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PHYTOCONGRESS - 2016

Indian Traditional Medicine - A Conglomeration of Ancient Knowledge and Modern Science

21 & 22 July 2016

Organized By

Centre for Advanced Research in Indian System of Medicine (CARISM)

SASTRA UNIVERSITY

Thirumalaisamudram, Thanjavur, Tamilnadu

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PHYTOCONGRESS – 2016

The International Conference Phytocongress – 2016 on the theme "Indian Traditional Medicine - A Conglomeration of Ancient Knowledge and Modern Science" was organized by Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA University, Thanjavur on 21 & 22 July 2016. This conference was focused on the vast, enviable traditional wealth of India, practices and usage of plant drugs, metals, minerals and animals by traditional medical practitioners during ancient times and the current status of traditional and synthetic drugs. This programme analyzed the requirements of modern times and how the same could be benefited through ancient wisdom. Sessions in the seminar covered a wide range of topics emphasizing the scientific merits involved in Ayurveda and Siddha medicines. These sessions highlighted the significance and importance of evidence based studies on traditional medicines and finding new experimental methodologies for their characterization using various sophisticated analytical techniques and their applications.

The modern tools of chemistry and biology, in particular allow scientists to detail the exact nature of the biological effects of natural compounds on the human body, as well as to uncover possible synergies, which holds much promise for the discovery of new therapies against many devastating diseases. With the technology at hand today, we can validate the science behind this ancient knowledge and unravel the mysteries behind why and how traditional medicine works. The challenge today is to integrate the best of the different healing traditions to meet the healthcare needs of contemporary society.

This International conference brought together leading academic scientists, researchers and research scholars to exchange and share their experiences and wisdom on all aspects of medicinal plants and natural products. It also provided an interdisciplinary platform for researchers, practitioners and educators to present and discuss the most recent innovations, trends, and concerns as well as practical challenges encountered and solutions to be adopted in the fields of medicinal plants and natural products.

This conference was inaugurated by Prof. Bhushan Patvarthan, University of Pune, and this Conference was convened by Prof. P. Brindha, Associate Dean and Co-ordinator, CARISM, SATRA University. Invited international speakers from USA, Australia and Malaysia and national speakers gave scintillating lectures covering various aspects on the theme of the conference. Altogether nearly 200 Scientists, Siddha and Ayurveda Researchers, Practitioners and Industrialists participated from various parts of India.

PROGRAMME SCHEDULE

21 July, 2016

9.00 – 10.30 am	**Prof. Bhushan Patwardhan** University of Pune Pune, India	Inaugural Lecture: Perspectives and prospective of traditional medicine: Phytopharmaceuticals and formulation development
Session I - Chemical perspectives of traditional drugs		
10.30 - 11.15 am	Dr. Suresh Govindaraghavan Network Nutrition IMCD Australia	Plenary Lecture I: Detecting adulteration in botanicals is akin to finding a needle in a haystack
11.15 – 11.30 am	**Tea Break**	
11.30 – 12.15 pm	**Dr. Shrikant Anant** University of Kansas Kansas City, USA	Plenary Lecture II: Phytomolecules in Cancer Therapy
12.15 - 1 .15 pm	**Paper presentations**	
1.15 – 2.00 pm	**Lunch Break**	
2.00 – 2.45 pm	**Prof. Mohammad Kamil** Zayed Complex for Herbal Research, Abu Dhabi	Plenary Lecture III: Modern sciences in the quality control of herbal medicine
2.45 – 3.30 pm	**Dr. K. Umamaheswari** School of Chemical and Biotechnology, SASTRA University. Thanjavur, India	Plenary Lecture IV: Unraveling the mechanism of anti diabetic action of Ayurvedic preparations
3.30 – 3.45 pm	**Tea Break**	
3.45 – 4.30 pm	**Paper Presentations**	
4.30 – 5.30 pm	**Poster Presentations**	

PROGRAMME SCHEDULE

22 July, 2016

Session II – Biochemical and biotechnological interventions in the quality control of traditional drugs		
9.00 - 9.45 am	**Prof. Intan Safinar Ismail** Institute of Bioscience, University Putra Malaysia	Plenary Lecture V: Standardization of herbal products through bioscientific approaches
9.45 – 10.30 am	**Prof. K. Shankar Rao** National Institute of Ayurveda Jaipur, Rajasthan	Plenary Lecture VI: Rasa Aushadhi (Herbo-Metallic preparations) and Nanotechnology
10.30 - 11.15 am	**Dr. N. Angayarkanni** Vision Research Foundation Sankara Nethralaya Chennai	Plenary Lecture VII: Biochemistry in the validation of Ayurvedic drugs
11.15 - 11.30 am	**Tea Break**	
11.30 – 12.15 pm	**Dr. R. Ilavarasan** Assistant Director(S-III) In-charge CSMRIASDD, CCRAS, Arumbakkam, Chennai	Plenary Lecture VIII: Need for the Standardization of Ayurvedic formulation
12.15 – 1.15 pm	**Poster presentations**	
1.15 – 2.00 pm	**Lunch Break**	
Session III – Perspectives and Prospectives of Traditional drugs		
2.00 – 2.45 pm	**Dr. Richa Shrivastava** Maharishi Ayurveda Products Pvt. Ltd. Noida- 201305, U.P.	Plenary Lecture IX: Contribution of modern sciences in enhancing the quality of Ayurvedic products
2.45 – 3.30 pm	Dr. L. Sivakumar General Manager SKM Siddha and Ayurveda Pvt. Ltd, Erode	Plenary Lecture X: Siddha Industry an overview
3.30 – 3.45 pm	Tea Break	
3.45 – 4.30 pm	Paper Presentations	
4.30 – 5.15 pm	Session IV – Industry academia interactions	
5.30 pm	Valedictory function	

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Research Article

THE AMELIORATING EFFECT OF *CENTELLA ASIATICA* ETHANOLIC EXTRACT ON LIVER OF ISONIAZID INTOXICATED ALBINO RATS

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ABSTRACT

Objective: Isoniazid (INH) is one of the commonly used drugs for treating tuberculosis. The main drawback of this drug is its toxic side effects on the liver. *Centella asiatica* (CA) has been used since time immemorial in the Ayurvedic system of medicine due to its wide range of medicinal properties.

