shrimp (*Artemia salina* Leach) was collected from local pet shops and hatched in a tank at a temperature around 37 ºC with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solution of the sample was prepared by dissolving required amount of extract in specific volume of pure dimethyl sulfoxide (DMSO). With the help of a pasteur pipette nauplii were exposed to different concentrations of the extracts.

Preparation of test groups

20 mg of sample was dissolved in 2 ml of DMSO to obtain a solution having concentration of 10 µg/ml. From that test solution different volumes were added to premarked glass vials or test tubes containing 5 ml of seawater and 10 shrimp nauplii, so as to make the final concentration of samples in the vials or test tubes 10 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml. Vincristine Sulphate and DMSO were used as positive and negative control respectively.

Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration. The median lethal concentration (LC_{50}) and 95% confidence intervals were determined as the measure of toxicity of the extract or fractions.

STATISTICAL ANALYSIS

Lethality assays were evaluated by Finney computer statistical program[26] to determine the LC_{50} values and 95% confidence intervals.

RESULTS

Antioxidant activity

n-hexane, chloroform, ethyl acetate extracts for the leaves of both the plants showed excellent antioxidant activity with the IC_{50} value of 56.27 µg/ml, 61.24 µg/ml, 50.32 µg/ml respectively for LUCY and 50.95 µg/ml, 57.81µg/ml, 51.77 µg/ml, respectively for LUAC compared with the standard ascorbic acid with IC_{50} value of 43.22 µg/ml (Table 2). As is observed in Table 1, the crude extracts of both the plants exhibited a concentration dependant DPPH radical scavenging activity.

Antibacterial test

Table 3 shows the antibacterial activity of LUCY & LUAC with the zone of inhibition value ranged from 5-13 mm by disc diffusion method. Among different fractions tested, n-hexane extracts of both plants exhibited potent inhibitory

Table 1: The concentration of antioxidant activity of leaves of LUCY and LUAC

Concentration (µg/ml)	Percent inhibition						
	Ascorbic acid	*Luffa cylindrica* L.			*Luffa acutangula*		
		Chloroform extract	n-hexane extract	Ethyl acetate extract	Chloroform extract	n-hexane extract	Ethyl acetate extract
20	25.26	28.14	24.27	23.77	19.84	22.78	24.01
40	58.23	37.73	29.72	43.36	46.00	47.21	43.36
60	81.00	51.89	42.85	69.23	50.00	73.84	64.03
80	86.05	69.23	73.62	76.92	70.96	75.38	79.02
100	86.53	83.23	76.92	41.53	76.92	76.15	80.63

Table 2: IC_{50} data of leaves of LUCY and LUAC compared to ascorbic acid

Plant extract	IC_{50} (µg/ml)		
	Luffa cylindrical L.	*Luffa actangula*	Ascorbic acid
Chloroform extract	61.24	57.81	43.22
N-hexane extract	56.27	50.95	
Ethyl acetate extract	50.32	51.77	

Table 3: *In vitro* antibacterial activity of leaves of LUCY and LUAC and standard kanamycin discs.

Name of bacteria	Zone of inhibition (mm)						
	Kanamycin (30µg/disc)	*Luffa cylindrica* L.			*Luffa actangula*		
		Chloroform extract	n-hexane extract	Ethyl acetate extract	Chloroform extract	n-hexane extract	Ethyl acetate extract
Gram positive bacteria							
Staphylococus aereus	26	7	8	–	9	13	7
Bacillus megaterium	30	8	11	7	7	13	6
Bacillus subtilis	23	7	10	–	8	12	6
Sarcina lutea	24	6	10	–	9	10	7
Gram negative bacteria							
Salmonella typhi	25	8	12	–	8	10	6
Escherichia coli	22	7	10	–	8	11	5
Shigella dysenteriae	25	6	9	7	10	13	–
Pseudomonas aeruginosa	25	7	11	7	7	11	5
Salmonella paratyphi	25	7	9	7	7	9	5
Shigella boydii	25	6	10	–	6	10	–
Vibrio mimicus	28	6	9	–	6	9	–
Vibrio parahemolyticus	26	6	8	–	7	10	5

(–) = No significant antibacterial activity.

activity followed by chloroform extracts whereas ethyl acetate extracts showed little or no activity on the tested microorganisms. The most sensitivity was observed in *Staphylococus aureus* (13 mm), *Bacillus megaterium* (13 mm) & *Shigella dysenteriae* (13 mm) by n-hexane extract of LUAC and in *Salmonella typhi* (12 mm) by the same extract of LUCY.

Cytotoxicity test

Cytotoxicity of leaves extracts of LUCY & LUAC is shown in Table 4 and LC_{50} value found with the range of 15.92 to 33.69 µg/mL compared to vincristine sulphate (data not shown). Ethyl acetate extract of LUCY and n-hexane extract of LUAC exhibited the potent cytotoxic activity with the LC_{50} value of 15.92 & 20.40 µg/ml, respectively.

DISCUSSION

Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea and herbs may contribute to shift the balance toward an adequate antioxidant status. Thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years.[27] In this study, antioxidant potential of n-hexane, chloroform and ethyl acetate extracts of both plants was evaluated based on the ability to scavenge the DPPH. This assay is highly important to provide information about the reactivity of organic compounds with stable free radicals,

Table 4: Effect of plant extracts on shrimp naupli

	Luffa cylindrica L.			*Luffa acutangula*		
	Chloroform extract	n-hexane extract	Ethyl acetate extract	Chloroform extract	n-hexane extract	Ethyl acetate extract
Log_{10} C	Percent mortality					
1.000	40	20	40	30	30	30
1.301	50	50	60	50	60	50
1.602	50	50	60	70	60	50
1.778	70	60	80	70	70	70
1.903	80	70	90	80	80	90
	LC_{50} (µg/ml)					
	20.61	33.69	15.92	21.25	20.40	23.09
	95% confidence interval					
	9.043-46.976	16.841-67.411	8.743-28.990	11.344-39.833	10.157-40.975	13.456-39.641

because of the odd number of electrons. DPPH shows a strong absorption band at 517 nm in visible spectrum (deep violet color). As the electron became paired of in the presence of free radical scavenging, the absorption vanishes and the resulting discoloration stoichiometrically coincides with the number of electrons taken up. The bleaching of DPPH absorption is representative of the capacity of the test drugs to scavenge the free radicals independently.[28] All extracts were found to exhibit antioxidant activity in the qualitative assay by displaying yellowish spots against purple background after the TLC plate being sprayed with the DPPH solution. In the quantitative assay the most promising antioxidant potential was observed with the ethyl acetate extract for LUCY and n-hexane extract for LUAC with an IC_{50} value of 50.32 µg/mL and 50.95 µg/mL respectively compared to 43.22 µg/ml for ascorbic acid (Table 2). This indicates that the plant extracts are electron donors so they can react with free radicals, convert them into more stable products and terminate the radical chain reaction. This may be important in protecting cellular DNA, lipids and proteins from free radical damage.[29] The percent inhibition of ethyl acetate extract at 100 µg/mL was about two fold less (41.53%) than that of chloroform extract in case of LUCY. The maximum inhibition or reduction of the DPPH absorbance with the chloroform extract for LUCY and ethyl acetate extract for LUAC was 83.23% and 80.63% respectively at the highest test concentration of 100 µg/ml. Standard ascorbic acid was found to have 86.53% activity at the concentration of 100 µg/ml. Our findings are in concordance with the previous studies of fruit & seed extracts of LUCY[8-9] and fruit extracts of LUAC.[13] Although the antioxidant activity of the extracts was considerably lower than the positive control, this is often the case with crude extracts. The positive control is a pure compound whereas the extracts are mixtures of several compounds. The compounds which were actually responsible for the antioxidant activities of the extracts were present in much lower concentrations than the concentrations of the crude extracts. Therefore, it could be assumed that isolation and purification of active constituents from these active extracts would lead to antioxidant compounds with comparable activity to that of the standard, Ascorbic acid.[30] So it is assumed that the antioxidant activity of the plant extracts depends on: the type and the polarity of the extracting solvent, the extracting technique, the purity of the active principle, the antioxidant test and the substrate used.[31]

It was observed that some phytochemicals such as alkaloids, flavonoids, glycosides, steroids and saponins were commonly present in all genus of Cucurbitaceae and some were found species specific.[32] So it is assumed that the antioxidant potential may be due to the common presence of various secondary metabolites. The essence of these secondary metabolites is also beneficial for maintenance of human health and chronic degenerative diseases.[32]

The zone of inhibition obtained of the n-hexane, chloroform & ethyl acetate extracts of LUCY and LUAC and the standard drug for the antibacterial activity are shown in Table 3 and found in the range of 5-13 mm against all tested bacteria. The n-hexane extracts of both the plants were found to have potent inhibitory activity against both gram positive and gram negative organisms followed by chloroform extract. This inhibitory activity may be attributed to be the presence of some active principles in them which are able to restirct the growth of bacteria. These active principles may inhibit protein synthesis of bacterial cell wall or alter the membrane function, inhibit protein synthesis or synthesis of purine and pyrimidines, hinder respiration or antagonize the metabolic pathways of microorganism leading to retardation of growth of bacteria. These active principles in these plants could be used as potent antibiotics.[33] Furthermore the ethyl acetate extracts of both the plants showed poor or no activity against tested organisms. The inhibitory activity to different microorganisms of LUCY supports with an earlier report in which the ethanolic extracts

of LUCY fruits were found to have significant antibacterial activity [10]. Highest antibacterial activity was shown by n-hexane extract of LUAC against *Staphylococus aereus* (13 mm), *Bacillus megaterium* (13 mm) & *Shigella dysenteriae* (13 mm) and of LUCY against *Salmonella typhi* (12 mm). The most resistant bacteria were *Escherichia coli* (5 mm), *Pseudomonas aeruginosa* (5 mm), *Salmonella paratyphi* (5 mm), *Vibrio parahemolyticus* (5 mm) observed in ethyl acetate extracts of LUAC. The results obtained in the present study revealed that Gram positive bacteria were more sensitive than Gram negative bacteria to the plant extracts. Various workers have already reported similar results.[34-36] The difference in sensitivity might be ascribed to the difference in morphological constitutions between Gram-positive and Gram-negative organisms. Many plant species present inhibition zones of differing diameters; however, size difference of the inhibition zone depends primarily upon many factors for e.g. diffusion capacity of substances (present in the extracts) in the agar medium, antimicrobial activity of diffused substances, growth and metabolic activity of microorganisms in the medium. Inhibition zone diameter can further be associated with polarities of substances which make up the tested extracts and also with cell wall composition of test organisms since Gram-positive bacteria present cell walls with lower lipid levels than do Gram-negative bacteria.[37]

The brine shrimp lethality assay (BSL) has been used extensively in the primary screening of the crude extracts as well as the isolated compounds to evaluate the toxicity towards brine shrimps, which could also provide an indication of possible cytotoxic properties of the test materials.[24] It has been established that the cytotoxic compounds generally exhibit significant activity in the BSL assay, and this assay can be recommended as a guide for the detection of antitumour and pesticidal compounds because of its simplicity and low cost.[38] Earlier reports in several plant extracts showed a good correlation of this bioassay with the cytotoxic activity.[39] The assay also exhibited a good correlation with cytotoxicity in cell lines such as 9KB, P388, L5178Y and L1210.[24],[40-42] In the present study all extracts and fractions of LUCY & LUAC displayed considerable general toxicity & different mortality rate towards shrimp nauplii (Table 4). The mortality rate of nauplii was found to be increased in concentration of each of the samples. It was found that very low concentrations of plant extracts (10 µg/mL) were detrimental to the shrimp nauplii. The LC_{50} values of the plant extracts/fractions were within the range of 15.92 to 33.69 µg/mL, which was compared to that of the positive control (vincristine sulphate) with the value of 0.91 µg/mL (data not shown) determined earlier in our laboratory.[43] Ethyl acetate extract of LUCY and n-hexane extract of LUAC showed the highest level of toxicity with the LC_{50} values of 15.92 and 20.40 µg/mL, respectively. Previous studies of LUAC have already demonstrated the occurrence of cytotoxic activity[15] although no similar observation was found in case of LUCY. The inhibitory effect of the extract might be due to the toxic compounds present in the active fraction that possess ovicidal and larvicidal properties. The metabolites either affected the embryonic development or slay the eggs.[44] So the cytotoxic effects of the plant extracts enunciate that it can be selected for further cell line assay because there is a correlation between cytotoxicity and activity against the brine shrimp nauplii using extracts.[44-45]

It was found that the antimicrobial activity of the extracts correlated strongly with the DPPH activity. This may be due to the availability of the antioxidative compounds to exert different inhibitory effect against tested organisms.[46] Moreover plant-derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells[47] and antitumor activity in experimental animals.[48] Antitumor activity of these antioxidants is either through induction of apoptosis[49] or by inhibition of neovascularization.[50] The implication of free radicals in tumors is well documented.[51-52]

Finally it was observed from Table 1, Table 2, Table 3 and Table 4 that LUCY & LUAC extracts were different in their antioxidant, antibacterial as well as cytotoxic activities depending on the extractive solvents used. This result agrees favourably with the suggestion of Oloke and Kolawole[53] that bioactive components of any medicinal plant may differ in their solubility depending on the extractive solvents used. Takazawa *et al*.[54] suggested that there is a need to employ broad range of extractive solvents in the extraction of possible phytochemicals from medicinal plants. Besides the variation of activities of the plant extracts is presumed to be due to the presence of different active compounds and their degree of nature to different solvents.

CONCLUSION

From the experiment it has shown that different extracts (n-hexane, chloroform and ethyl acetate) have been used *in vitro* to inhibit the growth of some disease causing bacteria. It can therefore be suggested that plant extracts have great potential as antimicrobial compounds against microorganisms and they can be used in the treatment of infectious diseases caused by resistant microorganisms. They can also be a source of natural antioxidants & anticancer agents. The data obtained showed that n-hexane extract for LUAC and ethyl acetate extract for LUCY are more potent at all of the activities tested in the present study except in antibacterial activity where n-hexane fraction showed strongest activity for both LUCY and LUAC. Due to their antibacterial and antioxidant activities LUCY &

LUAC extracts have promising potential as a source of natural antioxidant and antimicrobial agents. Therefore these results are encouraging enough to pursue characterization of these fractions in different other models in detail. Further studies may also be conducted to isolate & purify the active constituents to evaluate the cytotoxic activity in human cell line cultures.

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Antioxidant and Hepatoprotective Activity of *Lagenaria siceraria* Aerial parts

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ABSTRACT

Introduction: *Lagenaria siceraria* is traditionally used in liver disorders and various free radicals induced diseases. The present study was carried out to evaluate the antioxidant and hepatoprotective activities of the aerial parts of *L. siceraria* methanol extract (MELS). **Methods:** DPPH, nitric oxide, superoxide, hydrogen peroxide, lipid peroxide free radical scavenging activity, reductive ability and total phenolic and flavonoid content of MELS were determined. Hepatoprotective activity of the extract was investigated against carbon tetrachloride induced hepatotoxicity in rats. **Results:** The results explored significant *in vitro* antioxidant activity of MELS. It also showed potent hepatoprotective activity in rat, which was evident from its significant effect on the levels of serum biomarker enzymes and total protein & bilirubin. Significant improvement of the endogenous antioxidant status by the treatment of MELS further reflects its hepatoprotective potential, which was finally substantiated by the histological studies of the liver tissues. **Conclusion:** The results reveal potent hepatoprotective activity of MELS which is probably attributed to its significant free radical scavenging activity and high polyphenolic and flavonoid contents.

Key words: Antioxidant, Cucurbitaceae, Carbon tetrachloride, DPPH, Free radical, Hepatoprotective, *Lagenaria siceraria*.

INTRODUCTION

Free radicals generated either exogenously or endogenously in our body have been implicated in the pathophysiology of various clinical disorders, such as liver cirrhosis, inflammation, atherosclerosis, diabetes, cancer, neurodegenerative diseases, nephrotoxicity and also the process of aging.[1-5] The link between free radicals and diseases has led to considerable research into nontoxic drug that can scavange the free radicals. These radicals react with cell membrane, induce lipid peroxidation and are responsible for various deleterious effects in cells and tissues where they are generated.[6] The inhibition of free radical generation can serve as facile model for evaluating the activity of hepatoprotective agents. Several plant extracts and plant products have been found to possess significant *in vitro* and *in vivo* antioxidant property.[7-11]

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Lagenaria siceraria (Mol.) Standley, commonly known as bottle-gourd (in English), belongs to cucurbitaceae family. The plant is widely available throughout India as an edible vegetable. It is a climbing or trailing herb, with bottle or dumb-bell shaped fruits. Both of its aerial parts and fruits are commonly consumed as vegetable. Traditionally the plant is used as medicine in India, China, European countries, Brazil, Hawaiian island etc. for its cardiotonic, general tonic, hepatoprotective, diuretic properties.[12] Further, antihepatotoxic, analgesic and anti-inflammatory, hypolipidemic, antihyperglycemic, immunomodulatory and antioxidant activities of its fruit extract have been evaluated.[13-17] *Lagenaria siceraria* fruits are good source of vitamin B complex, ascorbic acid, fibers, proteins, cucurbitacins, saponins, fucosterols and compesterols, polyphenolics, flavones-C-glycoside.[13,14,18-20] Methanol extract of its leaves showed the presence of sterols, polyphenolics, flavonoids, saponin, protein and carbohydrates.[21] A novel protein, Lagenin has also been isolated from its seeds and it possesses antitumor, immunoprotective and antiproliferative properties.[22] Although extensive studies have been carried out on its fruits and seeds, pharmacology of the aerial parts of *L. siceraria* however has not been explored yet. The present investigation was therefore carried out to evaluate the antioxidant and hepatoprotective potential of methanol extract of *L. siceraria* aerial part (MELS).

MATERIALS AND METHODS

Plant Material

The aerial parts of *L. siceraria* was collected in November 2008, from Madanpur, West Bengal, India and identified by the Botanical Survey of India, Howrah, India. A voucher specimen (P/LS/1/08) was retained in our laboratory for further reference.

Preliminary Phytochemical Screening

Preliminary phytochemical screening was carried out by following the standard procedures.[23]

Preparation of Plant Extract

The aerial parts were dried under shade and powdered in a mechanical grinder. The powdered material was extracted with methanol using soxhlet apparatus. This extract was filtered and concentrated in *vacuo* in a Buchi evaporator, R-114 and kept in vacuum desiccators until use. The yield was 18.13% w/w with respect to dried powder. Various concentrations of methanol solution of MELS was used for *in vitro* antioxidant studies, while, aqueous suspension of MELS was prepared using 2% (v/v) Tween-80 for oral administration.

Animals

Healthy Wistar albino rats (180 g ± 20) were used for the present study. They were maintained at standard laboratory conditions and fed with commercial pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The animals were acclimatized to laboratory condition for one week before commencement of experiment. The experiments were performed following the animal ethics guidelines of Institutional Animals Ethics Committee.

Acute Toxicity Study

Healthy rats were starved overnight and then were divided into five groups ($n = 4$). Group I-IV animals were orally fed with MELS in increasing dose levels of 0.5, 1.0, 1.5 and 2.0 g/kg b.wt, while group V (untreated) served as control. The animals were observed continuously for first 2 h for any gross change in behavioral, neurological and autonomic profiles or any other symptoms of toxicity and mortality if any, and intermittently for the next 6 h and then again after 24 h, 48 h and 72 h for any lethality or death. One-tenth and one-fifth of the maximum safe dose of the extract tested for acute toxicity, were selected for the experiment.[24]

In vitro Antioxidant Activity Study

Various concentrations of MELS (10-160 µg/ml in methanol) was used for *in vitro* antioxidant studies on different models, DPPH, NO, SO, H_2O_2 and LPO. For reductive ability study, 100-800 µg/ml concentration of the extract was used. Butylated hydroxyl toluene (BHT) was used as standard.

Determination of DPPH Radical Scavenging Activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was measured using the method of Cotelle *et al*[25] with some modifications. 3 ml of reaction mixture containing 0.2 ml of DPPH (100 µM in methanol) and 2.8 ml of test or standard solution of various concentrations was incubated at 37 °C for 30 min and absorbance of the resulting solution was measured at 517 nm using Beckman model DU-40 spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the formula:

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Determination of Nitric oxide Scavenging Activity Assay

At physiological pH, sodium nitroprusside generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be measured by the Griess reaction.[26] 1 ml of 10 mM sodium nitroprusside was mixed with 1 ml of test or standard solution of different concentrations in phosphate buffer (pH 7.4) and the mixture was incubated at 25 ºC for 150 min. From the incubated mixture, 1 ml was taken out and 1 ml of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added to it. Absorbance of the chromophore formed by the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was read at 546 nm and percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Determination of Superoxide Radical Scavenging Activity

Superoxide anion scavenging activity was measured according to the method of Robak and Gryglewski[27] with some modifications. All the solutions were prepared in 100 mM phosphate buffer (pH 7.4). 1 ml of nitroblue tetrazolium (NBT, 156 µM), 1 ml of reduced nicotinamide adenine dinucleotide (NADH, 468 µM) and 3 ml of test/standard solution were mixed. The reaction was initiated by adding 100 µl of phenazine methosulphate (PMS, 60 µM). The reaction mixture was incubated at 25 ºC for 5 min, followed by measurement of absorbance at 560 nm. The percentage inhibition was calculated from the above formula.

Determination of Hydrogen peroxide Scavenging Activity

The hydrogen peroxide scavenging ability of the extract was determined according to the method of Ruch *et al*.[28] A solution

of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extract or standards (of different concentrations) in phosphate buffer (3.4 ml) was added to the H_2O_2 solution (0.6 ml, 40 mM). The absorbance of the reaction mixture was recorded at 230 nm after 10 min against a blank solution of phosphate buffer. Percentage of H_2O_2 scavenging was calculated using the above formula.

Determination of Inhibition of Lipid Peroxidation

Rat liver homogenate was used as the source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. Reaction mixture (0.5 ml) containing rat liver homogenate (0.1 ml, 25% w/v) in Tris–HCl buffer (40 mM, pH 7.0), KCl (30 mM), ferrous ion (0.16 mM) and ascorbic acid (0.06 mM) were incubated at 37 °C for 1 h in the presence or absence of the extracts or standards. The lipid peroxide formed was measured by TBARS formation according to the method of Ohkawa *et al.*[29] Incubation mixtures were treated with sodium dodecyl sulphate (SDS; 8.1%, 0.2 ml), thiobarbituric acid (TBA; 0.8%, 1.5 ml) and acetic acid (20%, 1.5 ml). The total volume was then made up to 4 ml with distilled water and kept on water bath for 30 min. After cooling, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and centrifuged at 4000 rpm for 10 min. The absorbance of the organic layer, containing the colored TBA-MDA complex, was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compound with those of the control, using the formula mentioned above.

Determination of Reductive Ability

Reducing power of the test samples was determined on the basis of the ability of their antioxidant principles to form colored complex with potassium ferricyanide, trichloro acetic acid (TCA) and $FeCl_3$, which is measured at 700 nm.[30] 1 ml of different concentrations of the extract or standard solution was mixed with potassium ferricyanide (2.5 ml, 1%) and 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50 °C for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was taken out and to this 2.5 ml water and 0.5 ml $FeCl_3$ (0.1%) were added and absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

Determination of Total Phenolic Compounds

The amount of total phenolic compounds in MELS was determined using Folin-Ciocalteu's reagent and sodium carbonate solution and the absorbence was measured at 760 nm.[31] A calibration curve of standard pyrocatechol was prepared and the results were expressed as mg of pyrocatechol equivalents/g of dry extract.

Determination of Total Flavonoid Content in the Extract

The total flavonoid content of MELS was determined spectrophotometrically.[32] Briefly 0.5 ml of 2% aluminium chloride in ethanol was mixed with same volume of extract (1.0 mg/ml). Absorption readings at 415 nm were taken after 1 h against a blank (ethanol). The total flavonoid content was determined using a standard curve with quercetin (0-50 mg/L). The mean of three readings was used and expressed as mg of quercetin equivalents/g of dry extract.

Hepatoprotective Activity Study

Experimental Design

After seven days of acclimatization, the healthy male rats were divided into five groups of six animals each. Treatment was done for 14 days. Group I served as control and received 2% Tween-80; 1 ml/kg. Group II-V received CCl_4 in liquid paraffin (1:2) (1 ml/kg i.p.) once in every 72 h. Group II was not treated with any drug and served as CCl_4 control. Group III and IV were orally administered 200 and 400 mg/kg MELS once daily. Group V received standard drug silymarin (25 mg/kg, p.o.).[33]

After 24 h of the last dose, blood was collected from retro-orbital plexus under ether anesthesia. The blood samples were allowed to clot and the serum was separated by centrifugation at 2500 g at 37 °C and was used for biochemical estimation. All the animals were then sacrificed and liver tissues were collected for the evaluation of *in vivo* antioxidant status and histopathological examination.

Estimation of Biochemical Parameters

Serum was analysed for various biochemical parameters like serum glutamic pyruvate transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT)[34] and alkaline phosphatase (ALP)[35] activities. The total protein concentration and total bilirubin were also measured by the method of Lowry *et al*[36] and Mallay and Evelyn[37] respectively. All the analysis were performed by using commercially available kits from Span Diagnostics Ltd.

Evaluation of Antioxidant Properties

For assessment of antioxidant activities, 25% (w/v) liver tissue homogenate for each animal was prepared using KCl solution (1.15% w/v) and centrifuged at 3000 g at 4 °C for 1 hr. The supernatant was used for the determination of lipid peroxidation (LPO)[29] (Ohkawa *et al.*, 1979) and endogenous antioxidant systems such as reduced glutathione (GSH),[38] superoxide dismutase (SOD)[39] and catalase[40] (CAT).

Histological Observation

For histological study, the liver tissues were collected and immediately fixed in 10% formalin, dehydrated in

gradual ethanol (50-100%), cleared in xylene and embedded in paraffin. Sections (4-5 µm) were prepared and then stained with hematoxylin-eosin dye for photomicroscopic observations.

Statistical Analysis

Values were presented as mean ± S.E.M. Data were statistically evaluated by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test using SPSS software. $P < 0.01$ were considered as statistically significant.

RESULTS

Preliminary phytochemical screening of MELS revealed the presence of polyphenolics, flavonoids, glycosides, triterpinoids and carbohydrates.

In acute toxicity study, MELS did not show any mortality or toxic effect upto the dose of 2 g/kg b.wt, accordingly 200 and 400 mg/kg b.wt were taken as low and high dose of MELS for the *in vivo* experiment.

In vitro Antioxidant Study

The methanol extract of *L. siceraria* aerial part was found to scavenge DPPH, nitric oxide, superoxide radical and hydrogen peroxide as well as inhibited lipid peroxidation *in vitro* in a concentration dependent manner. The concentrations of MELS needed for 50% scavenging of the ROS and lipid peroxidation (IC_{50} values) have been given in table 1. Reductive ability of the extract was also found to be concentration dependent, as like the standard (Figure1). The total phenolic and flavonoid contents of MELS were found to be 95.7 ± 3.96 mg pyrocatechol/g dry extract and 25.32 ± 1.48 mg quercetin equivalent/g dry extract respectively.

Table 1: Free radical scavenging activity of methanol extract of *L. siceraria* aerial parts (MELS) and Butylated hydroxyl toluene (BHT) on different *in vitro* models

In vitro models	IC_{50} Values (µg/ml)	
	MELS	BHT
DPPH	25.70 ± 1.02	18.05 ± 2.10
NO	52.20 ± 2.05	60.40 ± 3.33
SO	62.50 ± 3.65	120.50 ± 5.75
H_2O_2	46.50 ± 4.55	23.45 ± 2.56
LPO	43.85 ± 4.26	79.86 ± 3.33

DPPH: Diphenyl-2-picryl hydrazyl; NO: Nitric oxide; SO: Super oxide; H_2O_2: Hydrogen peroxide; LPO: Lipid peroxide.
% Inhibition = (Absorbance of control – Absorbance of test) × 100/Absorbance of control
IC_{50} Value: Concentration required for 50% inhibition of the free radical reactions
Values are mean ± SEM of triplicate determinations

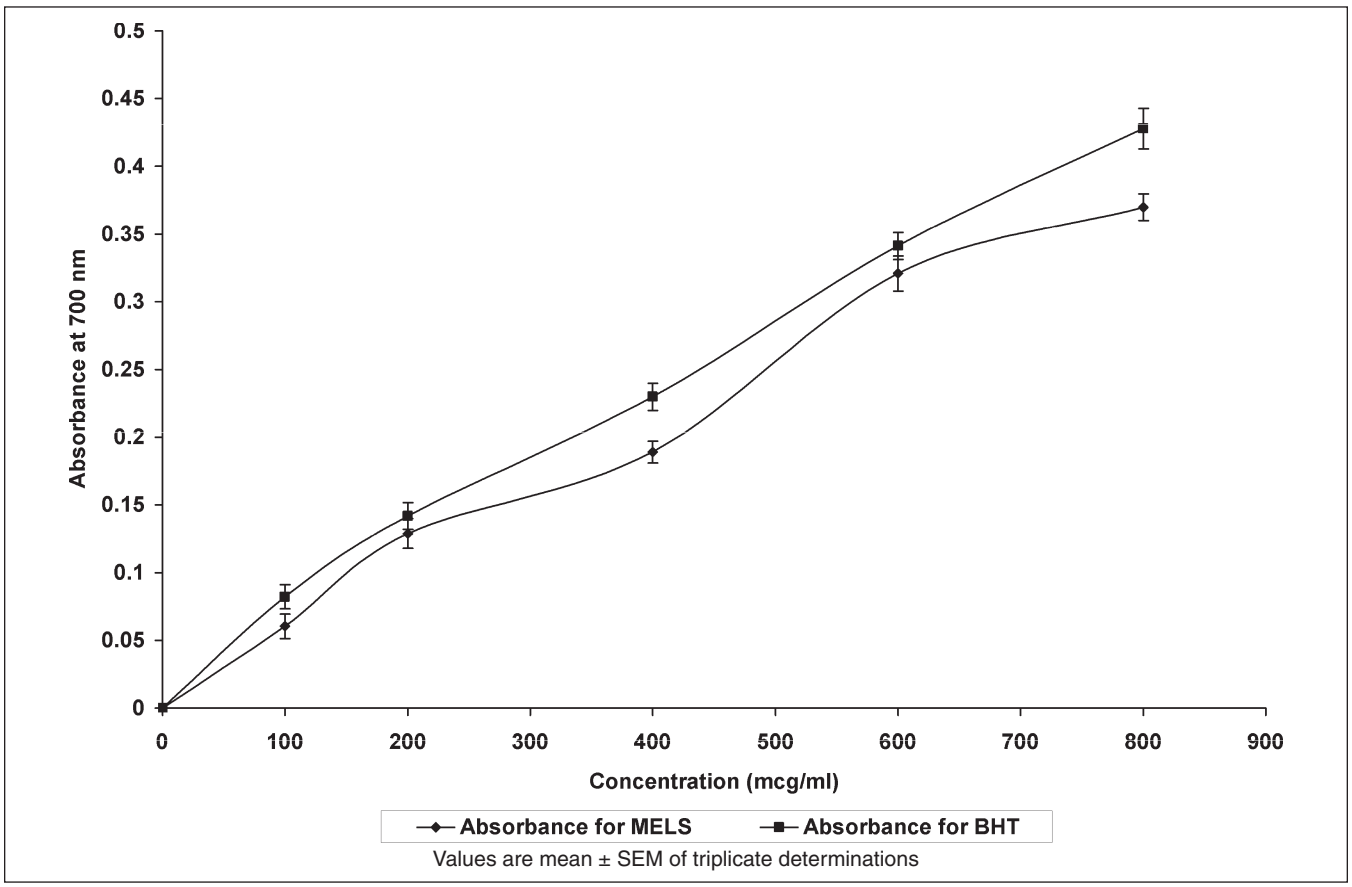


Figure1: Reductive ability of methanol extract of *L. siceraria* aerial parts (MELS) and Butylated hydroxyl toluene (BHT)

Hepatoprotective Activity Study

Administration of CCl_4 to the animals resulted in a marked elevation of serum transaminases (SGOT and SGPT), serum alkaline phosphatase (ALP) and total bilirubin (TB), when compared with those of normal control animals. However serum total protein level was decreased. The rats treated with methanol extract of *L. siceraria* and with silymarin showed a significant decrease ($p < 0.001$) in all the elevated serum marker levels, SGOT, SGPT, ALP and TB, and significant increase ($p < 0.001$) in total protein (Table 2) which showed the restoration of the level of liver function biochemistry to the near normal values.

Toxic dose of CCl_4 significantly reduced the activities of enzymic (CAT and SOD) and non-enzymic (GSH) antioxidant system and enhanced lipid peroxidation (LPO) level of liver tissue, as were found in group II animals. MELS treatment significantly raised both of the enzymic and non-enzymic antioxidant systems as was found in case of silymarin treated group, while the elevated LPO level were found to be reduced back to/towards the normal level in MELS as well as silymarin treated rats (Table 3).

Histological examination of liver sections of normal control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Figure 2A). Disarrangement of normal hepatocytes with centrilobuler necrosis, vacuolization of cytoplasm and fatty changes were observed in CCl_4 intoxicated rat livers (Figure 2B). The liver sections of the rats treated with MELS low and high dose (Figure 2C and Figure 2D) and silymarin (Figure 2E) showed a sign of protection against CCl_4 intoxication as evident by presence of normal hepatic cords and absence of necrosis with minimal inflammatory conditions around the central vein.

DISCUSSION

Excessive concentration of reactive oxygen species (ROS) and other radicals leads to oxidative stress in the body and that in turn is associated with a number of pathological conditions. Antioxidants can combat against this oxidative stress either by scavenging free radicals or by their potent reductive ability.[2] There are restrictions on the use of synthetic antioxidants, such as Butylated Hydroxy Toluene (BHT), Butylated Hydroxy Anisole (BHA), as they are suspected to be carcinogenic.[41,42] Natural antioxidants, therefore, have gained importance.