Methods: This study was designed to examine the protective effect of CA ethanolic leaf extract on the liver of INH intoxicated albino rats. The adverse effects induced by INH (50 mg/kg bw) administration on liver function markers (aspartate transaminase, alanine transaminase, alkaline phosphatase, total bilirubin, albumin, globulin, and protein) and their amelioration on treatment with various concentrations of CA (20, 40, 60, 100 mg/kg bw) or silymarin (50 mg/kg bw, administered before 1 hr of INH treatment for 30 days to rats) was studied. Moreover, the lipid peroxidation (thiobarbituric acid-reactive substances [TBARSs]), antioxidant status (TBARS, superoxide dismutase, catalase and glutathione-S-transferase), glycogen level, histological and ultrastructural (transmission electron microscopy) studies were carried out in liver tissues of rats treated with the most effective concentration to further support the possible effectiveness of CA on the liver of INH intoxicated rats.

Results: All the affected parameters were brought back to near normal levels, and the effective concentration of extract was found to be 100 mg/kg bw. The oxidant status, glycogen level, histology and ultrastructure of liver also subsequently supported the effectiveness of CA (100 mg/kg bw).

Conclusions: Thus, the overall results suggest that CA at 100 mg/kg bw can considerably reduce the toxic effects inflicted by INH intoxication on rat liver.

Keywords: Isoniazid, Antioxidant, *Centella asiatica*, Liver, Glycogen.

INTRODUCTION

Tuberculosis (TB) is a communicable chronic disease inflicted by the infection caused by the bacterium *Mycobacterium* TB. It is second in the line of mortality rate after HIV/AIDS. According to the 2013 Global TB Report by the World Health Organization, India had been accounted to comprise the world's quarter TB patient's burden. India and China alone were stated to have 26% and 12% of the total TB cases, respectively [1]. The most commonly administered first-line anti-TB drugs are isoniazid (INH), which is also known as INH or isotonic acid hydrazide, and rifampicin or rifampin. These drugs are orally administered either in combination or alone for more than 6 months [2-4]. INH is a known broad-spectrum antibiotic. It is commonly administered for treating *Mycobacterium* TB bacterial infections [5]. This first-line drug is clinically used since the year 1952 and is being used in treating as well as in chemoprophylaxis of TB. It is adversely correlated with hepatotoxicity [6-8]. INH is readily metabolized by *N*-acetyltransferase, forming acetylisoniazid. This then undergoes hydroxylation by cytochrome P450 enzymes, forming hepatotoxic intermediates like acetylhydrazine as well as isonicotinic acid. Acetylhydrazine is known for forming covalent cellular adducts. Acetylhydrazine gets further hydrolyzed to form hydrazine or acetylated products, called as diacetylhydrazine. Patients administered with INH are known to exhibit elevating levels of hydrazine in their blood serum [7]. Administration of a single dose of INH, the hydrazines produced can rapidly disseminate to all the organs without any favorable accumulation in a precise organ. Metabolism of hydrazines involves both enzymatic and nonenzymatic pathways. Humans possessing a slow acetylator genotype have a tendency to accumulate more hydrazine in their serum. This effect may be due to an impaired ability to metabolize and excrete the toxic secondary metabolites of INH [6,7]. Although several evidence-based reports exist on INH-induced hepatotoxicity, INH still remains to be a first-line drug for treating TB [8]. A study by Luntz and Smith also reported irregularities in carbohydrate metabolism accompanied with an increased blood glucose level in diabetic as well as in nondiabetic TB patients [9]. Thus, INH administered patients not only face the loss of normal hepatic function but are also challenged with abnormal carbohydrate metabolism, leading to the weakening of the overall health status of TB patients. Steps to ensure overall well-being must be prioritized in treating TB patients, ensuring both effective treatment and healthy metabolism for a speedy recovery.

Centella asiatica L. (Apiaceae) (CA) is commonly known as Asiatic pennywort or the Indian pennywort. It belongs to the Apiaceae (formerly known as Umbelliferae) family. It is characterized by slender, prostrate, glabrous, perennial creeping herb rooting at the nodes, with simple petiolate, palmately lobed leaves. It is extensively cultivated in Southeast Asia, India, China, and Sri Lanka as a vegetable and/or spice [10]. CA has been reported as possessing various pharmacological activities such as memory-enhancing, anti-inflammatory, antioxidant, immune boosting, antihypertension, antistress, and antiepilepsy activities. These properties have influenced the plant's utilization for treating diseases associated with skin, rheumatism, syphilis, hysteria, diarrhea, wounds, as well as ulcers [10-14]. Diverse health benefits of CA have led to the augmented usage of this plant in various foods and beverages [14]. An *in vitro* study has reported that CA ethanolic leaf extract possesses a strong antioxidant property and suggested that this plant can also act as an iron chelator [15]. Thus, therapeutic potential of CA has motivated us to scrutinize the effect of CA on INH-induced liver toxicity in Wistar albino rats, for evaluating the liver function markers, oxidative status, glycogen, histological as well as ultrastructural changes in the liver.

METHODS

Chemicals and reagents

INH and silymarin (SIL) were purchased from Sigma-Aldrich® Co. Ltd., USA. Methanol, HCL, sulfanilic acid, sodium nitrate, sodium carbonate,

copper sulfate and bovine serum albumin were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used for the biochemical estimations were of analytical grade and were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India.

Collection and identification of plant material

CA used in this study was collected freshly from the outskirts of Chidambaram, Cuddalore District. The plant was then identified at the Herbarium of Department of Botany, Annamalai University (Herbarium No. DDE/HER/44). All the plant materials were washed thoroughly under running tap water for removing any dirt and/or other debris. The cleaned plant materials were then spread under a clean shade for drying. The final dried plant material was milled to a coarse powder using a mechanical grinder and stored in an air-tight container.

Ethanolic leaf extraction of CA

Approximately, 1 kg of powdered CA was used for ethanolic extraction using a Soxhlet apparatus. The dark green extract was subjected to an ultracentrifugation, followed by microfiltration. The final clear dark extract was then concentrated in a rotary evaporator under reduced pressure (10-15 mmHg) at 40°C to obtain the crude ethanol extract. The final dried extract was lyophilized and was stored in a glass vial at −20°C for further use.