DPPH is a relatively stable nitrogen centered free radical, which is widely used to evaluate free radical scavenging property of natural antioxidants. An antioxidant can scavenge DPPH radical by donating H atom and forming corresponding non-radical hydrazine.[43] In the present study, MELS was found to scavenge DPPH in a concentration dependent manner. Further, MELS effectively scavenged superoxide, nitric oxide, hydrogen peroxide and lipid

Table.2. Effect of Methanol extract of *L. siceraria* (MELS) on serum enzyme levels and Total bilirubin and Total protein of CCl_4 intoxicated rats

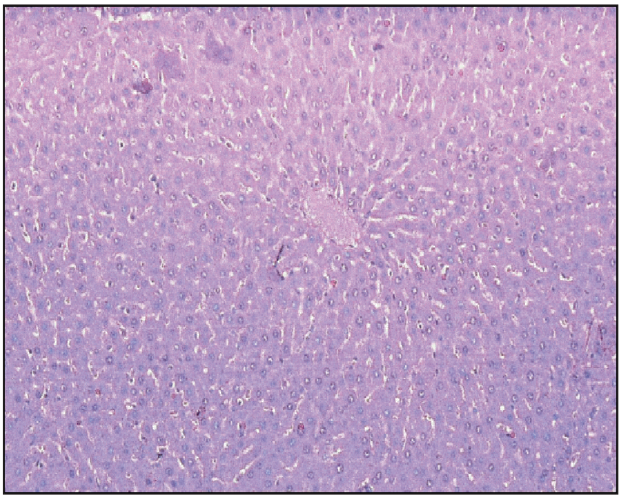
Groups	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)	Total bilirubin (mg/dl)	Total protein (g/dl)
Normal Control	74.63 ± 1.43	113.21 ± 2.41	31.64 ± 1.57	0.83 ± 0.04	7.48 ± 0.06
CCl_4 Control	199.08 ± 4.62[a,#]	247.85 ± 4.28[a,#]	90.01 ± 2.27[a,#]	2.66 ± 0.06[a,#]	4.19 ± 0.04[a,#]
MELS (200 mg/kg)	90.55 ± 2.33[b,*]	156.62 ± 2.40[b,*]	45.48 ± 1.75[b,*]	1.43 ± 0.04[b,*]	6.95 ± 0.05[b,*]
MELS (400 mg/kg)	72.30 ± 1.18[b,*]	127.23 ± 2.23[b,*]	32.89 ± 1.40[b,*]	1.24 ± 0.03[b,*]	7.35 ± 0.03[b,*]
Silymarin (20 mg/kg)	80.95 ± 1.51[b,*]	140.22 ± 2.17[b,*]	40.06 ± 1.84[b,*]	1.01 ± 0.04[b,*]	7.08 ± 0.04[b,*]

Values are Mean ± S.E.M.; $n = 6$ in each group. Drug treatment was done for 14 days.
[a]CCl_4 control group *vs* normal control group, [#]$p < 0.001$;
[b]Treated groups *vs* CCl_4 control group, *$p < 0.001$; where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test.

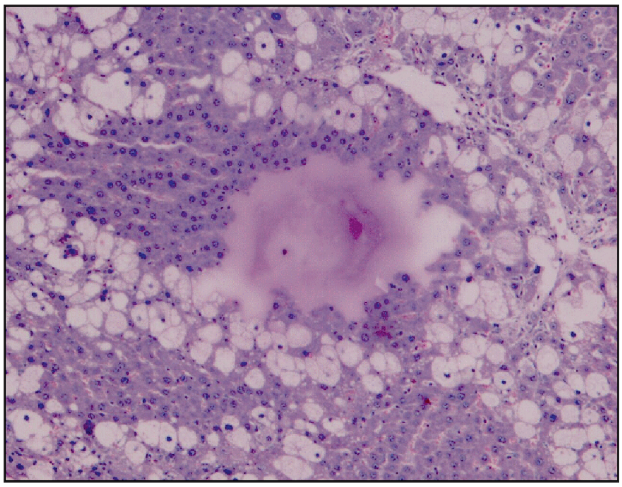
Table.3. Effect of Methanol extract of *L. siceraria* (MELS) on LPO, GSH, SOD and CAT levels of CCl_4 intoxicated rats

Groups	LPO (nM/mg wet tissue)	GSH (µg/mg wet tissue)	SOD level (Units/mg wet tissue)	CAT level (µM of H_2O_2 decomposed/min/ mg wet tissue)
Normal Control	98.91 ± 1.91	64.05 ± 1.48	1.50 ± 0.03	3.90 ± 0.05
CCl_4 Control	297.85 ± 5.12[a,##]	28.06 ± 1.62[a,##]	0.79 ± 0.02[a,##]	1.14 ± 0.03[a,##]
MELS (200 mg/kg)	122.08 ± 1.84[b,**]	58.72 ± 1.99[b,**]	1.01 ± 0.04[b,**]	1.99 ± 0.05[b,**]
MELS (400 mg/kg)	92.86 ± 1.54[b,**]	78.71± 1.68[b,**]	1.31 ± 0.03[b,**]	3.04 ± 0.05[b,**]
Silymarin (20 mg/kg)	100.32 ± 2.91[b,**]	70.07 ± 2.11[b,**]	1.45 ± 0.03[b,**]	3.00 ± 0.07[b,**]

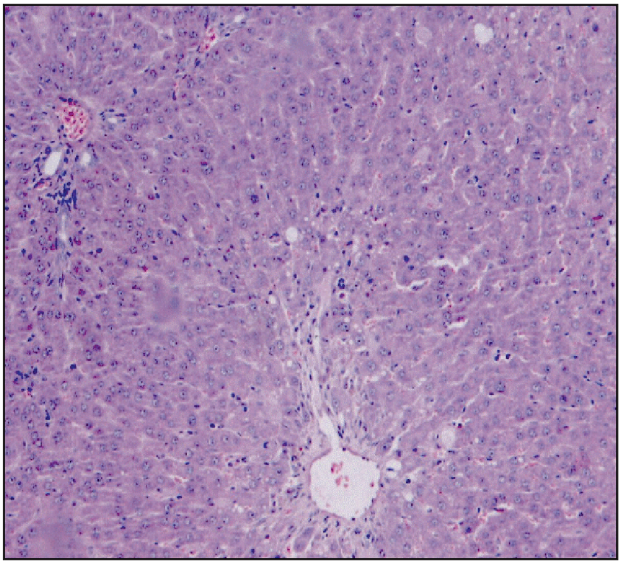
LPO: Lipidperoxide; GSH: reduced Glutathione; SOD: Superoxide dismutase; CAT: Catalase.
Values are Mean ± S.E.M.; $n = 6$ in each group. Drug treatment was done for 14 days.
[a]CCl_4 control group *vs* normal control group, [#] $p < 0.001$;
[b]Treated groups *vs* CCl_4 control group, [*]$p < 0.001$; where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test.



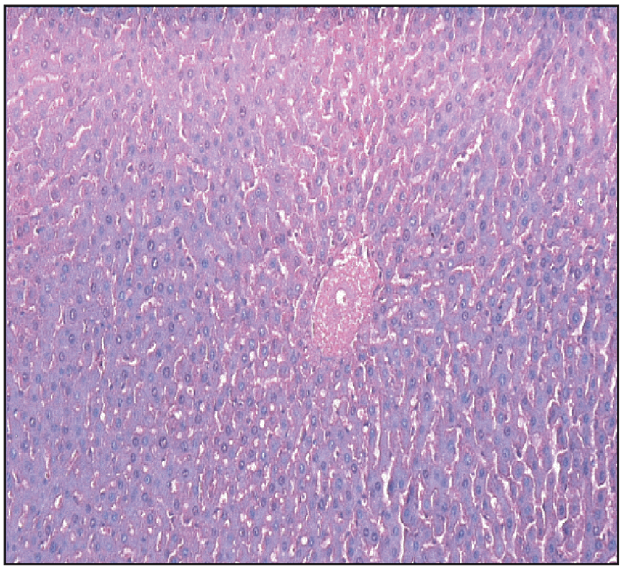
A. Normal Control group



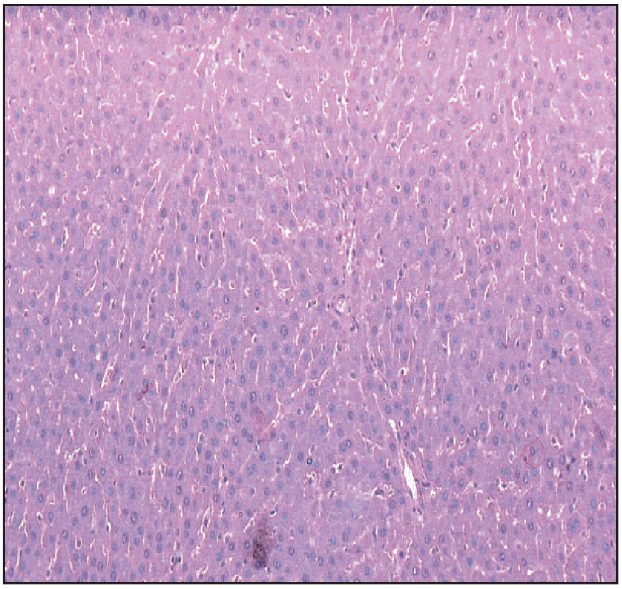
B. CCl_4 Control group



C. MELS treated group (Low dose)



D. MELS treated group (High dose)



E. Standard treated group

Figure 2: Histological observations of 14 days experimental rat liver

peroxide *in vitro*. These radicals are generated inside our body during the normal metabolism or in presence of xenobiotics and removed by the body's innate antioxidant defense mechanism. However, under pathologic conditions, there is an imbalance between ROS and antioxidant defense, which leads to oxidative modifications in the cellular membrane or major intracellular molecules. In addition to ROS, the potential mechanism of cellular oxidative damage by nitric oxide is the nitration of the tyrosine residues of proteins, peroxidation of the lipids, degradation of DNA and oligonucleosomal fragments. Increased level of lipid peroxide can give rise to the formation of carbonyl compounds like malondialdehyde (MDA) which are highly cytotoxic.[2,29] In the present study, the dose dependent scavenging of both ROS as well as nitric oxide radicals indicates the potent antioxidant property of MELS. The significant antioxidant activity of the extract thus suggests the possible therapeutic value of this plant.

Potent reductive ability of the extract implies its capability of donating hydrogen atom in a dose dependent manner. The high phenolic and flavonoid content of the extract may be the contributing factor towards its antioxidant activity. Because of the presence of the hydroxyl groups phenolic constituents can function as hydrogen donor. Antioxidant potential of flavonoids against oxidative stress is the interesting subject of many investigations.[8,9,11,44]

The hepatotoxicity induced by CCl_4 is due to its metabolite trichloromethyl free radical ($CCl_3^{\cdot}$), that alkylates cellular proteins and other macromolecules and finally result in cell death. Hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from the liver into the blood.[45] The present study revealed a significant increase in the activities of SPT, SGOT ALP and total bilirubin levels on exposure to CCl_4, indicating considerable hepatic injury. Administration of MELS attenuated the increased serum marker enzyme levels as well as total bilirubin and caused a subsequent recovery towards normalization almost like that of silymarin treatment. Decreased total protein content in the CCl_4 control group was also restored to near normal value by MELS supplementation**.** Stabilization of serum total bilirubin and total protein levels by MELS treatment is a clear indication of the improvement of functional status of the hepatic cells.[46]

The body's innate defense mechanism consists of a set of endogenous antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD) and non-enzymatic system such as reduced glutathione. In CCl_4 induced hepatotoxicity, the balance between ROS production and this antioxidant system is lost, hence the oxidative stress results. This was reflected by the reduced level of SOD and CAT and the exhausted GSH content in CCl_4 control group animals. Improvement in the antioxidant status to/towards normal level by the treatment with MELS as like silymarin treated animals implies the hepatoprotective effect of the extract. The free radicals generated in the metabolism of CCl_4 react with the unsaturated lipid of the cell and initiates the chain reactions of lipid peroxidation, which may cause peroxidative tissue damage in inflammation, cancer, aging, ulcer, cirrhosis and atherosclerosis. Increased lipid peroxidation in CCl_4 control group indicates tissue damage and failure of antioxidant defense mechanisms. However significant decrease in lipid peroxidation in the treated groups further potentiates its antioxidant activity *in vivo*.[2,47]

The protective effect of MELS in liver injury can further be concluded by the histological examinations of the liver tissues.

Hence the present investigation infers potent antioxidant and hepatoprotective activity of MELS, which may be due to its good amount of phenolic and flavonoid contents; however, further research is going on in our laboratory to isolate and purify the bioactive principle(s) from the methanol extract of *L. siceraria* aerial parts.

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Phytochemical Screening and *In vitro* Anti-inflammatory Activity of the Stem of *Coleus forskohlii*.

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ABSTRACT

The extracts of *Coleus forskohlii* prepared by using hexane, chloroform, methanol, 80% methanol and water as solvents were screened for secondary metabolites and for its *invitro* anti-inflammatory activity. The extracts revealed the presence of alkaloids, phenols, tannins, proteins, carbohydrates, saponins, glycosides and cardiac glycosides and most of these compounds were observed in the aqueous, 80% methanol and methanolic extracts. DPPH antioxidant assay and the *in vitro* anti-inflammatory activity assays *viz.*, BSA anti-denaturation and HRBC membrane stabilization assay indicated that the aqueous and methanolic extracts of *Coleus forskohlii* possess constituents with anti-inflammatory properties. TLC profile of the methanolic extract confirmed the presence of forskolin, which is a major bioactive compound isolated from the roots of *Coleus forskohlii*. Of all the extracts that were tested for their *in vitro* anti-inflammatory activity, methanolic and aqueous extracts showed maximum activity.

Key words: *Coleus forskohlii*, anti-inflammatory, DPPH and TLC.

INTRODUCTION

In Ayurveda, *Coleus species* have been used to treat spasmodic pain, digestive problems, heart disease, convulsions, and painful urination.[1] Since the 1970s, research work was predominantly carried out on forskohlin, that was extracted from the tuberous roots of *Coleus forskohlii*. Although most studies recently have used the isolated forskohlin, it is believed that the whole of *Coleus forskohlii* plant may be more effective, due to the presence of multiple compounds that can act synergistically. *Coleus forskohlii* is a perennial member of the mint (Lamiaceae) family and grows in the subtropical temperature climates in India, Nepal, Sri Lanka and Thailand.[2] Inflammation covers a sequence of reparative and defensive reactions to tissue injury, caused either due to infection, autoimmune stimuli or mechanical injury. It involves several types of compounds such as plasma proteins, vasoactive amines, tissue digestive enzymes, biologically derived oxidants and eicosanoids.

As a result of the undesirable side effects, like gastric lesions, caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as anti-inflammatory and analgesic agents have not been successful in all the cases. Therefore, new anti-inflammatory and analgesic drugs lacking those effects are being searched all over the world as alternatives to NSAIDs and opiates.[3] The herbs used in Ayurveda and other traditional methods of treatment can be used as good source of molecules having anti-inflammatory activity and which have lower adverse effects compared to the NSAIDs presently being used. There is a renewed interest in identifying plant constituents that can suppress the inflammatory responses, which is a complex array of enzyme activation, mediator release, cell migration, tissue breakdown and repair.

In the present study, the phytochemical screening of *Coleus forskohlii* shoot extract in various solvents was performed and the anti-inflammatory activity of the extracts was tested using BSA anti-denaturation assay and HRBC membrane stabilization assays. TLC profiling of the methanolic extract was also performed along with the root extract of *Coleus forskohlii*, and an attempt was made to evaluate the anti-inflammatory activity of the shoot extract of *Coleus forskohlii*.

MATERIALS AND METHODS

The dried shoot part of the plant (*Coleus forskohlii*) collected was pulverized and 10 g was refluxed with hexane,

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chloroform, methanol, 80% methanol and water in the ratio 1:10(w/v). The crude extracts were collected in amber coloured sample bottles and stored. All chemicals and reagents used including the solvents were of analytical grade.

Phytochemical screening

Chemical analysis was carried out in the hexane, chloroform, methanolic and water extracts of the shoot of *Coleus forskohlii* using standard procedures to identify constituents, as described by Harborne (1984), Trease and Evans (1979), and Sofowara (1993).[4-6]

DPPH Antioxidant assay

The DPPH antioxidant assay was carried out by adopting the method of Blois (1984).[7] The extracts (5 mL) were dried in vacuum oven and redissolved in 50% methanol. This was used for the DPPH assay. 50 µL (400µg/mL) and 100 µL (800µg/mL) of each fraction was taken and made up to 1 mL with 50% methanol. To this, 1 mL of DPPH (1 mM) was added. The mixture was left for 20 minutes in dark at room temperature. Absorbance was measured after 20 minutes at 517 nm. Control was taken without the extracts.

Anti-inflammatory assays

BSA anti denaturation assay:[8] 5 mL of each extract was dried in vacuum oven and redissolved in 5 mL of isosaline. Then, 1 mg/mL of all extracts were made from the above-mentioned stock solution. To 1.8 mL of 1% of BSA solution, 0.2 mL of extract solution in isosaline was added. The pH was adjusted to 6.5 using 1N HCl. This solution was incubated at 37 ºC for 20 minutes and then heated to 57 ºC for 10 to 15 minutes. After cooling, turbidity was measured at 660 nm. Control was taken without the extracts.

HRBC membrane stabilization assay:[9] Blood was collected freshly and mixed with equal volume of Alsever solution. It was then centrifuged at 3000 rpm for 15 minutes. The packed cells were washed with isosaline and a 10% suspension was made with isosaline. To 0.5 mL of extract, 1 mL phosphate buffer, 2 mL hyposaline and 0.5 mL HRBC suspension were added. This was incubated for 30 minutes at 37 ºC and then centrifuged at 3000 rpm for 20 minutes. Absorbance was measured at 560 nm. Control was taken without the extract.

TLC: Thin layer chromatography was performed with Toluene: Ethyl acetate: Methanol as solvent system and Anisaldehyde-H_2SO_4 as spraying reagent and the Rf values were compared with standard forskolin.