Percentage yield of CA extract

The percentage yield of the CA ethanolic extract was determined gravimetrically, i.e., by measuring the dry weight of the final crude extract (X) and also the dry weight of plant powder utilized for the extraction (Y), using the following formula,

Percentage yield = X/Y × 100.

Experimental animals

Male Wistar albino rats of body weight 180-200 g were used for this study. The animals were maintained at Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar, India. The animals were fed on a staple standard pellet diet and water *ad libitum*. The animals were housed in polycarbonate cages under controlled conditions of temperature (23±2°C) and humidity (65-70%) with a 12 hrs light/dark cycle. All the protocols of this study were approved by the Institutional Animal Ethics Committee and Guidelines of Annamalai University (160/1999/CPCSEA; Proposal number: 1021, dated 06.08.2013).

Experimental design

A total of 48 rats were randomly divided into eight groups, each comprising six rats. All the treatments were oral and test drug was administered using an intragastric tube daily for 30 days [16,17]. Group 1 received only vehicle (0.5% dimethyl sulfoxide) and served as a control. Group 2 was administered INH (50 mg/kg bw) alone. Group 3-6 received INH (50 mg/kg bw) and CA at various concentrations (20, 40, 60 and 100 mg/kg bw). Group 7 received CA alone with the highest concentration (100 mg/kg bw) and was assigned as a positive control. Group 8 acted as an internal control and received INH (50 mg/kg bw) and SIL (50 mg/kg bw). Both CA and SIL were administered 1 hr before INH administration.

The experiment was terminated at the end of 30 days, and the animals were fasted overnight, weighed and sacrificed by cervical dislocation. Fresh blood was centrifuged to collect serum for biochemical parameters. A portion of freshly dissected rat liver from each group was stored in 3% glutaraldehyde solution (prepared in 0.1 M phosphate buffer; pH 7.2). A portion of liver tissue from each was washed with ice cold saline and stored in a 10% buffered formalin solution.

Preparation of serum and plasma

Blood samples were collected and centrifuged at ambient temperature for 30 minutes to separate serum (2000 ×*g* for 10 minutes). Plasma samples were collected by centrifuging blood with anticoagulant at 2000 ×*g* for 20 minutes. Then the supernatant comprising the plasma was carefully separated and utilized for further analysis.

Biochemical parameters

The total serum protein (TSP) concentration was determined using Folin-Ciocalteu reagent, as described by the method of Lowry *et al*. and the albumin concentration was carried out by the method of Doumas *et al*., which utilizes bromocresol green dye at pH 4.2, to form green colored complex [18,19]. The globulin concentration was derived from the difference between albumin concentration and the total protein concentration. Activities of serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined according to the method of Reitman and Frankel [20]. Alkaline phosphatase (ALP) serum activity was determined as described by King and Armstrong [21]. The total serum bilirubin level was measured using Van den Bergh reaction, as described by the method of Malloy and Evelyn [22].

Biochemical studies were carried out in blood serum. The concentration of serum thiobarbituric acid-reactive substances (TBARS) was analyzed according to the method of Hogberg *et al*. [23]. The serum superoxide dismutase (SOD) was carried out by the method of Kakkar *et al*. [24]. The serum catalase (CAT) was measured by the method of Sinha [25]. The serum glutathione-S-transferase (GST) was determined according to the method described by Habig *et al.* [26]. Glycogen content in liver was determined as described by Morales *et al*. [27].

Histological examination

The liver tissues for the control and effective concentration treated animals were subjected to histological studies. The tissue samples were fixed for 48 hrs in 10% buffered formalin, followed by dehydrating the tissues by passing through a different mixture of ethyl alcohol and water, and was finally cleaned with xylene and embedded in paraffin for sectioning. Sections of the 5-6 µm thickness of tissues were prepared using a rotary microtome. These sections were fixed on glass slides and stained with hematoxylin and eosin dye and were observed microscopically.

Transmission electron microscopic (TEM) study of liver tissues

A portion of freshly dissected rat liver was sliced into 1 mm^3 and stored in 3% glutaraldehyde (EM grade) in 0.1 M phosphate buffer (pH 7.2) for fixing (48 hrs at 2-4°C). The samples were washed with 0.1 M phosphate buffer (pH 7.2), post fixed in 1% osmium tetroxide prepared in 0.1 M phosphate buffer (pH 7.2), dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections with 40-60 nm thickness were cut using Leica ultramicrotome with a diamond knife (DiATOME). The ultrathin sections were taken on a copper grid and stained with (double metallic) uranyl acetate and Reynold's solution (sodium citrate and lead mitrate). The sections were examined using a Philips Tecnai T12 (120 kV) Electron Microscope (Netherland). The images were acquired using a Gatan Image Filter (GIF; Ultrascan 10000 slow scan) with CCD camera 4 K × 4 K chip and were processed using Gatan software (T12).

Statistical analysis

Statistical comparisons were performed by one-way analysis of variance followed by the Duncan's multiple range test, SPSS software version 16.0 (SPSS Inc. Released 2007, SPSS for Windows, Version 16.0, SPSS Inc., Chicago, Ill, USA). Values are represented as mean ± standard deviation and $p<0.05$ was considered statistically significant.

RESULTS

Percentage yield of plant extract

Table 1 shows that the percentage yield of CA ethanolic extract and was found to be 1.089%.

Evaluation of serum and plasma biochemical parameters (liver function test)

The changes in the level of liver function (TSP, albumin, globulin, activities of serum ALT, AST and ALP and total bilirubin) in INH

(Group 2) and CA extract treated rats (Group 3-6) are summarized in Fig. 1. A significant decrease ($p<0.05$) in the levels of TSP and albumin and increase ($p<0.05$) in the activities of ALT, AST, ALP, globulin, and total bilirubin was observed in INH-treated rats (Group 2) when compared to control rats (Group 1). These levels were found to be restored in the CA extract pretreated rats (Group 3-6) in a concentration-dependent manner, with 100 mg/kg bw (Group 6) being the most effective concentration. Similar results were observed in SIL (50 mg/kg bw) treated INH intoxicated rats (Group 8).

Evaluation of liver oxidant status and glycogen levels

In INH-treated rats (Group 2), the concentration of TBARS in liver was found to be significantly increased ($p<0.05$) as compared to control animals (Group 1). A significant decrease ($p<0.05$) in the levels of SOD, CAT, and GST were also observed in the liver of INH-treated rats (Group 2). These levels were found to be restored in the CA extract pretreated rats with a concentration of 100 mg/kg bw (Group 6). Similar results were observed in SIL (50 mg/kg bw) treated INH intoxicated rats (Group 8) (Table 2). Further, the glycogen reserve in liver was found to be significantly depleted ($p<0.05$) in INH-treated rats (Group 2) compared to control animals (Group 1). The glycogen reserve was restored in the CA extract pretreated rats with a concentration of 100 mg/kg bw (Group 6). Similar results were observed in SIL (50 mg/kg bw) treated INH intoxicated rats (Group 8) (Fig. 2).

Histological and ultrastructural examination of liver tissues

The histological examination of liver tissue photomicrograph of INH (50 mg/kg bw) treated rats (Group 2) revealed the numerous changes such as cellular damage, inflammation, vascularization, hypertrophy, loss of structural integrity of cells as well as loss in characteristic tissue organization. Near normal cellular architecture with normal hepatocyte arrangement, sinusoids, mild central vein congestion were observed in CA (100 mg/kg bw) treated INH intoxicated (Group 6) rat liver. Similar results were observed in SIL (50 mg/kg bw) treated INH intoxicated rats (Group 8) (Fig. 3).

The analysis of INH-treated rat (Group 2) liver ultramicrographs obtained from TEM revealed the presence of cell membrane enfolding, damaged nuclear membrane, nuclear chromatin condensation, regression of mitochondrial cisternae, and the presence of fat droplets with vacuoles. Near normal hepatocyte ultrastructure with euchromatic nucleus, prominent nuclear membrane, prominent nucleolus, many mitochondria, rough endoplasmic reticulum, appearance of glycogen granules and less vacuolated cytoplasm were observed in CA ethanolic extract administered (100 mg/kg bw) INH intoxicated rat hepatocytes (Group 6). Similar effects were observed in SIL (50 mg/kg bw) treated INH intoxicated rat (Group 8) hepatocyte (Fig. 4).

Table 1: Percentage yield of plant extract

Plant	Solvent	Method	Weight of crude extract (g)	% yield
CA	Ethanol	Soxhlet extraction	10.89	1.089

CA: *Centella asiatica*

DISCUSSION

The present experiment was carried out to evaluate the effectiveness of CA leaves extract on INH intoxicated albino rats. The previous finding by Antony *et al*. confirmed that CA alcoholic extract has hepatoprotective effect in carbon tetrachloride and cadmium-induced liver injuries [28,29]. Abdulla *et al*. acute toxicity work revealed that the ethanolic extract of CA at a dose ranging from 2 to 5 g/kg bw for 14 days showed no manifestation of any significantly noticeable signs of toxicity [30]. SIL is a flavonolignan obtained from the "milk thistle" plant, *Silybum marianum*, which is a reference drug used widely for hepatoprotection [31].

The liver is the central organ engaged in detoxification of various products such as hormones, xenobiotics, toxins, and drugs. During detoxification of xenobiotics, the metabolites generated can lead to hepatocyte damage [32]. INH is metabolized in the liver into hydrazine and acetylisoniazid, which is followed by hydrolysis

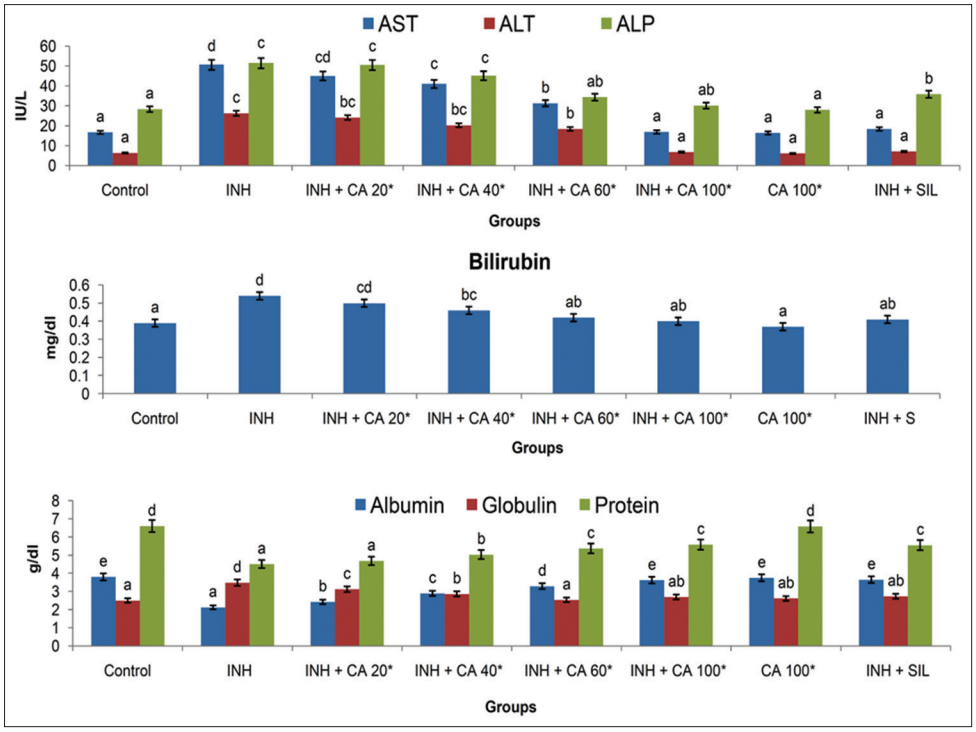

Fig. 1: Effect of extract on liver function tests in control and experimental animals. Values are expressed as mean±standard deviation (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). (INH: isoniazid 50mg/kg/bw/day; CA: *Centella asiatica* ethanolic leaves extract; SIL: Silymarin 50mg/kg/bw/day; *mg/kg bw/day)