RESULTS AND DISCUSSION

Phytochemical analysis

Coleus forskohlii is one of the most significant potential medicinal crops of the future with its therapeutic properties being scientifically authenticated recently. The roots have been established as the source of forskolin, which is popularly used in medicinal preparations, against asthma, cardiac disorders, eye diseases, hypertension, cancer, and gastric disorders. Forskolin is reported to be responsible for virtually all pharmacological activities attributed to *Coleus forskohlii* and the extracts of this constituent has been used in nearly all existing studies though other plant constituents, such as volatile oils, diterpenoids and coleonols, are also reported to contribute to the pharmacological activity and adsorption of forskolin.[10] Forecasts of the requirements of forskolin for drug development indicate the need for a sustained supply of root material in quantities that could threaten the survival of the species in nature. Concern for species conservation and for a sustained supply of the root material led to the consideration of developing *C. forskohlii* as a medicinal crop. The focus on the development studies, led to an increased yield of root tubers, due to its cultivation as a source for forskolin. In the present study, the possibility of the utilization of the shoot was considered and thus evaluated for the levels of forskolin and for its *in vitro* anti-inflammatory properties.

Phytochemical-screening results (Table 1) of the powdered sample extracted in water, hexane, chloroform, methanol and 80% methanol showed the presence of all the constituents in aqueous and methanol, whereas the chloroform extract

Table 1: Phytochemical analysis of extracts

Chemical constituents	Water	80% Methanol	Methanol	Chloroform	Hexane
Flavonoids	+	+	+	–	–
Alkaloids	+	+	+	–	–
Phenols	+	+	+	–	–
Tannins	+	+	+	–	–
Proteins	+	+	+	+	–
Carbohydrates	+	+	+	+	–
Saponins	+	+	+	–	–
Glycosides	+	+	+	–	–
Cardiac glycosides	+	+	+	+	+

showed the presence of proteins, carbohydrates and cardiac glycosides and the hexane extracts was positive for cardiac glycosides, indicating that water and methanol were able to extract most of the phytochemicals present in the shoot. The TLC chromatogram of the methanolic extract of *Coleus forskohlii* shoot showed similar profile, when compared with the chromatogram of the methanolic extract of *Coleus forskohlii* root (Figure 1), which demonstrates that the shoot parts of *Coleus forskohlii* contained similar chemical constituents as the root portion. The Rf value of 0.589 (red band) (Table 3) corresponded with the Rf of standard forskolin, specified in the literature, confirming the presence of forskolin in both the extracts. Further isolation and identification of the other constituents separated on TLC, is needed by bioassay guided fractionation.

DPPH Assay

The results of the free radical scavenging potential of different extracts tested by DPPH method is given in Table 2. Reduction of the DPPH radicals were observed by a decrease in absorbance and change in the color to yellow that denotes the quenching of the free radical was higher by the compounds in the methanolic extract compared to other extracts. These observations clearly indicate a close linkage between active compounds and antioxidant activity. The high activity of the water and methanolic extracts are generally attributed to the presence of flavonoids and polyphenolics, as the majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins. In addition, to the above compounds found in natural foods, vitamins C and E, b-carotene and alpha-tocopherol are known to possess antioxidant potential.

The extracts prepared with water, 80% methanol and methanol showed maximum activity. The concentration and percentage of scavenging the DPPH radical by the solvent extracts are as follows: Aqueous extract 400 µg/mL (78.2%) and 800 µg/mL (100%), 80% methanol extract 400 µg/mL (97.5%) and 800 µg/mL (101.1%) and methanolic extract 400 µg/mL (86.1%) and 800 µg/mL (87.5%), whereas the chloroform and hexane fractions showed low activity. The standard, BHA, is reported to show an IC_{50} value of 53.27 mg/mL,[11] whereas the aqueous and methanolic extracts had an IC_{50} value less than 400 µg/mL.

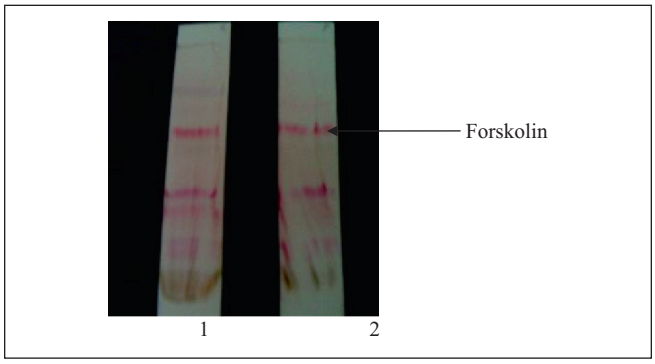

Figure 1: TLC chromatograms of methanolic extracts of *Coleus forskohlii* root (1) and shoot (2) respectively.

Table 2: HRBC membrane stabilization of extracts, standard Forskolin and drug

Concentration	% stabilization by methanolic extract		% stabilization by Diclofenac
	Met Extract	Std Forskolin	
50 µg/mL	77.6	24.32	68.09
100 µg/mL	79.1	30.78	80.48
250µg/mL	80.4	37.73	82.74
500 µg/mL	69.2	28.65	88.21

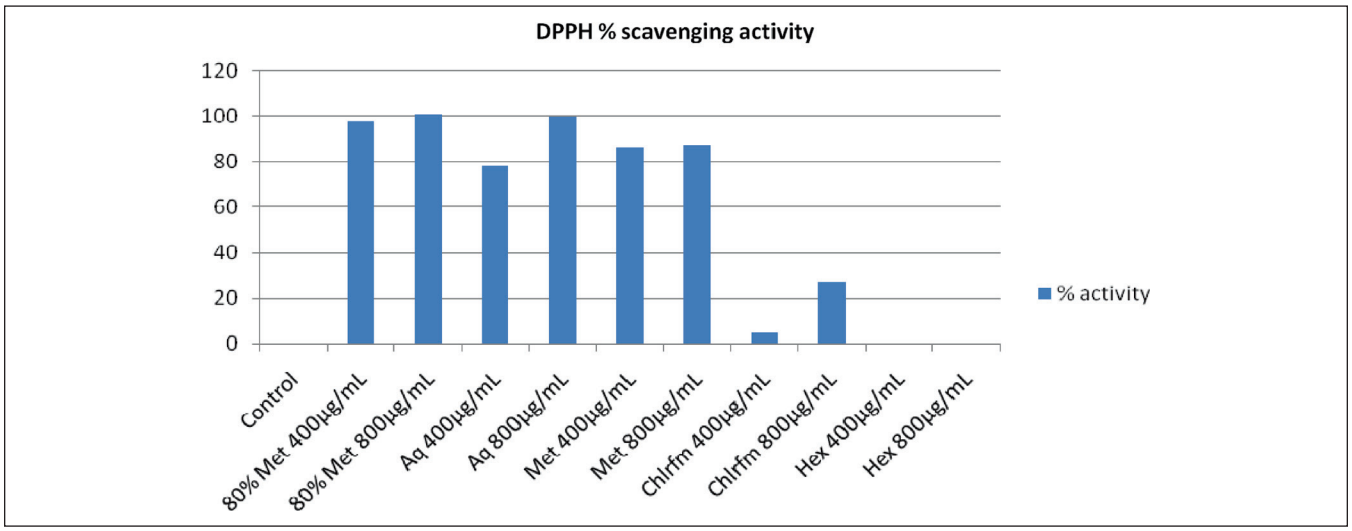

Figure 2: DPPH % scavenging activity of all the extracts.

BSA anti denaturation assay

The inhibitory effect on protein (BSA) denaturation by the extracts is shown in Table 3. All the extracts were tested at 200 µg/mL concentration. The aqueous, methanolic and hexane fractions showed good activity, whereas the chloroform extract showed comparatively lower activity. At 200 µg/mL concentration, aqueous extract showed 69.67% inhibition of denaturation followed by methanol (66.80%); 80% methanol (65.84%), hexane (65.16%) and chloroform (34.69%). Denaturation of proteins is well-documented cause of inflammation and rheumatoid arthritis. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation.[12] When BSA is heated, it undergoes denaturation and expresses antigens associated with type III hypersensitive reaction and which are related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus. Thus, this assay was applied for the detecting compounds, which can stabilize the protein from denaturation process. Several nonsteroidal anti-inflammatory drugs such as Indomethacin, Ibufenac, Diclofenac sodium, salicylic acid and flufenamic acid prevent denaturation of BSA at pathological pH (6.2-6.5).[13]

Table 3: Rf values from chromatogram

Rf value	Color of the band
0.465	Pink
0.589	Red (Forskolin)
0.643	Light red
0.684	Light brown
0.821	Dark pink
0.890	Brown

HRBC membrane stabilization assay

After the initial screening, it was found that the methanolic extract showed activity similar to Diclofenac, the standard anti-inflammatory drug used, for treating inflammation. Various concentrations of the methanolic extracts in iso-saline were tested and it was observed that at 250 µg/mL, both Diclofenac and the extract showed similar effects (Table 2). The analogous activity makes the extract a potential candidate for further studies.

Among all the solvent extracts, methanolic and aqueous extract showed significant anti-inflammatory activity in a concentration dependent manner. Methanolic extract at a concentration of 250 µg/mL showed 80.4% protection of HRBC in hypotonic solution. All the results were compared with standard diclofenac. According to Chou, the erythrocyte membrane can be considered to be related to lysosomal membranes and thus stabilization of the membrane is a significant criterion for assessing the effect of any extract/drug.[14] The methanolic extracts exhibited relatively higher stabilization effect by inhibiting the hypotonicity-induced lyses of erythrocyte membrane. The inflammatory responses get limited by preventing the release of lysosomal constituents of activated neutrophils thereby the damage to the tissue is reduced. Some of the non-steroidal anti-inflammatory drugs are known to possess membrane stabilization properties, which may contribute to the potency of their anti-inflammatory effect. The exact mechanism of stabilization of the membrane by the extract is not known, but it can be observed that the osmotic loss of intracellular electrolytes and fluid components was inhibited under induced hemolysis. According to Iwueke, plant extracts have the potential to stimulate or enhance the efflux of these intracellular

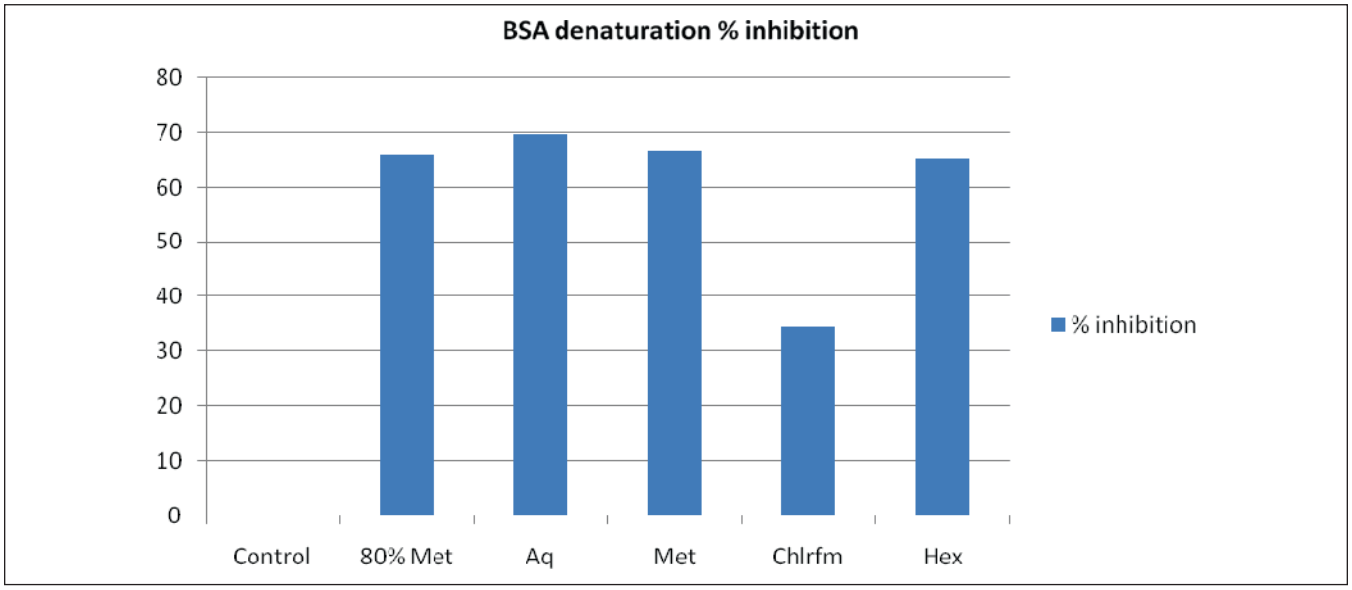

Figure 3: BSA denaturation % inhibition.

components.[15] Since inflammation responses amplify any disease conditions, it can be suggested that the shoot of *Coleus forskohlii* can also be used to reduce the inflammatory responses that is activated in most disease condition, in addition, to the root of *Coleus forskohlii* that is generally used.

The anti-inflammatory effect of the test samples were assessed by *in vitro* assay using the modified assay based on the method of Mizushima and Kobayashi.[16] The data obtained is summarized in Table 4. The analysis revealed that the methanolic extract of the shoot of *Coleus forskohlii* was more effective than 80% methanol, water, chloroform and hexane extracts, and similar to the levels of standard forskolin. Engprasert *et al.,* reported that forskolin is synthesized from Isopentenyl-diphosphate; a common biosynthetic precursor *via* a non-mevalonate pathway by geranyl-geranyl pyrophosphate synthase and is primarily synthesized in the leaves and subsequently accumulated in the stems and roots.[17]

The need for biologically active compounds with low profiles of adverse reactions compared to pharmacological drugs has triggered an extensive investigation of herbal phytochemicals and their mechanisms of action. The net biological activity is determined by the outcome of the multiple cellular effects exerted by a phytochemical and a better understanding of these cellular effects is vital to properly utilize the phytochemicals, as promising agents for promoting health and preventing disease.

CONCLUSION

The shoot extract of *Coleus forskohlii* showed potent antioxidant and anti-inflammatory activities, and TLC confirms the presence of forskolin and other constituents that is similar to that present in the root extracts. Further studies related to the active constituents on lipid-derived eicosanoids, enzyme expression (COX-2, lipoxygenase) and cytokines are necessary to understand the mechanism of action in relation to the observed anti-inflammatory activity.

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Antidiabetic and Hypolipidemic Activity of *Citrus medica* Linn. Seed Extract in Streptozotocin Induced Diabetic Rats

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ABSTRACT

Introduction: The objective of the present investigation was to evaluate the antidiabetic and hypolipidemic activity of petroleum ether extract of *Citrus medica* Linn. seeds in streptozotocin (STZ) induced diabetic model in rats. **Methods:** The study was carried out using ~~albino~~ rats of either sex weighing ~~150-200~~ gm. One group was selected as control group (buffer alone) and four groups of STZ induced diabetic rats (n = 5 in each group) were administered vehicle (1% tween 80), seed extract (200 and 400 mg/kg, p.o.) of *C. medica* Linn. and standard drug glibenclamide (5 mg/kg) for 15 days after 10 days of single dose of STZ (60 mg/kg) intraperitoneal administration. Blood samples were collected by retro-orbital puncture and were analyzed for blood glucose, serum cholesterol, triglycerides, HDL, LDL and VLDL on days 0, 3, 10 and 25 by using diagnostic kit. **Results:** The petroleum ether extract of *C. medica* Linn. seeds (200 and 400 mg/kg, p.o.) induced significant reduction ($p < 0.05$) of fasting blood glucose, serum cholesterol, serum triglycerides, LDL and VLDL in dose dependent manner after 15 days of drug administration. Though 200 mg/kg/day seed extract for 15 days was not showing any change in HDL level, while 400 mg/kg/day dose significantly increased HDL level in diabetic rats. **Conclusion:** So it is concluded that *C. medica* Linn. seeds have significant antidiabetic, hypocholesterolemic and hypolipidemic activity.

Key words: Antidiabetic, hypolipidemic, *Citrus medica* Linn., blood glucose.

INTRODUCTION

Diabetes mellitus is a chronic disease that has a significant impact on health, quality of life as well as on the health care system. It is a disease characterized by elevated blood glucose levels and disturbances in carbohydrate, fat and protein metabolism and by complications like retinopathy, microangiopathy and nephropathy.[1] These metabolic abnormalities result, in part, from a deficiency of the blood sugar lowering hormone insulin; this deficiency in insulin results in type 1 diabetes or insulin dependent diabetes mellitus (IDDM). Type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM) is a result of hyperglycemia caused by overproduction of glucose at the hepatic level or because of abnormal β cell function or insulin resistance at target cells.[2]

In diabetic rats, the impaired utilization of carbohydrate leads to accelerated lipolysis, resulting in hyperlipidemia.[3] NIDDM has also been associated with an increased risk of premature arteriosclerosis due to increase in triglycerides and low density lipoprotein levels. About 70-80% deaths in diabetic patients are due to vascular diseases. Thus an ideal treatment for diabetes would be a drug that not only controls the blood sugar level but also prevents the development of arteriosclerosis and other complications of diabetes.[4] Currently available synthetic antidiabetic agents produce serious side effects like hypoglycemic coma, hepatorenal disturbances and are unsafe during pregnancy.[5-7]

There has been an increasing demand for the use of natural products with antidiabetic and antihyperlipidemic activity. This is largely because insulin cannot be used orally and insulin injections are associated with the risk of hypoglycemia and impairment of hepatic and other body functions. The undesirable side effects of synthetic hypoglycemic drugs,

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and the fact that they are not suitable for use during pregnancy, have made researchers look towards hypoglycemic drugs of plant origin.[8-10]

Several members of the genus *Citrus* (Rutaceae) are being used traditionally in a wide variety of ethnomedical remedies. One among them is *Citrus medica* Linn. It is commonly known as matulunga in Sanskrit, citron in English and bijapura or bara nimbu in Hindi.[13] Ripe fruits of *C. medica* Linn. are potent anti-scorbutic, tonic, stomachic, used in vomiting and expellant of poison. Seeds are used in piles, biliousness, inflammations and as vermifuge, stimulant and cardiac tonic.[14] The objective of the present study was to evaluate the hypoglycemic and hypolipidemic activity of petroleum ether extract of seeds of *C. medica* Linn. in streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Plant material

The seeds of *C. medica* Linn. were collected locally. The plant was identified as *C. medica* Linn. by an authorized taxonomist at National Botanical Research Institute, Lucknow. Voucher specimen number 97840 has been deposited in the same institute.