Table 2: Effect of CA on TBARS, SOD, CAT, and GST in liver of control and experimental groups of rats

Groups	Control	INH (50 mg/kg bw)	INH (50 mg/kg bw) + CA (100 mg/kg bw)	CA (100 mg/kg bw)	INH (50 mg/kg bw) + SIL (50 mg/kg bw)
TBARS[1]	0.76±0.13[a]	4.38±0.27[d]	1.32±0.23[c]	0.78±0.05[a]	1.09±0.07[b]
SOD[2]	7.90±0.25[c]	3.82±0.30[a]	6.93±0.60[b]	8.05±0.41[c]	6.94±0.47[b]
CAT[3]	78.14±3.62[c]	43.24±2.80[a]	56.57±4.64[b]	80.94±1.46[c]	59.00±2.65[b]
GST[4]	8.81±0.67[cb]	6.31±0.47[a]	7.49±0.62[b]	9.01±0.34[d]	8.27±0.45[c]

Values are expressed as mean±SD (n=6). Values not sharing a common superscript letter differ significantly at P<0.05 (DMRT). DMRT: Duncan's multiple range test, SD: Standard deviation, [1]TBARS in tissues were expressed as nmoles/100 g wet tissue, [2]SOD for tissues were expressed as 50% inhibition of nitroblue tetrazolium reduced in 1 minute/mg protein, [3]CAT for tissues were expressed as µmoles of H_2O_2 consumed/minute/mg protein, [4]GST for tissue were expressed as CDNB-GSH conjugate formed/minute/mg protein. TBARS: Thiobarbituric acid-reactive substances, SOD: Superoxide dismutase, CAT: Catalase, GST: Glutathione-S-transferase, INH: Isoniazid 50 mg/kg/bw/day, CA: *Centella asiatica* ethanolic leaves extract, SIL: Silymarin 50 mg/kg/bw/day

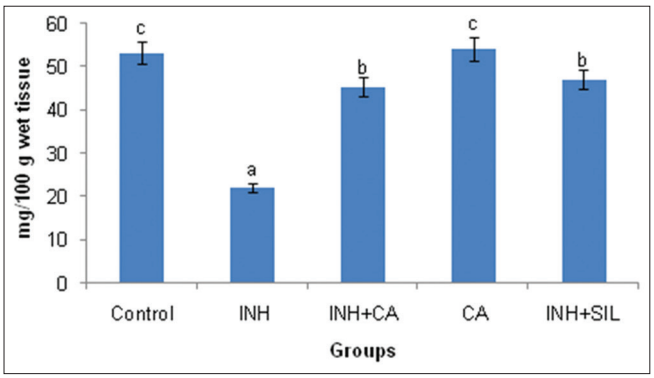

Fig. 2: Effect of *Centella asiatica* leaves ethanolic extract on liver glycogen in control and experimental rats. Values are expressed as mean±standard deviation (n=6). Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT). (INH: Isoniazid 50 mg/kg/bw/day; CA: *Centella asiatica* ethanolic leaves extract; SIL: Silymarin 50 mg/kg/bw/day; *mg/kg bw/day)

forming acetylhydrazine and undergoing oxidization by cytochrome 450 to produce hepatotoxic intermediaries [7]. These hepatotoxic intermediates such as acetylhydrazine and hydrazine can inflict cellular damages in liver causing hepatotoxicity [7]. Liver specific enzymes such as aminotransferases are engaged in the catalytic reactions facilitating the interconversion of amino acid and α-keto acid, by transferring amino groups. Damage to hepatocytes leads to the release of aminotransferases enzymes, AST and ALT, into the blood circulation. Hepatotoxicity also causes cirrhotic liver conditions, leading to defects in biliary functioning and elevated bilirubin release in the blood circulation [33]. ALP is a marker enzyme, which is found increased in the liver disorders. Elevation in the levels of serum ALP is an indication of cellular leakage and also the loss of functional integrity of hepatocyte cell membrane [34]. The healing of liver cells may be the reason for the decrease in the levels of AST, ALT, ALP, and bilirubin in pretreated CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) treated INH intoxicated groups to near normal levels. These effects may be owed to the improvement in the functioning of the rat liver and stabilization of biliary dysfunction of INH-treated rat liver to near normal. The increase in reactive oxygen species (ROS) accompanied by a reduction in the levels of SOD, CAT, and GST in liver tissues also directly influence the elevation of parameter levels of serum such as AST, ALT, and ALP in INH-treated rats. Liver cells face damage due to increase in TBARS level and decrease in SOD, CAT and GST, leading to an increased cell membrane damage and leakage of cellular contents into the circulatory system The enzymes CAT and SOD are previously reported to get inactivated by hydrazine, which results in significant increase in the endogenous levels of H_2O_2 in the liver cells [35]. INH is also well known to inflict cellular damage by aggravating oxidative stress in the organ system. These effects also cause dysfunction of the native hepatic antioxidant defense system [36]. Thus, the depletion of the antioxidant defenses accompanied with an increase in free radical production, leads to an imbalance in the overall prooxidant-antioxidant status, leading to oxidative stress-induced cell death in the liver in INH-treated rats [37]. CA ethanolic leaf extract has previously been reported to possess very good *in vitro* antioxidant as well as free radical scavenging activity. This may be attributed due to the presence of various antioxidant compounds present in the extract [15]. The elevated levels of TBARS and decreased levels of SOD, CAT, and GST in liver tissues were found to be restored in CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) pretreated INH groups to near normal levels. Thus, from the findings of this study, it is evident that INH cause damage to the membrane of liver cells by ROS lead to increase in lipid peroxidation (TBARS), causing depletion of cellular antioxidants (SOD, CAT, and GST) and leading to cell membrane damage and leakage of cellular enzymes such as like AST, ALT, and ALP into the serum. These adverse effects were successfully contemplated by pretreatment with CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) in INH intoxicated rats.

Albumin is biosynthesized in the liver and is also the main protein found in the blood. Decrease in the albumin level indicates dysfunction of the liver [38]. Various studies have indicated a decrease in albumin level, and an increase in serum total protein and serum globulin in TB-treated rats [32,34-36]. TSP level helps in differentiating between normal and damaged liver condition, as most of the serum proteins such as albumins and globulins are biosynthesized in the liver [38]. During any hepatocellular injury, the total protein may decline a little, but always a sharp decrease in albumin and increase in globulin levels are always observed [25]. The current findings reveal a decrease in the TSP and serum albumin, and an increase in serum globulin. This may be the result of damage as well as dysfunction of liver by administration of INH. Increase in the antioxidative status of the liver observed on pretreatment with CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) in INH intoxicated groups might be the reason behind the restored levels of TSP, serum albumin, and globulin to near normal. A decrease in glycogen level was observed in INH (50 mg/kg bw) treated rats and was in agreement with the findings of Jadhav and Mateenuddin [39], and Sankar *et al*. [40]. This result reflects that the carbohydrate metabolism of the rats is affected by INH administration. The pretreatment of CA extract (100 mg/kg bw) in INH intoxicated groups successfully restored the depleted glycogen level in hepatic tissues to near normal. Pretreatment with standard drug SIL (50 mg/kg bw) in INH intoxicated rats also considerably elevated glycogen level but was not as effective as CA extract (100 mg/kg bw) pretreatment. The effectiveness of CA extract may be due to the synergic effect of various phytochemicals, which help in the restoration of carbohydrate metabolism in INH-treated rats.

Sever distortion in the hepatocellular organization was observed in INH (50 mg/kg bw) treated rats. This observation was in agreement with the results of Jadhav and Mateenuddin [39], and Sankar *et al*. [40]. These histological damages were clearly reflected in ultrastructural damages in liver section along with depletion of glycogen granules in INH-treated rats. These adverse effects on histology and/or ultrastructural changes in INH administered liver sections were found to be restored on CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) pretreatment. This may be due to the presence of various antioxidant and anti-inflammatory compounds present in the extract, which could restore the normal functioning of hepatocytes.

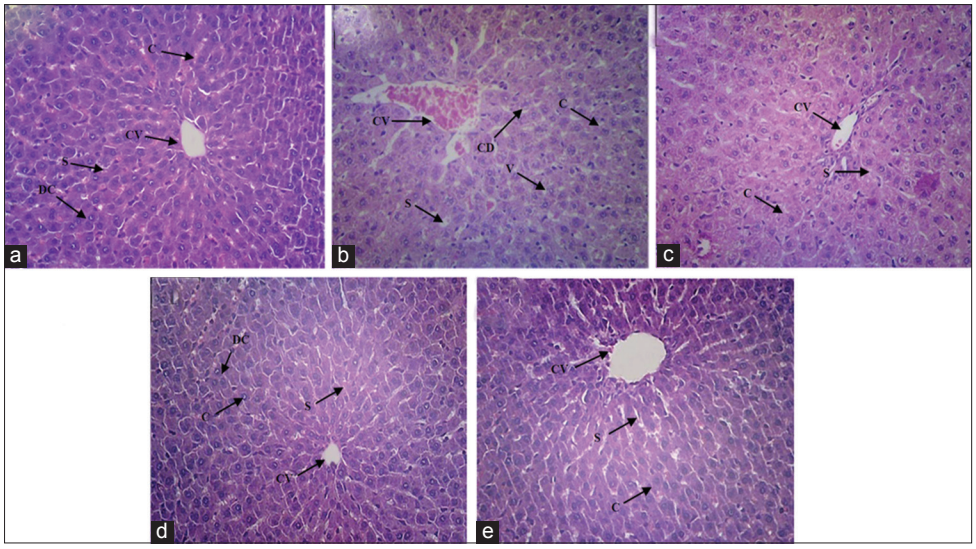

Fig. 3: Photomicrographs of histological changes in H and E stained liver sections of control and experimental rats (×200). (a and d) Control and *Centella asiatica* ethanolic treated rat liver showed clear central vein (CV) with hexagonal cells, embedded in connective tissue. All hepatocytes are radiantly arranged as trabecules from the central portal vein, separated by sinusoids (S). The hepatocytes contain clear spheroidal nucleus with distinct nucleolus as well as peripheral chromatin condensation (C). Few cells contain two nucleuses (cells undergoing division) was also observed (DC), (b) Isoniazid (INH) treated rat liver showed distorted cellular organization accompanied with vacuoles (V), hypertrophic cells, cellular damage (CD), inflammation, widening of inter cellular sinusoids (S) and congestion in central vein (CV). The nucleoli are not distinct and the nucleuses appeared to have more condensed chromatin (C), (c and e) CA ethanolic extract or silymarin oral pretreated INH intoxicated rats liver showed near normal hepatocyte arrangement and sinusoids (S) accompanied with mild central vein congestion (CV). The nucleoli and nucleolus also appeared to be nearly distinct with less condensed chromatin (C)