Preparation of the extract

The collected seeds were washed thoroughly under running tap water, dried under sun and coarsely powdered. The petroleum ether extract of course powder was prepared by Soxhlet extraction at 40 ºC for 3-4 h, and then the solvent was recovered at 40 ºC under reduced pressure. The extract was oily, pale yellow coloured liquid, stored at room temperature till used in the experiment.

Animals

Healthy adult ~~malc~~ Wistar rats between 2-3 months of age and weighing 150-175 g were used for the study. Housed individually in polypropylene cages, maintained under standard conditions (12-h light and 12-h dark cycle; 25 ± 5 ºC; 35-60% humidity), the animals were fed with standard rat pellet diet and provided water *ad libitum*. The study protocol was approved by Institutional Animal Ethics Committee and the CPCSEA.

Experimental design and induction of diabetes

The animals irrespective of sex were distributed into five groups (with six animals in each group) as follows: (I) control group, (II) diabetic control group, (III) diabetic group treated with 200 mg/kg/day of *Citrus medica* Linn. extract, (IV) diabetic group treated with 400 mg/kg/day of *Citrus medica* Linn. extract, and (V) diabetic group treated with Glibenclamide 5 mg/kg/day.

Animals of groups II, III, IV and V were rendered diabetic by a single intraperitoneal (i.p.) injection of 60 mg/kg of streptozotocin (STZ) freshly prepared in 0.1 M of citrate buffer (pH 4.5). Animals of group I were injected with buffer alone. Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined after 72 h and after10 days of STZ injection. The threshold value of fasting plasma glucose to diagnose diabetes was taken as >126 mg/dl.[13] Only those rats that were found to have permanent NIDDM were used for the study.

Ten days after the STZ injection, animals of group III received 200 mg/kg/day, group ~~III~~ IV received 400 mg/kg/day of *Citrus medica* Linn. extract and group V received Glibenclamide 5 mg/kg/day for 15 days. The *Citrus medica* Linn. extract and Glibenclamide were administered orally as a suspension in 1% v/v tween 80. While animals of group II were received only 1% v/v of tween 80.

Collection of blood and estimation of biochemical parameters

Blood was withdrawn from the retroorbital sinus under ether inhalation anesthesia. The fasting blood glucose levels and lipid profile were determined on day 0 and after day 3, 10, and 25. Fasting blood glucose was determined by Modified Roeschlau's Method.[14] The blood sugar level was measured by using Agappe diagnostics kit (Ernakulan, Kerala). The blood sugar, serum triglyceride and total cholesterol levels were measured by spectrophotometric methods using Agappe diagnostic kits (Ernakulan, Kerala). HDL (high density lipoproteins)-cholesterol was determined using the diagnostic kit of Piramal Healthcare, Mumbai. LDL (low density lipoproteins)-cholesterol and VLDL (very low density lipoproteins)-cholesterol were calculated by the equations (Friendewald's Formula):[15]

$$\text{LDL (mg/dl)} = \frac{\text{Total Cholesterol} - \text{HDL Cholesterol} - \text{Triglyceride}}{5}$$

$$\text{VLDL (mg/dl)} = \text{Total cholesterol} - \text{HDL} - \text{LDL}$$

Statistical analysis

The data was analyzed by one-way ANOVA followed by Tukeys's multiple comparison test. Values were considered to be significant at $P \leq 0.05$.

RESULTS

A single dose of STZ at a dose of 60 mg/kg body weight causes a significant diabetogenic response in rats. The increase in glucose level in diabetic groups (II-V) was found to be highly significant ($p < 0.001$) when compared to normal control group I. The diabetes was maintained even after ten days of STZ administration. Changes in blood

glucose of various groups are presented in Table 1. *C. medica* Linn. seed extract caused dose dependent significant reduction of fasting blood glucose in groups III and IV as compared to the diabetic control group. The antihyperglycemic effect of 400 mg/kg/day of extract for 15 days, being comparable to that of glibenclamide. Glibenclamide showed a 28.1% decrease in glucose level as compared to the diabetic control group. Administration of vehicle to STZ induced diabetic rats resulted in an increase in the level of triglycerides, total cholesterol, LDL, and VLDL, and decreased HDL, even after 25 days; same effect in rats of group III to V till tenth day of STZ administration. Continuous administrations of the petroleum ether extract (200 and 400 mg/kg/day for 15 days) of *C. medica* Linn. seeds lead to significant decrease ($p < 0.05$) in the level of triglycerides, total cholesterol, LDL, and VLDL in the diabetic rats, while it increased the level of HDL (Table 2).

DISCUSSION

Effective blood glucose control is the key for preventing or reversing diabetic complications and improving the quality of life in patients with diabetes. Thus, sustained reduction in hyperglycemia will decrease the risk of developing microvascular complications ~~and most likely reduce the risk of microvascular complications~~.[16] STZ is widely used to induce ~~diabetesin~~ experimental animals. As STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells, NO has been proposed to contribute to STZ induced DNA damage.[17,18] STZ was also found to generate reactive oxygen species which contribute to DNA fragmentation and evoke deleterious changes in the cells.[19,20] The present investigation studied the hypoglycemic and hypolipidemic potential of *C. medica* Linn. seeds in STZ-induced diabetic rats. *C. medica* Linn. seeds extract has shown significant decrease of fasting blood

Table1: Effect of *C. medica* extract on fasting blood sugar level in diabetic rats

Group	Treatment	Fasting blood sugar level (mg/dl)			
		0 day	3rd day	10th day	25th day
1.	Control	116.342 ± 2.512	110.419 ± 4.384	110.943 ± 3.703	124.688 ± 3.424
2.	Diabetic Control	107.912 ± 3.769	204.679 ± 4.893##	177.9 ± 5.012##	181.162 ± 3.577##
3.	200 mg extract	108.02 ± 3.758	207.904 ± 4.212##	200.512 ± 2.327##	138.695 ± 2.224*
4.	400 mg extract	118.747 ± 2.251	214.874 ± 4.666##	205.864 ± 1.848##	132.755 ± 1.425**
5.	Glibenclamide	116.206 ± 3.664	188.296 ± 5.87##	184.408 ± 6.087##	130.116 ± 1.689**

Value represents mean ± SEM ($n = 6$); #$p < 0.05$, ##$p < 0.001$, significant when compared to normal control group (Group I); *$p < 0.05$, **$p < 0.001$, significant when compared to diabetic control group (Group II); One way ANOVA followed by Tukeys's multiple comparision test.

Table2: Effect of *C. medica* extract on lipid profile in STZ-induced diabetic rats

Parameters	Period	Experimental Groups				
		Normal control	Diabetic control	*C. medica* extract		Glibenclamide
				200 mg/kg	400 mg/kg	5 mg/kg
TCH	0 day	100.38 ± 1.65	99.74 ± 1.82	111.4 ± 2.52	115.97 ± 1.98	107.76 ± 1.87
	3 days	101.93 ± 1.87	176.45 ± 4.74##	191.01 ± 1.94##	189.73 ± 1.96##	189.79 ± 1.26##
	10 days	101.20 ± 2.17	186.10 ± 1.52##	190.60 ± 1.73##	191.59 ± 2.35##	192.13 ± 1.73##
	25 days	102.92 ± 1.99	187.95 ± 1.36##	141.37 ± 2.72*	136.34 ± 3.54**	108.72 ± 1.08**
TG	0 day	81.79 ± 3.70	91.59 ± 2.77	93.07 ± 2.34	109.70 ± 6.52	98.46 ± 6.184
	3 days	82.89 ± 2.52	224.50 ± 6.59##	200.95 ± 5.16##	213.44 ± 3.46##	207.15 ± 4.47##
	10 days	86.20 ± 3.18	206.63 ± 4.72##	204.25 ± 4.1##	212.99 ± 9.10##	210.23 ± 5.32##
	25 days	79.57 ± 2.7	213.27 ± 4.54##	155.62 ± 3.41*	147.05 ± 3.53*	102.51 ± 2.18**
HDL	0 day	39.42 ± 1.30	35.13 ± 1.0	31.11 ± 1.45	35.86 ± 0.85	38.67 ± 1.21
	3 days	38.77 ± 1.60	29.82 ± 1.07#	27.65 ± 2.28#	29.71 ± 1.10#	30.79 ± 1.28#
	10 days	34.74 ± 1.30	30.27 ± 0.72#	26.59 ± 0.80#	28.31 ± 0.61#	30.72 ± 1.32#
	25 days	36.82 ± 1.11	32.46 ± 0.88#	27.04 ± 0.9	31.88 ± 1.11*	33.35 ± 1.42*
LDL	0 day	44.60 ± 2.70	46.28 ± 2.46	61.38 ± 4.03	58.162 ± 2.932	49.56 ± 1.50
	3 days	44.28 ± 2.6	101.73 ± 4.74##	123.16 ± 1.59##	117.33 ± 2.66##	117.56 ± 2.49##
	10 days	49.19 ± 1.78	114.49 ± 1.52##	123.15 ± 2.37##	120.67 ± 3.38##	119.37 ± 3.1##
	25 days	50.18 ± 3.14	113.84 ± 2.39##	108.20 ± 2.56	94.45 ± 2.60*	90.86 ± 1.59*
VLDL	0 day	16.25 ± 0.58	18.32 ± 0.55	18.61 ± 0.46	21.94 ± 1.30	19.53 ± 1.36
	3 days	15.85 ± 0.76	44.9 ± 1.31##	40.18 ± 1.03##	42.68 ± 0.69##	41.43 ± 0.89##
	10 days	17.27 ± 0.63	41.32 ± 0.95##	40.85 ± 0.82##	42.59 ± 1.82##	42.032 ± 1.604##
	25 days	15.85 ± 0.54	42.65 ± 0.90##	31.02 ± 0.76*	29.01 ± 0.80*	27 .49 ± 0.43*

Value represents mean ± SEM ($n = 6$); #$p < 0.05$, ##$p < 0.001$, significant when compared to normal control group (Group I); *$p < 0.05$, **$p < 0.001$, significant when compared to diabetic control group (Group II); One way ANOVA followed by Tukeys's multiple comparision test.

sugar levels at doses of 200 and 400 mg/kg/day in diabetic rats as compared to sugar levels in untreated diabetic rats.

The level of serum lipids are usually elevated in diabetes mellitus and such an elevation represents a risk factor for coronary heart disease. This abnormally high level of serum lipids is mainly due to the uninhibited actions of lipolytic hormones on the fat depots, mainly due to the action of insulin. Under normal circumstances, insulin activates the enzyme lipoprotein lipase, which hydrolyses triglycerides. However, in diabetic state, lipoprotein lipase is not activated due to insulin deficiency, resulting in hypertriglyceridemia[21] and insulin deficiency is also associated with hypercholesterolemia due to metabolic abnormalities.[22] In our study also, the diabetic rats showed hypercholesterolemia and hypertriglyceridemia and the treatment with plant extract significantly decreased both cholesterol and triglyceride levels, the extract also decreased LDL and VLDL levels and increased useful HDL level in diabetic rats. This implies that the petroleum ether extract of *C. medica* Linn. seeds may prevent or be helpful in reducing the complications of lipid profile seen in many diabetics in whom hyperglycemia and hypercholesterolemia coexist quite often.[23]

Possibly the petroleum ether extract may lead to regeneration of the β-cells of the pancreas and potentiation of insulin secretion from surviving β-cells; the increase in insulin secretion and the consequent decrease in blood glucose level may lead to inhibition of lipid peroxidation and control of lipolytic hormones. A number of other plants have also been reported to have antihyperglycemic, antihyperlipidemic, and insulin stimulatory effects.[24,25]

It is well known that LDL plays an important role in arteriosclerosis and that hypercholesterolemia is associated with a defect relating to the lack of LDL receptors. The decrease of cholesterol and LDL levels achieved by administration of *C. medica* Linn. seeds extract, demonstrates a possible protection against hypercholesterolemia and the harm this condition brings about.

As the chemical constituents and medicinal uses of *C. medica* Linn. seeds have been reported, the observed effects could be attributed to the presence of various phytoconstituents including limonin, limonol and nomilinic acid present in the seeds.[26]

CONCLUSION

From the results of the present study, it can be concluded that *C. medica* Linn. seeds are potent antihyperglycemic and hypolipidemic agent. The biological efficacy may be even higher after the isolation and purification of the compounds. Further studies are needed to identify the chemical constituents that may be responsible for the above activities.

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Evaluation of Antioxidant and Free Radical Scavenging Activity of *Samadera indica* Using *In vitro* Models.

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ABSTRACT

Antioxidant has gained importance in the current scenario as it has an ability to trap free radicals which are produced during the degenerative diseases. Natural antioxidant is considered superior to synthetic as it is safe and produces a prominent action. *Samadera indica* a bitter plant widely distributed throughout India, and mostly found in evergreen forest of Western Ghats and along river shore, the bitterness is due to the presence of flavanoids like Quassinoids. Literatures showed the presence of Quassinoids and therefore, plant may show the antioxidant activity which is not yet ruled out. Hence the present study was focused out to evaluate the antioxidant activity of *Samadera indica* by in-vitro models. **Method:** The plant leaf was extracted using methanol and acetone as solvent. The extracts were evaluated for antioxidant activity and free radical scavenging assay using DPPH, ABTS radical scavenging assay, FRAP, DCF/AAPH assay and was expressed as IC_{50}. **Result:** All the methods showed a prominent antioxidant activity but were comparatively lower than standard Quercetin. Antioxidant activity of extracts produced increased scavenging activity in a dose dependent manner. **Conclusion:** The evaluation of antioxidant activity of *Samadera indica* concludes that the plant extracts showed activity and this may be due to the presence of flavanoids. Hence, further work should be done on the isolation and identification of other antioxidant components of *Samadera indica.*

Key words: Antioxidant, Free radical scavenging, Samadera indica, Free radical

INTRODUCTION

Since ancient times for the world population, natural source serves as primary element for health and healing and till dated many constituents from this natural source are yet to be explored. This has made the scientist to look-in these plants for new constituents in order to treat many infectious diseases. Reports reveal that most of the medicinal plants possess antioxidant property, which play an important role in the prevention of various degenerative diseases and have potential benefits to the society.[1] Naturally occurring antioxidants like whole grains, fruits and vegetables are considered as primary source. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk that can scavenge free radical.[2,3] Some studies highlighted that phenolic compounds such as flavanoids, phenolic acid and tannins are much more potent antioxidants than vitamin C and vitamin E.[4] The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources, these free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease like anti-inflammatory, anti-carcinogenic, and anti-atherosclerotic activities. Phenolic compounds viz. phenolic acids, polyphenols and flavanoids scavenge free radicals like peroxide, hydro peroxide or lipid peroxyl. Other studies also showed that there is a high degree of correlation between the total antioxidant activity of some fruits and their phenolic contents.[5] This naturally occurring antioxidant showed greater advantages over currently available synthetic source namely butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters. As the synthetic source have low solubility, may prompt or cause negative effects and produced only moderate effect.[3] Hence the study was focused to examine out a natural antioxidant activity from *Samadera indica.*

Samadera indica Gaetrn (Simaroubaceae) is a bitter plant widely distributed throughout India, and mostly found in evergreen forest of Western Ghats and along river shore.[6] The bitterness of this plant is due to the presence of Quassinoids,

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which is a group of degraded triterpenes found in the family Simaroubaceae, that show wide variety of biological activities such as antitumor,[7] antifeedant,[8] phytotoxic,[9] antiviral,[10] and antihelmintic[11] etc. Samadera is used to vitiate diseases such as vata, kapha, arthritis, constipation and skin diseases like leprosy, scabies, purities, erysipelas.[6] Reports have suggested that some quassinoids like brusatol, glaucarubinone and quassin showed the presence of anti-malarial activity.[12] Samadera was also studied for insect anti-feedant and growth regulating activities of quassinoids. In the present study four Quassinoids such as indaquassin C, samaderins C, B and A was isolated from the Samadera and were tested for anti-feedant and growth regulating activities and Indaquassin C was found to be the most effective antifeedant.[13] Literatures shows the presence of Quassinoids, and the plant may show the presence of antioxidant activity which is not yet found out. Hence, the present study was oriented to examine the antioxidant activity of plant extracts *in vitro* using various model systems.

MATERIALS AND METHODS

Collection and Authentication of Plant Materials

The plant was collected from the locally growing area mostly from Ernakulam district, Kerala during the month of February. It was then botanically authenticated by taxonomist and a voucher specimen is currently deposited in the Department of Pharmacognosy, Amrita School of Pharmacy, Kochi. The leaves were separated, dried, coarsely powdered passed through sieve no 40 and stored in a closed container for further use.[14] All regents used were of analytical grades.