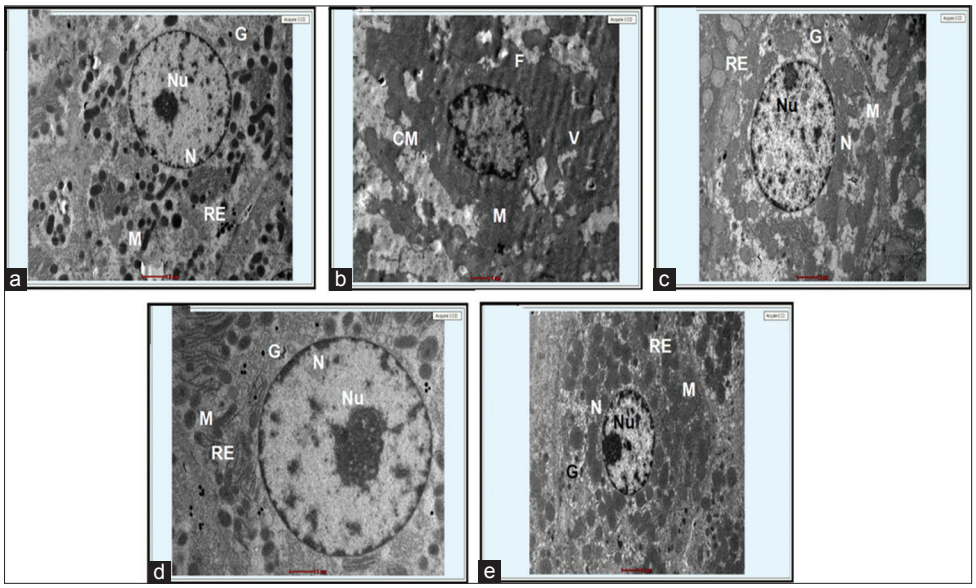

Fig. 4: Liver ultramicrographs of control and experimental rats. (a and d) Control and *Centella asiatica* ethanolic treated rats liver showed a normal hepatocyte with euchromatic nucleus (N) with prominent nuclear membrane, prominent nucleolus (Nu), many mitochondria (M) having normal cristae, rough endoplasmic reticulum (RE), and glycogen granules (G). Transmission electron microscopic (TEM) magnification for A, ×12,000; TEM magnification for D, ×15,000, (b) Isoniazid (INH) treated rat liver showed hepatocyte with enfolding of cell membrane (CM), damaged nuclear membrane, condensation of nuclear chromatins and pyknotic nucleus (N), regression of mitochondrial cisternae (M), and presence of fat droplets (F) with vacuole (V). TEM magnification, ×15,000, (c and e) CA ethanolic extract or silymarin oral pretreated INH intoxicated rats liver showed hepatocyte with euchromatic nucleus (N) with prominent nuclear membrane, prominent nucleolus (Nu), many mitochondria (M), rough endoplasmic reticulum (RE), glycogen granules (G) with less vacuolated cytoplasm. TEM magnification, ×12,000

CONCLUSION

This study jointly reveals that the pretreatment of INH-treated rats with CA leaves ethanolic extract at a concentration of 100 mg/kg bw can substantially reduce the side effects caused by INH, which is one of the first-line drugs for TB patients. This also provides future avenues to study the molecular mechanisms associated with the protective mechanism of the CA ethanolic extract on INH intoxication. Isolation and characterization of the potent molecules responsible for this protective effect can help in developing new lead compounds which

could be useful in pharmaceutical companies in producing more effective drugs that can contribute in reducing the side effects of commercially available drugs and also enhance overall human health.

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Research Article

EVALUATION OF WOUND HEALING POTENTIAL OF ETHANOL AND AQUEOUS EXTRACT OF *CENTRATHERUM PUNCTATUM* CASS. (ASTERACEAE) IN RATS

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ABSTRACT

Objectives: The development of effective treatments for infected wound healing process is one of the important problems of medicine for many years. From the literature review, it is evident that Asteraceae members are rich in wound healing molecules, and they have well-proven mechanism of wound healing activity. In the present research, a common Asteraceae member called *Centratherum punctatum* was selected and screened for its wound healing potentials.

Methods: The aqueous and ethanol extract of *C. punctatum* Cass. formulated as ointment (5%, 10% and 20%) was studied for its wound healing potency on experimentally-induced wound in rats. Wounds were inflicted on Wistar rats using excision, incision and dead space model. The extract was tested on eight groups of Albino rats, consisting of six animals each, were placed individually in cages, and all animals were experimentally wounded. Group I served as disease control and received only simple ointment base (petroleum jelly). Group II treated as standard control and applied with 2% povidone iodine ointment. Groups III, IV and V were applied with 5%, 10% and 20% w/w ethanol extract ointment daily and Groups VI, VII and VIII were applied with 5%, 10% and 20% w/w aqueous extract ointment daily. The effects of extract ointment on the rate of wound healing were assessed by the rate of wound closure, period of epithelialization, wound breaking strength, weights of the granulation tissue, and histopathology of the granulation tissues.

Results: In excision wound, the wounds treated with the aqueous extract showed a higher rate of contraction during the treatment period as compared to diseased control group and ethanol extract. In incision wound, a significant increase in the wound breaking strength was observed in aqueous extract of *C. punctatum* treated animals as compared with the controls and ointment of ethanol extract. In dead space wound, there was a significant increase in the weight of the granulation tissue in the animals treated with the aqueous extract at a concentration of 20%.

Conclusions: The data of this study indicated that the aqueous extract of *C. punctatum* Cass. possesses better wound healing activity than the ethanol extract and it can be used to treat different types of wounds in human beings too.

Keywords: *Centratherum punctatum* Cass. Ethanol and aqueous extract at three different concentration, Excision, Incision and dead space wound model.

INTRODUCTION

The restorative efficacies of many native plants, for diverse diseases, have been described by conventional herbal remedy practitioners [1]. Natural products are a spring of synthetic and traditional herbal medicines which are still the leading health-care system in some parts of the world [2]. The presence of various life-supporting constituents in plants has urged scientists to investigate these plants with a view to determine potential wound healing properties.

Centratherum punctatum a traditional drug belonging to the family Asteraceae. *C. punctatum* Cass. is one among 33 species of the type genus *Centratherum* and is a perennial bushy plant of 45-60 cm height. It has a well-branched stem with revitalizing scented foliage and purple flower heads. Recently, a vital oil containing nearly 59 different compounds has been isolated from the leaves of this plant [3]. Centratherin, sesquiterpene lactone, has been isolated from *C. punctatum* but its medicinal properties have not yet been established conclusively [4]. The phytochemical constituents present in different extracts of *C. punctatum* in solvents such as petroleum ether, chloroform, ethanol, and water were, respectively, used to identify the bioactive compounds from the aerial parts of *C. punctatum* and screened for their phytochemical constituents [5]. The micromorphological features of *C. punctatum* were analyzed to localize the active molecules histochemically [6]. The antimicrobial activity of ethanol and aqueous extracts of *C. punctatum* Cass. was also evaluated [7]. An attempt was made to study proteases in floral extracts of *C. punctatum* which may play a role in wound healing property of the plant [8].

Wound healing is the process of repair that follows injury to the skin and other soft tissues. Following injury, an inflammatory response occurs and the cells below the dermis (the deepest skin layer) begin to increase collagen (connective tissue) production. Later, the epithelial tissue (the outer skin layer) is regenerated. There are three stages in the process of wound healing: Inflammation, proliferation, and remodeling. Impaired wound healing due to infection or metabolic complications can result into severe morbidity leading to long hospitalization of patients. The aim of treating wounds is to shorten the time taken for healing and to reduce risks of undesired complications.