Preparation of crude extract

The coarse powder (50 gm) was extracted by soxhletation process. The powder was first defatted with n-hexane and then allowed to dry. The marc thus obtained was extracted for 72 hrs with both acetone and methanol. The resulting solvents were removed under reduced pressure and resulting semisolid, which was vacuum dried using rotary flash evaporator to get a solid residue and named it as Acetone Extract of *Samadera indica* (AESI) and Methanol Extract of Samadera indica (MESI). The dried extract thus obtained was used for assessment of antioxidant activity.

Preliminary Phytochemical Analysis

The dried extracts of AESI and MESI were used to analyze qualitatively various phytoconstituent like alkaloids, proteins, steroids, saponins, flavanoids, phenolic compounds and tannins. Phenolics: 2 ml of filtrate + 2 ml Ferric chloride, blue precipitate indicated presence of phenolics. Saponins (frothing test): 0.5 ml filtrate +5 ml distilled water; frothing persistence indicated presence of saponins. Alkaloids: 2 ml of filtrate +1% hydrochloride +Dragendroff reagent, orange precipitate indicate the presence of alkaloids. Flavanoids; 5 ml dilute ammonia was added to a portion of filtrate +concentrated sulphuric acid; yellow colour indicates presence of flavanoids. Steroids (Liebermann-Burchard reaction): 2 ml filtrate +2 ml acetic anhydride +concentrated sulphuric acid; green color indicates the presence of steroids. Terpenoids: 4 ml of filtrate +concentrated sulphuric acid 3 ml was added to form a layer; reddish brown coloration interface indicates the presence of terpenoids. Cardiac glycosides (Keller-Killiani test): 2 ml filtrate + 1 ml of glacial acetic acid + Ferric Chloride +concentrated sulphuric; brown colour indicates the presence of cardiac glycosides.[15]

DPPH free radical scavenging activity

The radical scavenging activity of all the plant extracts was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (2,2-diphenyl-1- picryl hydrazyl), which offers a convenient and accurate method for titrating the oxidisable groups of natural or synthetic antioxidants.[2,16] 0.1 mM solution of DPPH in ethanol was prepared; 1 ml of the solution was added to 2.5 ml of MESI and AESI in ethanol at different concentrations (100-500 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 518 nm by using a UV-Visible Spectrophotometer (Schimadzu UV-Visible 1700) and compared with Quercetin as the standard. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.[17-20] The percentage DPPH scavenging effect was calculated using the following equation:

$$\text{Percentage DPPH Scavenging Activity} = \left[1 - \left(\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right)\right] \times 100$$

Where, $\text{Abs}_{\text{control}}$ is the absorbance of control at 518 nm;

$\text{Abs}_{\text{sample}}$ is the absorbance of sample extract/standard at 518 nm.

Test was performed in triplicate and the results were averaged.

ABTS radical scavenging assay

$ABTS^{+}$ the oxidant is generated by per sulfate oxidation of 2, 2- azinobis (3-ethylbenzoline-6-sulphonic acid) – $ABTS^{2+}$.[21-23] $ABTS^{+}$ radical was freshly prepared by adding 4.9 mM ammonium persulfate solution to 14 mM ABTS solution and kept for 16 hrs in dark. After 16 hrs, this solution was diluted with ethanol (99.5%) to yield an absorbance of 0.70 ± 0.02 at 734 nm and the same was used for the assay. To 950 µl of ABTS radical solution, added 50 µl of extract solutions (100-500 µg/ml) and the

reaction mixture was vortexed for 10 sec. After 6 minutes the absorbance was recorded at 734 nm and compared with the control ABTS solution.[1,2] Percentage inhibition was calculated from the formula:

Percentage ABTS Scavenging Activity =

$$\left[1 - \left(\frac{Abs_{sample}}{Abs_{control}}\right)\right] \times 100$$

Where, $Abs_{control}$ is the absorbance of ABTS radical + methanol;

Abs_{sample} is the absorbance of ABTS radical + sample extract/standard.

Test was performed in triplicate and the results were averaged.

Ferric reducing ability of plasma (FRAP) expressed as a function of time

Ferric reducing ability of plasma is one of the method which directly analyses total antioxidant. FRAP agent was prepared by mixing acetate buffer (500 mM/l) with tripyridyltriazine (TPTZ) (10 mM/l) and 2.5 ml of ferric chloride (20 mM/l) solution. The reaction mixture contained freshly prepared FRAP reagent warmed to 37 °C, added to test along with water. Absorbance of this solution was taken at 593 nm, just after 4 min from the time of addition of FRAP reagent. An increase in absorbance indicated enhanced reducing potential of plasma. Quantitative calculation for each sample was done using an equation obtained from the standard curve of Fe^{++}-TPTZ.[24,25] The equation used was:

$$\text{Absorbance} = 0.274 \times \mu M \text{ of } Fe^{++} + 0.114 \; [R^2 = 0.974]$$

DCF/AAPH Assay

A water soluble azo initiator [2, 2'-diazobis (2-amidinopropane) dihydrochloride (AAPH)] produced the peroxyl radicals while a spectophotometric analysis of 2, 7- dichlorofluorescin-diacetate (DCF) monitored the scavenging activity of the plant extracts.[26] The activation of DCF was achieved by mixing DCF and NaOH and allowing the mixture to stand for 20 min before adding 18.25 ml of sodium phosphate buffer (25 mM, pH 7.2). The reaction mixture contained 10 µl of extract (diluted to final concentrations of 500, 250, 100, 50 and 25 µg/ml), 170 µl activated DCF solution and 20 µl of 600 mM AAPH (adjusted to a final concentration of 60 mM). The reaction was initiated by adding the AAPH solution. After 10 min, the absorbance was read at 490 nm using a spectrophotometer. Results were calculated over 60 min from the area under the curve and % inhibition was determined from the comparison to the buffer control curve recorded with each reading.[27,28]

Statistical analysis

All experimental measurements were carried out in triplicate and were expressed as average of three analyses ± standard deviation. Statistical analyzes was performed by t-test.

RESULT AND DISCUSSION

Preliminary phytochemical analysis

Preliminary phytochemical screening of AESI and MESI revealed the presence of alkaloids, tannins, phenolic compound and flavanoids.

Antioxidant activity

The antioxidant activity of AESI and MESI in various concentrations was evaluated using in-vitro models. It was observed that the test compounds scavenged free radicals in concentration dependent manner in all the models. The antioxidant activity was expressed as IC_{50} (the amount of antioxidant needed to decrease the radical concentration by 50%), which is negatively related to antioxidant activity. The lower the EC50 value, the higher is the antioxidant activity of the tested sample.

DPPH free radical scavenging activity

DPPH is a rapid, simple and inexpensive method to measure antioxidant capacity of plant extracts involves the use of the free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity.[5] It is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow colored product, diphenyl picryl hydrazine, with the addition of all fractions in a concentration-dependent manner. From table 1, it shows that all the concentration of AESI and MESI demonstrated the H-donating activity. The EC_{50} value of AESI and MESI were 3454 µg/ml and 5497 µg/ml respectively. This activity was found to be less than the standard Quercetin 17.84 µg/ml (figure 1).

ABTS radical scavenging assay

Proton radical scavenging is an important attribute of antioxidants. The method is based on the technique that ABTS react with potassium per sulphate and produces a blue green color due to the formation of ABTS radical cation ($ABTS^+$). The colored radical formed will be converted to colorless radical in the presence of antioxidant radical and it is measured at 734 nm.[21-23] The table 2 shows that free radical scavenging activity increases with the concentration of the extracts AESI and MESI (100-500µg/ml). The radical scavenging activity of AESI and MESI showed the free radical scavenging activity and the EC_{50} value were found to be 980.5 µg/ml and 1123 µg/ml respectively (table 2). But the activity was found to be less than quercetin, has EC_{50} value 70.28 µg/ml (figure 2).

Ferric reducing ability of plasma (FRAP) expressed as a function of time

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6).[24] Table 3 and figure 3 showed that both AESI and MESI showed a good antioxidant activity.

Table 1: Percentage Scavenging Activity of AESI and MESI by DPPH Scavenging Radical Method

Standard Concentration(µg/ml)	Percentage inhibition of Quercetin	Concentration of extracts (µg/ml)	Percentage inhibition of AESI	Percentage inhibition of MESI
20	53.1294 ± 0.1462225	100	13.72751 ± 0.2403619	7.137693 ± 0.102587
40	64.47514 ± 0.07229092	200	17.35342 ± 0.1763765	9.249325 ± 0.08292265
60	71.02309 ± 0.08344647	300	20.52277 ± 0.0809797	11.7947 ± 0.08947238
80	76.94708 ± 0.06601271	400	23.01868 ± 0.1000132	13.87589 ± 0.2142842
100	81.29971 ± 0.27206	500	27.31042 ± 0.1545977	17.4942 ± 0.1646634
IC_{50}	17.84 µg/ml		3454 µg/ml	5497 µg/ml

Values are mean ± SD (n = 3).

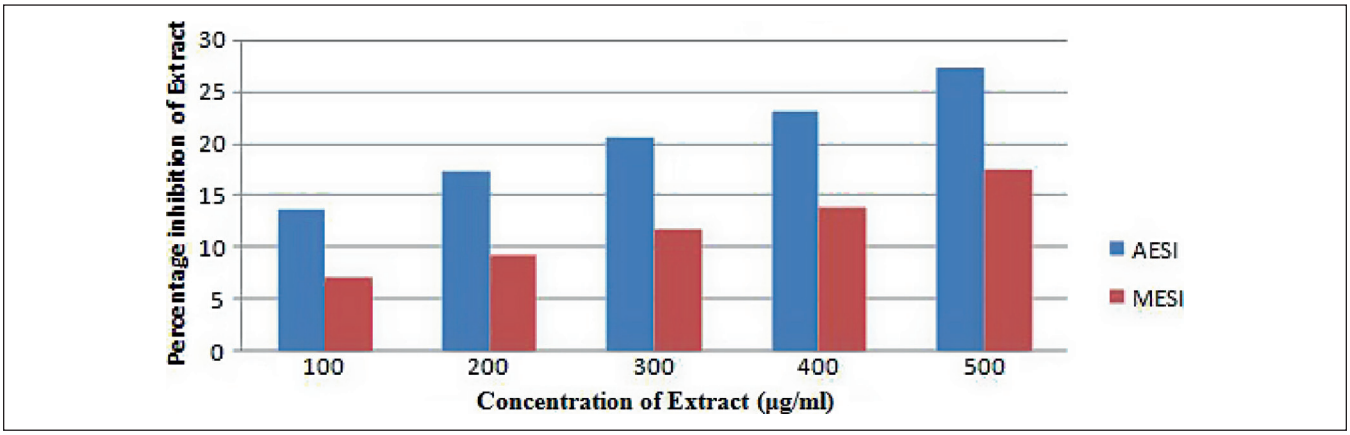

Figure 1: DPPH Radical Scavenging Method

Table 2: Free Radical Scavenging Activity of AESI and MESI by ABTS Radical Scavenging Assay

Concentration of Quercetin (µg/ml)	Percentage inhibition of Quercetin	Concentration of Extract (µg/ml)	Percentage inhibition of Extract	
			AESI	MESI
20	72.30 ± 0.149	100	7.605016 ± 0.1445431	13.73194 ± 0.1786582
40	76.95132 ± 0.09134215	200	12.23478 ± 0.2854078	16.77395 ± 0.1160129
60	82.1294 ± 0.2458645	300	17.03142 ± 0.1569847	21.93773 ± 0.2274192
80	86.73056 ± 0.1121264	400	22.00448 ± 0.1404752	26.34816 ± 0.1408803
100	91.02179 ± 0.1242456	500	25.48992 ± 0.1468061	27.54017 ± 0.2528417
IC_{50}	70.28 µg/ml		980.5 µg/ml	1123 µg/ml

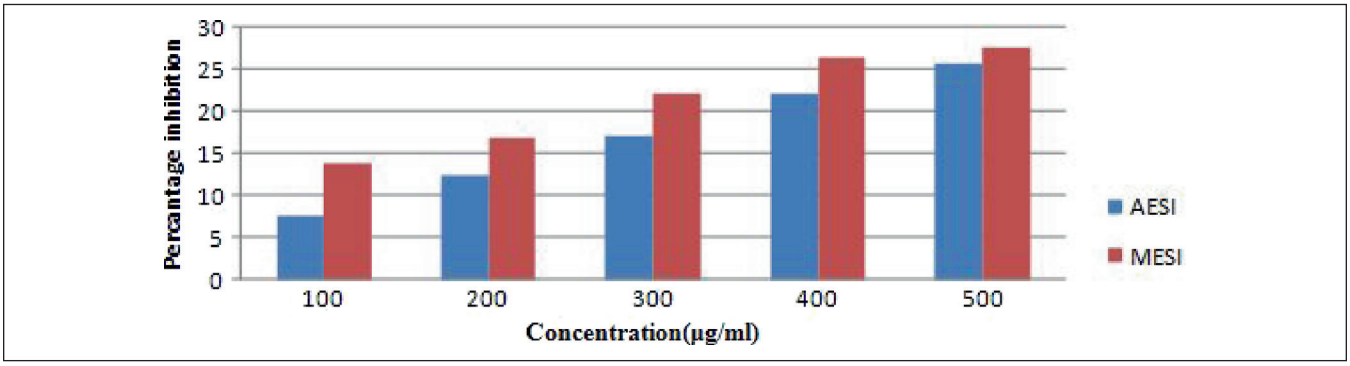

Figure 2: ABTS Radical Scavenging Assay

DCF/AAPH Assay

The azo scavenging activity of the extracts AESI and MESI were examined and it showed a dose dependent activity with concentration. But the antioxidant activity was comparatively less than the standard quercetin. The data's are represented in Table 4 and figure 4.

CONCLUSION

The traditional medicines have gained place in humans' health care from ancient times, as it is safe and possess prominent action against many degenerative diseases. A common theme that underlines the etiology of degenerative disease is free

Table 3: Ferric reducing ability of plasma (FRAP) expressed as a function of time

Concentration (µg/ml)	Absorbance	
	Acetone	Methanol
10	0.06363333 ± 0.0002333323	0.2034 ± 0.000608278
20	0.1357 ± 0.0006027716	0.2089333 ± 0.0002403693
30	0.1944 ± 0.0004163319	0.2959667 ± 0.002596361
40	0.4128667 ± 0.000218582	0.3569 ± 0.001703911
50	0.5369666 ± 0.001047741	0.6745334 ± 0.001245434

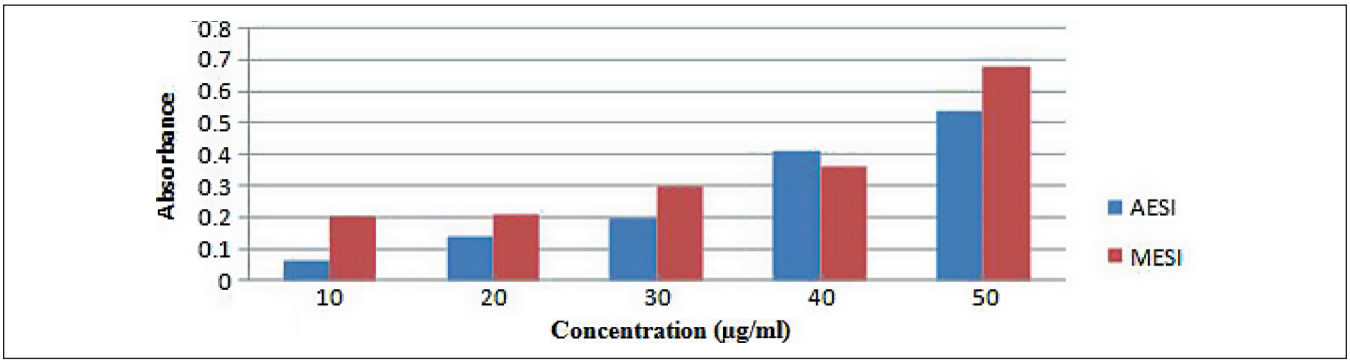

Figure 3: Ferric reducing ability of plasma (FRAP) expressed as a function of time

Table 4: Free Radical Scavenging Activity of AESI and MESI by DCP/AAPH Assay

Concentration (µg/ml)	Quercetin	Concentration of Extract (µg/ml)	AESI	MESI
10	11.66 ± 0.13	100	35.19 ± 0.06	29.54 ± 0.07
20	17.95 ± 0.08	200	39.33 ± 0.07	34.51 ± 0.05
30	49.41 ± 0.11	300	40.33 ± 0.05	39.32 ± 0.09
40	53.57 ± 0.24	400	42.77 ± 0.10	45.20 ± 0.18
50	65.54 ± 0.07	500	43.88 ± 0.16	46.93 ± 0.07
IC-50	31.47 µg/ml		628.4 µg/ml	437.2 µg/ml

Values are Mean ± SEM (n = 3)

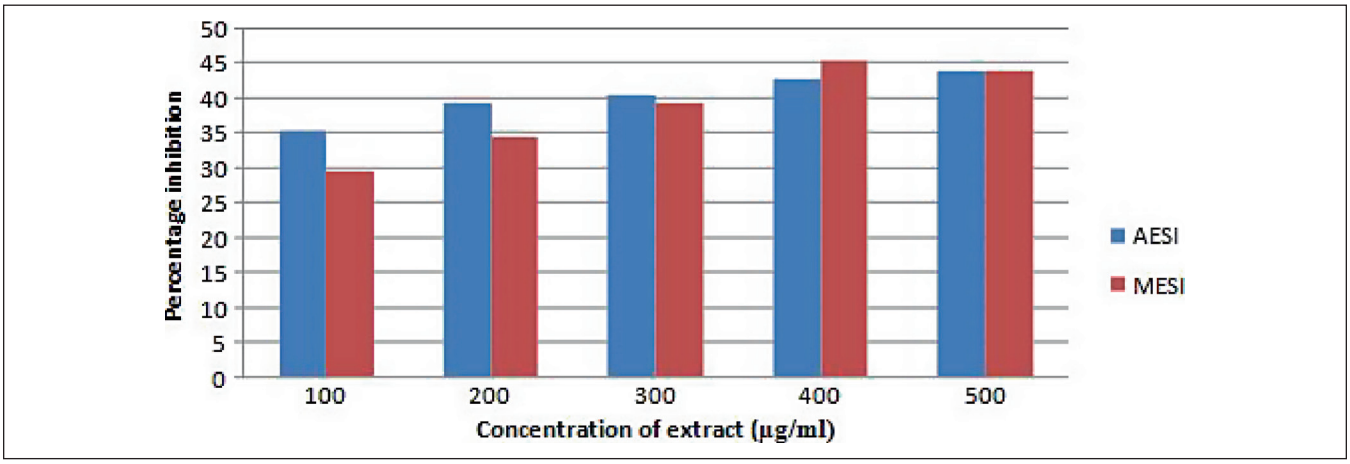

Figure 4: DCF/AAPH Assay

radical stress and antioxidants play a key role in inhibiting and scavenging free radicals, thereby protecting from the threat. Researchers are focusing on natural antioxidants and the use of it is increasing at an alarming rate as it is safe and posses a prominent activity than synthetic ones. The present study demonstrated that both acetone and methanolic extracts of *Samadera indica* exerts antioxidant capacity and hence can be used for free radical stress. The preliminary phytochemical screening exhibited the presence of alkaloids, tannins, phenolic compound and flavanoids. It can be also supported by literatures for the presence of Quassinoids like samaderine A, B and C. Indaquassin brusatol, glaucarubinone and quassin, which demonstrated the antimalarial activity. Hence from the present study we speculate that the antioxidant capacity of *Samadera indica* may be due to the presence of tritereponoids; Quassinoids. Therefore, a potent antioxidant can be isolated and identified from the extracts of these plants and can be used as a natural antioxidant.

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Antimicrobial Activity of *Ruellia tuberosa* L. (Whole Plant)

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ABSTRACT

The present communication attempts to study the antimicrobial activity of successive extraction using n-hexane, chloroform, ethyl acetate, alcohol and separate aqueous extract from whole plant of ***Ruellia tuberosa*** **L.** against different bacterial and fungal organisms (ATCC, MTCC) using disc diffusion method. The chloroform, ethyl acetate, alcohol and aqueous extracts were active against all the bacteria tested and showed significant antibacterial properties. The aqueous extract exhibited less activity against fungal organisms. Thus it may be informed that ***Ruellia tuberosa*** **L.** extract may be used to treat oral bacterial diseases.

Key words: *Ruellia tuberosa,* Antimicrobial activity, Successive extractions, MIC

INTRODUCTION

Since ancient time, plants have been a veritable source of drugs. Recent research work on herbal medicinal plants is intensified and information on these plants be exchanged. This thought will go a long way in the scientific exploration of medicinal plants for the benefit of man and is likely to decrease the dependence on drugs. Today plant compounds are providing the models for 50% of western drugs.[1] The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternative, offering profound therapeutic benefits and more affordable treatment. Some studies have concentrated exclusively on oils on microorganism. While these data are useful, the reports are not directly comparable due to methodological difference such as choice of plant extracts, test microorganism and antimicrobial test methods.[2]

Ruellia tuberosa **L.** is a Minnie root medicinal plant; a tropical plant widely distributed in South East Asia. It belongs to the family of Acanthaceae. It is a tropical perennial growing up to a height of 6.5" with a hairy stem. The simple leaves are opposite and elliptic; the plant flowers after the start of the rainy season. It has thick finger like roots. The big bisexual flowers are violet in colour. The ripe fruits are in a pod with 7-8 seeds each, burst open, when they get wet and the black seeds are hurdled away.[3,4] ***Ruellia tuberosa*** **L.** is medicinally used as an anthelmentic, against joint pains and strained muscles. In folk medicine, it has been used as diuretic, antidiabetic, antipyretic, analgesic, anti-hypersensitive, thirst-quenching, and antidotal agent. It has also recently been incorporated as a component in herbal drink in Taiwan,[5,6] and traditionally used for reducing toxicity, healing urine tract inflammation,[7] However, very few chemical constituents and pharmacological activities have been reported for this species.[5,8,9]

The present communication attempts to study the antimicrobial activity of successive extraction using n-hexane, chloroform, ethyl acetate, alcohol and separate aqueous extract from whole plant of ***Ruellia tuberosa*** **L.** against different bacterial and fungal organisms (ATCC, MTCC) using disc diffusion method. The scope of this study is to analyze antimicrobial compound and to generate data for this plant crude extract on which little information exist.

MATERIALS AND METHODS

Plant materials

The fresh plant materials of ***Ruellia tuberosa*** **L.** (Acanthaceae) were collected in July 2008 from district of Tamilnadu, India, and identified with the help of the Flora of Presidency of Madras[10,11] and a dried specimen (No: 00628) was deposited in museum of CSMDRIA Chennai.

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Extract preparation

The whole plant of ***Ruellia tuberosa* L.** was collected and allowed to dry at room temperature in the laboratory for a period of 2 weeks in sun shade, coarsely powdered and weighed. Then it was soaked with n-hexane for 48 hrs, the extract was filtered, the filtrate was concentrated by distillation over boil water batty and the last traces of solvent were removed under vacuum. Extraction was repeated with n-hexane and the residue was then extracted twice with dichloromethane, ethyl acetate and methanol respectively and successively. Aqueous extract was separately taken by using soxlet apparatus and concentrated under reduced pressure (T < 40 ºC). The yield of n-hexane was 134.34%, for chloroform 16.05%, yield with ethyl acetate 39.85%, yield with alcohol 56.63% and aqueous 83.06% per kg of plant powder. One gram from each successive extract were weighted in dry clean bottle and diluted by using 10% solution of dimethylsulfoxide (DMSO), dimethlyformamide (DMF), to make the concentration of 100 mg/ml. The aqueous extract is diluted by using saline (0.9%). The diluted solution was used for further antimicrobial work.

Microorganism

Both Gram positive and Gram negative bacteria's and fungus (ATCC and MTCC) were used as test organism for this study. They were obtained from the stock cultures of department of microbiology CSMDRIA & from SRM medical college hospital and also from Dr. Jeorge Mosses Lab, Chennai, India. **Gram positive bacteria** such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Shigella sonnei,* and *Protease*. **Gram negative bacteria** such as *Salmonella, Staphylococcus spp, Seratia, Bacillus spp* and *Protease* and fungus like *Saceromyces cervesiae, Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Candida albicans,* and *Candida tropicallis*. The organisms were sub cultured on to nutrient agar in order to determine their viability. The identity of each test organism was confirmed by using standard culture, morphological and biochemical techniques as described.[12] Stock cultures were maintained on nutrient agar slants at 4 ºC and then sub-cultured in nutrient broth at 37 ºC prior to each antimicrobial test. Inoculants of the test organisms were standardized by methods.[13] This was done by suspending 5 colonies of a 24 hrs culture in 5 ml of nutrient broth and comparing the turbidity with that of 0.5 Mac farina standards after incubating at 35 ºC for 2 hrs. Then the plant extract fractions were subjected to antimicrobial assay using disc diffusion method.[3,4,14,15]

Anti-microbial assay

Muller Hinton Agar (Hi media) for bacteria and Saber Dextrose Agar (Hi-media) for fungus were prepared according to the manufacturer's instructions. Muller Hinton Agar (MHA) contains Beef-2 g, casein acid hydrolysate 17.5 g, starch 105 g and agar 17 g; pH 7.4 ± 0.2. MHA (38 g) was weighed and dissolved in 1000 ml of distilled water. Sabouraud Dextrose Agar (SDA) was used for cultivation of fungi and particularly pathogenic fungi associated with skin infections. It contains Peptone –10 g, dextrose 40 g and agar 15 g; pH 5.6 ± 0.2. SDA (65 g) was dissolved in 1000 ml of distilled water. The medium was sterilized by autoclaving at 121 ºC for 15 minutes at 15 psi pressure and was used for tests. Sterile molten cool (45 ºC) agar was poured aseptically into sterile petridishes (15 ml each) and the plates were allowed to solidify at room temperature in sterile condition. After gelling and drying, the plates were seeded with appropriate micro organisms by streaking evenly on to the surface of the medium with a sterile cotton swab or pouring the appropriate microorganism on the surface of dry agar plate present in peptone broth. Care was taken for the even distribution of culture all over the plate. The inoculums were allowed to dry for 5 minutes. The discs of 6 mm diameter were prepared from Whatmann filter paper No. 1 and were sterilized in a hot air oven at 160 ºC for 1 hrs. The discs were then impregnated with the extracts and solvent DMSO, Amikacin, Gatifloxacin, Ciprofloxacin, Amphitrosine, discs were used as standard. Each disc contained 5 µg of corresponding standards. Sterile Whattman No 1 filter paper with 100 mg/ml were placed on to the agar with flamed forceps and gently pressed down to ensure contact along with the diluted extract, one appropriate control dry disc also placed at the center. Then the plates were incubated below 37 ºC for 24 hrs to allow perfusion of drugs being tested. The next day the zones of inhibition were measured with a measuring scale. This experiment was carried out in triplicate for their conformation. The results were read by the presence or absence of zone of inhibition. The lowest concentration of the each extract that inhibited the organisms was recorded as the MIC. This experimental procedure was repeated using several dilution of different successive extract until the minimal inhibitory zone was obtained.

RESULT AND DISCUSSION

Table 1 shows that preliminary antimicrobial test for ***Ruellia tuberosa* L.** whole plant successively extracted by n-hexane, chloroform, ethyl acetate and alcohol, and separate aqueous extract by the disc diffusion method. **Table: 2** shows that *Escherichia coli* have no characteristic zone of minimal inhibition for all extracts. It is indicated that *Escherichia coli* does not show any growth inhibition for all the extract at any concentration. Chloroform extract has zone of minimal inhibition of 1.0 cm at (50 µl), ethyl acetate extract has 1.0 cm at (200 µl) and alcohol extract has 0.8 cm at (100 µl) but hexane shows no characteristic zone of minimal inhibition at any concentration for *Pseudomonas aeruginosa.*

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

Name of the Micro organism	H	C	E	M	A	Con
Gram positive bacteria						
Escherichia coli ATCC – 73	–	–	–	–	–	Ak
Pseudomonas aeruginosa ATCC – 25583	–	+	+	+	–	Ak
Klebsiella pneumoniae ATCC – 700693	–	+	+	+	+	Ak
Shigella sonnei ATCC – 29508	+	+	+	+	+	Gt
Protease ATCC -9484	–	+	+	–	–	Cf
Gram negative bacteria						
Salmonella ATCC -10749	–	+	–	–	–	Ak
Staphylococcus spp ATCC – 25923	–	+	+	+	–	Ak
Seratia ATCC -14460	–	+	+	+	+	Gt
Bacillus spp ATCC – 6633	+	+	+	+	+	Gt
Fungus						
Saceromyces cervesiae	–	–	–	–	+	A
Aspergillus niger ATCC – 2587	–	–	–	–	–	A
Aspergillus fumigatus MTCC – 2551	–	–	–	–	–	A
Aspergillus flavus MTCC – 1884	–	–	–	–	–	A
Candida albicans	–	–	–	–	–	A
Candida tropicallis	–	–	–	–	–	A

H: n-Hexane, C: Chloroform, E: Ethyl acetate, M: Alcohol, A: Aqueous, Con: Control Ak = Amikacin, Gt = Gatifloxacin, Cf = Ciprofloxacin, A = Amphitrosine, (+) = Positive results, (–) = Negative results.

Table 2: Antibacterial effect of successive extract of n-Hexane, Chloroform, Ethyl acetate, and Alcohol extract of from whole plant of *Ruellia tuberosa* L. for Gram positive bacteria (100 mg/ml)

Name of the Bacterial organism	Zone of minimal inhibition (cm)										
	Successive extracts										
	Control	n-Hexane (µl)	Chloroform (µl)			Ethyl acetate (µl)		Alcohol (µl)			
		100	10	25	50	100	50	200	25	50	100	800
Escherichia coli	2.0	–	–	–	–	–	–	–	–	–	–	–
Pseudomonas aeruginosa	3.0	–	–	–	1.0	–	–	1.0	–	–	0.8	–
Klebsiella pneumoniae	3.0	–	2.2	–	–	–	0.8	–	0.8	–	–	–
Shigella sonnei	3.5	1.2	–	–	–	0.8	0.8	–	–	–	–	1.2
Protease	1.5	–	–	–	–	0.8	–	–	–	–	–	–

It is indicated that the growth of *Pseudomonas aeruginosa* is inhibited at 50 µl for chloroform, 200 µl for ethyl acetate and 100 µl for alcohol extract. More over *Pseudomonas aeruginosa* does not show any growth inhibition for hexane extract at any concentration. Chloroform extract has zone of minimal inhibition of 2.2 cm at (10 µl), ethyl acetate extract has 0.8 cm at (50 µl) and alcohol extract has 0.8 cm at (25 µl) but hexane extract show no characteristic zone of minimal inhibition at any concentration for *Klebsiella pneumoniae*. It is indicated that the growth of *Klebsiella pneumoniae is* inhibited at 10 µl for chloroform, 50 µl for ethyl acetate and 25 µl for alcohol extract. More over *Klebsiella pneumoniae* does not show any growth inhibition for hexane extract at any concentration. Hexane extract has zone of minimal inhibition of 1.2 cm at (100 µl), chloroform extract has 0.8 cm at (100 µl), ethyl acetate extract has 0.8 cm at (50 µl) and alcohol extract has 1.2 cm at (800 µl) concentration for *Shigella sonnei*. It is indicated that the growth of *Shigella sonnei is* inhibited at 100 µl for hexane, 100 µl for chloroform, 50 µl for ethyl acetate and 800 µl for alcohol extract. **Table: 3**

Table 3: Antibacterial effect of Aqueous extract from whole plant of *Ruellia tuberosa* L. for Gram positive bacteria (100 mg/ml)

Name of the Bacterial organism	Zone of minimal inhibition		
	Separate aqueous extracts		
	Control	10 µl	100 µl
Escherichia coli	2.0 cm	–	–
Pseudomonas aeruginosa	3.0 cm	–	–
Klebsiella pneumoniae	3.0 cm	1.3 cm	–
Shigella sonnei	3.5 cm	–	0.5 cm
Protease	1.5 cm	–	–

shows that aqueous extract shows no characteristic zone of minimal inhibition at any concentration for *Escherichia coli, pseudomonas aeruginosa* and *Protease, but* aqueous extract has 1.3 cm at (10 µl) for *Klebsiella pneumoniae* and 0.5 cm at (100 µl) concentration for *Shigella sonnei*. It is indicated that the growth of *Klebsiella pneumoniae* and *Shigella sonnei* are inhibited at 10 µl and 100 µl for aqueous extract. **Table: 4**

Table 4: Antibacterial effect of successive extract of Hexane, Chloroform, Ethyl acetate, and Alcohol extract from whole plant of *Ruellia tuberosa* L. for Gram negative bacteria (100 mg/ml)

Name of the Bacterial organism	Zone of minimal inhibition								
	Successive extracts								
	Control	n-Hexane (µl)		Chloroform (µl)		Ethyl acetate (µl)		Alcohol (µl)	
		25	10	100	1600	100	400	10	50	100
Salmonella	3.0 (cm)	–	0.8	–	–	–	–	–	–	–
Staphylococcus	4.0 (cm)	–	–	1.1	–	–	1.2	–	1.8	–
Seratia	3.2 (cm)	–	–	0.8	–	0.9	–	–	–	0.6
Bacillus	4.0 (cm)	0.9	–	–	0.8	–	1.2	0.8	–	–

shows that chloroform extract has zone of minimal inhibition of 0.8 cm at (10 µl) but hexane, ethyl acetate, and alcohol extract shows no characteristic zone of minimal inhibition at any concentration for *Salmonella*. It is indicated that the growth of *Salmonella* is inhibited at 10 µl for hexane. Chloroform extract has zone of minimal inhibition of 1.1 cm at (100 µl), ethyl acetate extract has 1.2 cm at (400 µl) and alcohol extract has 1.8 cm at (50 µl) but hexane extract shows no characteristic zone minimal inhibition at any concentration for *Staphylococcus spp*. It is indicated that the growth of *Staphylococcus spp* is inhibited at 100 µl for chloroform, 400 µl for ethyl acetate and 50µl for alcohol extract. More over *Staphylococcus spp* does not show any growth inhibition for hexane extract at any concentration. Chloroform extract has zone of minimal inhibition of 0.8 cm at (100 µl), ethyl acetate extract has 0.9 cm at (100 µl) and alcohol extract has 0.6 cm at (100 µl), but hexane and aqueous extract shows no characteristic zone minimal inhibition at any concentration for *Seratia*. It is indicated that the growth of *Seratia* is inhibited at 100 µl for chloroform, 100 µl for ethyl acetate and 100 µl for alcohol extract. More over *Seratia* does not show any growth inhibition for hexane extract at any concentration. Hexane extract has zone of minimal inhibition of 0.9 cm at (25 µl), chloroform extract has 0.8 cm at (1600 µl), ethyl acetate extract has 1.2 cm at (100 µl) and alcohol extract has 0.8 cm at (10 µl) concentration for *Bacillus spp*. It is indicated that the growth of *Bacillus spp* is inhibited at 25 µl for hexane, 1600 µl for chloroform, 100 µl for ethyl acetate and 10 µl for alcohol extract. **Table: 5** shows that aqueous extract shows no characteristic zone minimal inhibition at any concentration for *Salmonella and Staphylococcus spp,* but aqueous extract has 0.6 cm at (100 µl) for *Seratia* and 0.8 cm at (10 µl) concentration for *Bacillus spp*. It is indicated that *Seratia* and *Bacillus* shows resistance to aqueous extract at any concentration. It is indicated that there is no inhibition for growth of *salmonell*a and *staphylococcus spp*, but there is growth inhibition for *Seratia* at 100 µl and *Bacillus spp* at 10 µl for aqueous extract. **Table: 6** Shows that no characteristic zones of minimal inhibition for Hexane, Chloroform, Ethyl acetate, Alcohol extract for *Saceromyces cervesiae, Aspergillus niger. Aspergillus fumigatus, Aspergillus flavus, Candida albicans* and *Candida tropicallis*. It is indicated that the *Saceromyces cervesiae, Aspergillus niger. Aspergillus fumigatus, Aspergillus flavus, Candida albicans* and *Candida tropicallis* does not show any growth inhibition for hexane, Chloroform, Ethyl acetate, and Alcohol extract at any concentration. **Table: 7** Shows that no characteristic zone of minimal inhibition for *Aspergillus niger. Aspergillus fumigatus, Aspergillus flavus, Candida albicans* and *Candida tropicallis* for Aqueous extract but *Saceromyces cervesiae* has zone of minimal inhibition of 0.8 cm at (10 µl). It is indicated that the *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Candida albicans* and *Candida tropicallis* does not show any growth inhibition for aqueous extract at any concentration, but there is growth inhibition for *Saceromyces cervesiae* at 10 µl for aqueous extract. All fractions showed a very good level of broad spectrum antibacterial activity tested at a concentration 500 µgm/ml particularly good activity was observed in the ethyl acetate and alcohol fractions from the whole plant extract. None of the fractions demonstrated antifungal activity expects aqueous extract from the whole plant for *Saceromyces cervesiae*. Though no valuable activity was observed against fungi there results may provide scientific support for some uses of the plant in traditional medicine. Chloroform and hexane extract showed less activity than that of ethyl acetate and alcohol extract at the tested concentration against bacterial strain.

Table 5: Antibacterial effect of separate Aqueous extract from whole plant of *Ruellia tuberosa* L. for Gram negative bacteria (100 mg/ml)

Name of the Bacterial organism	Zone of minimal inhibition		
	Separate aqueous extracts		
	Control (µl)	10 (µl)	100 (µl)
Salmonella	3.0 cm	–	–
Staphylococcus	4.0 cm	–	–
Seratia	3.2 cm	–	0.6 cm
Bacillus	4.0 cm	0.8 cm	–

Table 6: Antifungal effect of successive extract of Hexane, Chloroform, Ethyl acetate, Alcohol extract of from whole plant of *Ruellia tuberosa* L. for fungus (100 mg/ml)

Name of the Fungal organism	Zone of minimal inhibition				
	n-Hexane, Chloroform, Ethyl acetate, Alcohol extract				
	Control (µl)	10 (µl)	25 (µl)	50 (µl)	100 (µl)
Saceromyces cervesiae	1.2 cm	–	–	–	–
Aspergillus niger	1.2 cm	–	–	–	–
Aspergillus fumigatus	2..0 cm	–	–	–	–
Aspergillus flavus	1.1 cm	–	–	–	–
Candida albicans	1.1 cm	–	–	–	–
Candida tropicallis	1.1 cm	–	–	–	–

Table 7: Antifungal effect of separate extract of Aqueous from whole plant of *Ruellia tuberosa* L. for fungus (100 mg/ml)

Name of the Fungal organism	Zone of minimal inhibition				
	Aqueous extract				
	Control (µl)	10 (µl)	25 (µl)	50 (µl)	100 (µl)
Saceromyces cervesiae	1.2 cm	0.8 cm	–	–	–
Aspergillus niger	1.2 cm	–	–	–	–
Aspergillus fumigatus	2..0 cm	–	–	–	–
Aspergillus flavus	1.1 cm	–	–	–	–
Candida albicans	1.1 cm	–	–	–	–
Candida tropicallis	1.1 cm	–	–	–	–

CONCLUSION

In conclusion data presented in this study explain and justify the use of ***Ruellia tuberosa* L.** extract in the treatment of diarrhoea, typhoid, cholera, chronic jaundice, fever, headache, skin disease etc. all the extractives showed a range of activity against all the tested bacteria drastically improved the level of activity for ethyl acetate and alcohol fractions exhibiting in all cases at better levels of activity. Though no activity was observed against fungi, these results may prove scientific support for some uses of the plant in traditional medicine. The present work has shown that the whole plant of ***Ruellia tuberosa* L.** studied is potentially good source of antimicrobial agents and that further investigations are carried to support the view that traditional use in medicine and also assisting primarily health care.

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Anti-inflammatory Effect of Ethanolic Extract of *Ficus bengalensis* Linn. in Carrageenan Induced Paw Edema In Rats

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ABSTRACT

Ficus bengalensis of family Moraceae was extensively utilized by the tradipractitioners for its various ethnopharmacological activities. The aim of this study was to evaluate, experimentally, the antiinflammatory effect of ethanolic extract of the bark of *F. bengalensis* in carrageenan induced paw edema in rats at a dose level of 50, 100 and 200 mg/kg, orally. The extract was administered for the antiinflammatory activity 1 h prior to carrageenan injection in the subplantar region. Paw edema was measured by plethysmometer on 1st and 3rd h, after carrageenan injection. *F. bengalensis* extracts at all the doses significantly prevented the inflammation in dose dependent manner which was comparable to that of diclofenac sodium (5 mg/kg, intraperitoneally). The phytochemical analysis of *F. bengalensis* extracts revealed the presence of antioxidant phytochemicals such as flavonoids and tannins, and thus the antiinflammatory action of *F. bengalensis* extracts may be subsequent to its *in vivo* antioxidant activity. *F. bengalensis* extracts eliminate the systemic oxidative stress produced by carrageenan injection.

Key words: Antiinflammatory, antioxidant, *Ficus bengalensis*.

INTRODUCTION

Ficus bengalensis Linn. (Moraceae) is popularly known as "*Banyan*." It is a large tree, widely distributed in tropical and subtropical regions. The bark and aerial roots of this plant are frequently used as a folk medicine, particularly in conditions like ulcer, leprosy, sepsis, diarrhoea, dysentery, diabetes, gonorrhea and piles.[1,2] The milky juice from its aerial roots possesses aphrodisiac and antiinflammatory activity[3] and its paste is applied externally in conditions like pain, bruises, rheumatism, cracked feet and gum inflammation.[4] Some workers have demonstrated antioxidant effect of *F. bengalensis* in *in vitro* studies.[5] Numbers of plants having antioxidant property were found effective in preventing the development of inflammation and thus indicated in inflammatory conditions.[6] The protection offered by such plants has been attributed to their antioxidant property. Hence, it was contemplated that ethanolic extract of *F. bengalensis* may exhibit antiinflammatory effect in rats, particularly in view of its traditional use in inflammatory conditions and *in vitro* antioxidant property.

Therefore, the present study demonstrated the influence of ethanolic extract of the bark of *F. bengalensis* against carrageenan induced paw edema in rats. Further to delineate the antiinflammatory effect to the antioxidant activity, oxidative stress parameters were assessed.

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MATERIALS AND METHODS

Plant material

The bark of ***F. bengalensis*** was collected in July, 1999. The plant material was authenticated and deposited at Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University Campus, Nagpur, Maharashtra, India.

Preparation of ethanolic extract of *Ficus bengalensis* (FBE)

The dried coarse powder of the bark of ***F. bengalensis*** was defatted by extracting with petroleum ether (60-80 °C)

in the Soxhlet apparatus continuously for 72 h, and the phytochemicals were then extracted by using 90%, v/v ethanol. The concentrated ethanolic extract was solvent dried under reduced pressure and subsequently air dried until constant weight (extraction yield 13%, w/w). The extract was suspended in 0.1%, Na-CMC for oral administration.

Preliminary phytochemical screening

FBE was subjected to the preliminary phytochemical screening as per standard procedures.[7]

Animals

Sprague Dawley rats (220-225 g) of either sex were used. Animals were kept at the departmental animal house at 25 ± 2 °C, under relative humidity of 55-65% and light and dark cycles of 12 h. Animals were provided with a standard pellet diet (Goldmohar brand, Lipton India Ltd.) and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethical Committee constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi.

Doses and treatments

Diclofenac sodium was a gift sample from Zim Laboratories Ltd., while thiobarbituric acid and carrageenan (Lambda grade) were purchased from Sigma Aldrich, USA. Pyrogallol and trichloroacetic acid were purchased from LOBA Chemicals, India.

Acute oral toxicity study

FBE was tested at 2000 mg/kg, orally for its cut off LD_{50} dose as per OECD guidelines.[8] No toxicity of any nature or mortality could be observed in the subsequent 14 days after receiving this dose and hence the maximum employed dose of extract was 200 mg/kg (10% of the cut off level of LD_{50}).

Grouping and treatments

Sprague Dawley rats (220-225 g) of either sex were divided into five different groups containing 5 animals each. Group I received 0.1 ml of normal saline (in the subplantar region) while group II, III, IV and V received 0.1 ml of 1%, w/v carrageenan (in the subplantar region). During the treatment, group II received vehicle of the extract (5 ml/kg, 0.1% Na-CMC, orally), while group III, IV and V received FBE (50, 100 and 200 mg/kg, orally). Group VI was administered diclofenac sodium (5 mg/kg, intraperitoneally) as standard antiinflammatory agent. The vehicle, extracts and diclofenac sodium were administered 1 h before carrageenan administration.

Assessment of the antiinflammatory activity in carrageenan induced paw edema[9]

The increase in the paw volume was recorded on plethysmometer (UGO Basile, Italy) at 1st and 3rd h after administration of carrageenan. The results are expressed in terms of mean increase in paw volume at 1st and 3rd h and antiinflammatory activity was expressed in terms of percent inhibition of paw edema at 3rd h.

Assessment of oxidative stress parameters in blood

Estimation of lipid peroxidation (LPO)

Malondialdehyde formation was estimated by the method of Stocks and Dormandy, absorbance was measured at 532 nm.[10]

Estimation of superoxide dismutase (SOD)

SOD was estimated by the method of Marklund and Marklund.[11]

Estimation of catalase (CAT)

Catalase was estimated by Aebi method.[12]

Statistical analysis

The data were analyzed by one way ANOVA followed by Dunnett's multiple comparison test. A difference of $P < 0.05$ was considered significant in all the cases.

RESULTS

Phytochemical screening

The phytochemical screening of FBE indicates the presence of flavonoids, carbohydrates, glycosides and tannins.

Assessment of antiinflammatory activity

One way ANOVA revealed a significant ($p < 0.0001$) influence of FBE on the carrageenan induced inflammation in rat paw. Post hoc Dunnett test indicated that the dose of 50 mg/kg produced significantly less effect while higher doses 100 and 200 mg/kg produced maximum effect when compared to vehicle. This effect of FBE was comparable to that of diclofenac sodium, a standard antiinflammatory agent (Table 1).

Assessment of in vivo antioxidant activity

One way ANOVA revealed a significant influence of FBE on oxidative stress parameters in blood. Carrageenan administration increased systemic oxidative stress after 3rd h as shown by increased LPO and activities of SOD and CAT. Post hoc Dunnett test indicated that FBE administration reduced the lipid peroxidation and restored the activities of SOD and CAT at all doses (Figure 1, 2, and 3).

Table 1: Assessment of the antiinflammatory activity of FBE in carrageenan induced paw edema

Treatment	Dose (mg/kg)	Mean increase in paw volume		Percent inhibition of paw edema at 3rd h
		1st h	3rd h	
Vehicle	–	0.70 ± 0.02	0.73 ± 0.04	–
FBE	50	0.55 ± 0.02*	0.50 ± 0.008*	31.50
FBE	100	0.36 ± 0.01#	0.32 ± 0.002#	56.16
FBE	200	0.28 ± 0.006@	0.22 ± 0.01@	69.86
Diclofenac sodium	5	0.22 ± 0.01@	0.20 ± 0.02@	72.60

Each value is presented as mean ± SEM (n = 5 rats; one-way ANOVA followed by Post hoc Dunnett test). *p < 0.05, #p < 0.05, @p < 0.05 compared with the vehicle.

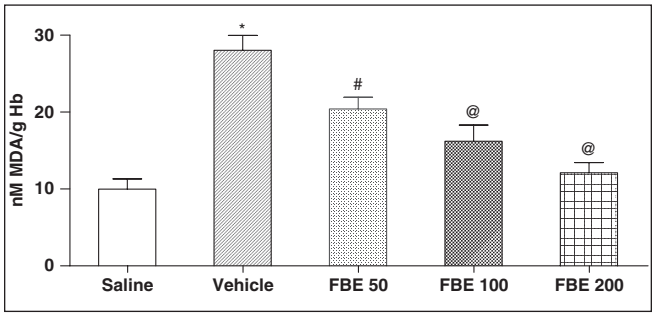

Figure 1: Estimation of lipid peroxidation (LPO)
*Each value is presented as mean ± SEM (n = 5 rats; one-way ANOVA followed by Post hoc Dunnett test). *p < 0.001, #p < 0.01, @p < 0.001 compared with the vehicle.*

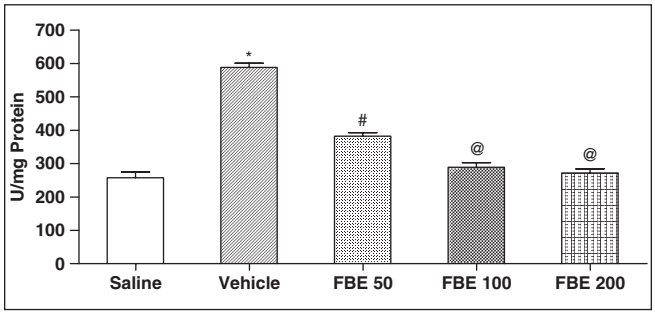

Figure 3: Estimation of catalase (CAT)
*Each value is presented as mean ± SEM (n = 5 rats; one-way ANOVA followed by Post hoc Dunnett test). *p < 0.001, #p < 0.01, @p < 0.001 compared with the vehicle.*

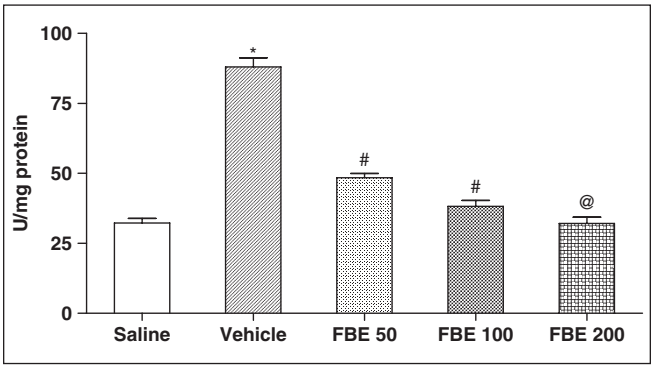

Figure 2: Estimation of superoxide dismutase (SOD)
*Each value is presented as mean ± SEM (n = 5 rats; one-way ANOVA followed by Post hoc Dunnett test). *p < 0.001, #p < 0.01, @p < 0.001 compared with the vehicle.*

DISCUSSION AND CONCLUSION

The results of the present study showed that FBE treatment prevented carrageenan induced inflammation and development of edema in rat paw. This effect of FBE was very much comparable to that of diclofenac sodium. Since, FBE exhibited its antiinflammatory effect at both 1st and 3rd h, it is possible that FBE might be influencing both the stages of inflammation i.e. release of histamine at 1st h and release of bradykinin and prostaglandins and other inflammatory mediators at 3rd h after administration of carrageenan. In addition, FBE treatment also attenuated the carrageenan induced rise in LPO and increase in SOD and CAT activity suggesting *in vivo* antioxidant action of FBE against free radicals generated in the process of inflammation. The observed *in vivo* antioxidant activity of FBE is substantiated by the fact that most of the herbal antiinflammatory agents also possess antioxidant activity.[5,6]

The phytochemical screening of FBE has shown that it contained flavonoids, terpenes and tannins. It has been earlier reported that the bark of this plant contains three flavonoids, two are the forms of leucoanthocyanidin and the remaining is leucoanthocyanin.[13] The antioxidant activities of flavonoids, terpenes and tannins, in general, are well demonstrated[14] and they are often found effective in inflammatory disorders. Further, many of the terpenoids from the other plants sources have been reported to impair the release of autocoids in inflammation.[15]

Therefore, it appears that the observed antiinflammatory effect by FBE is either directly attributed to its terpenoid content or subsequent to the antioxidant effect shown by the flavonoids, terpenes or tannins present in FBE. However, the activity guided phytochemical analysis would only reveal the exact phytochemical responsible for the *in vivo* antioxidant activity and antiinflammatory effect of FBE.

Concludingly, these preliminary studies revealed that the ethanolic extract of the bark of *Ficus bengalensis* eliminated the systemic oxidative stress produced by carrageenan and prevented the consequent inflammation in rats.

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