This study was undertaken to determine the wound healing potential of the ethanolic extracts of *C. punctatum*. Wistar albino rats to generate preclinical data a step toward the development of formulations and trial in clinical setting.

METHODS

Collection of plant material and identification

Aerial parts of *C. punctatum* Cass. for the proposed study were collected from herbal garden of Srimad Andavan Arts and Science College, Tiruchirappalli. Care was taken to select healthy plants. The identity of the plant specimen was confirmed using Flora of Presidency of Madras [9]. The botanical identity was confirmed by comparing with the herbarium specimen deposited at Royal botanical Garden Kew (Voucher specimen number K000373089).

Preparation of ethanol and aqueous extracts

About 500 g of coarsely powdered plant materials were taken in an aspirator bottle and soaked in ethanol for 48 hrs at room temperature. After 48 hrs, the filtered solvent was distilled off and residues obtained were subjected to further studies. 500 g of coarsely powdered plant materials were taken and boiled with distilled water in the ratio of 1:6 till the total volume reduces to half the quantity. The water extract was filtered through a 420 mm stainless steel filter, cooled and transferred to screw capped glass vials.

Preparation of test ointment

The ointment was prepared following the methods described by trituration method using deaginous bases [10]. 100 g of petroleum jelly was mixed with 5%, 10%, 20% w/w of extract of *C. punctatum* by stirring continuously in a magnetic stirrer at 800 rpm for 1 h. Mixing was continued until a uniform dispersion was formed. Three types of ointment formulations were prepared using both the ethanol and aqueous extract, viz., ointment containing 5% (w/w), ointment containing 10% (w/w), and ointment containing 20% (w/w). Povidone iodine cream (2% w/w) manufactured by Cipla (Batch No. JMT95 Manufactured: February 2013, Expiry: January 2016) was used as a standard. Ointment was prepared separately for ethanol and aqueous extract with concentrations as specified.

Evaluation of wound healing activity of various prepared formulation

Selection and procurement of animals

Wistar albino male rats weighing between 200 and 350 g were procured and used for this study. The rats were fed with commercial pelleted rat chow purchased from Sai Durga Feeds and Foods, Bengaluru, India and water *ad libitum*. The animals were housed in a clean polypropylene cages bedded with husk, under a 12 hrs light/12 hrs dark cycle maintained at 25°C. All animals were handled with utmost care. Experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (IAEC). The experiments were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experimental Animal (CPCSEA) New Delhi (Reg. No. 817/04/ac/CPCSEA), SASTRA University, Thanjavur (IAEC Approval No: 113/SASTRA/IAEC/RPP).

Wound healing models

The effect of ethanol and aqueous extract ointment were evaluated on excision, incision and dead space wound models in rats at dose levels of 5%, 10% and 20% w/w body weight.

Experimental design for all wound models

Wistar albino rats were divided into eight groups, each comprising six rats. Outlines of the treatments given to each group of rats were as follows:

- Group I served as disease control and received only simple ointment base (petroleum jelly).
- Group II treated as standard control and applied with 2% povidone iodine ointment.
- Groups III, IV and V were applied with 5%, 10% and 20% w/w ethanol extract ointment daily (for both excision and incision wound model).
    - Groups III, IV and V were applied with ethanol extract ointment daily at a dose level of 100 mg, 200 mg and 400 mg/kg. p.o. (for dead space wound model).
- Groups III, IV and V were applied with 5%, 10% and 20% w/w aqueous extract ointment daily (for both excision and incision wound model).
    - Groups III, IV and V were applied with aqueous extract ointment daily at a dose level of 100 mg, 200 mg and 400 mg/kg.p.o. (For dead space wound model).

Excision wound model [11]

Creation of wound

About 1 week after acclimatization, the experimental animals were subjected to wound healing activity studies. All the surgical interventions were carried out under sterile conditions. Animals were anesthetized by ketamine (100 mg/kg, i.p.) before and during creation of the wounds. The animals were allowed to recover and housed individually in their cages and monitored for respiration and temperature. The animals were maintained under standard husbandry conditions and on a uniform diet and managed throughout the experimental period. Animals were closely observed for any infections; those who show signs of infection are separated and excluded from the study. The animals were periodically weighed before and after the experiments.

The precise skin area was shaved with an electric clipper and the imagined area of the wound to be created was outlined on the back of the animals 1 day before the experiment. A full thickness of the excision wound of circular area approx. 500 mm^2 and 2 mm^2 depth were made on the shaved back of the rats. The wounds were left undressed to the open environment and observed throughout the study period. Hemostasis is achieved by blotting the wound with a cotton swab soaked in normal saline [12]. To all the experimental animals including diseased control, standard, Groups III, IV and V and Groups VI, VII and VIII ointment, standard drug and test drug of different doses were applied once daily for 18 days, starting from the day of wounding.

The treatment was given as per the treatment schedule till complete epithelization. The selected model was employed to study epithelization period, percentage of wound closure, wound surface microbial load count and levels of antioxidant enzymes were taken as major criteria to assess the wound healing potential.

Wound contraction

The progressive reduction in the wound area is monitored planimetrically by tracing the raw wound boundaries initially on a sterilized transparency paper sheet in mm^2 without causing any damage to the wound area, and then, the wound area recorded is measured using a graph paper at every 3-6 day interval.

Wound contraction, which contributes to wound closure, is expressed as a reduction in the percentage of the original wound size is studied starting from the day of operation until the day of complete epithelialization and evaluated to calculate the degree of wound healing. Percentage of wound contraction is calculated as:

$$\text{Percentage of wound contraction} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

Period of epithelialization

The period of epithelialization is expressed as the number of days required for falling of the scar (dead-tissue remnants) without any residual raw wound is considered as the end point of epithelialization [13].

Wound surface microbial load count

The microbial load of the wound area was determined using the swab method [14].

Collection of blood sample and granuloma tissue

At the end of the experimental period, animals were sacrificed by CO_2 inhalation euthanasia. The regenerated tissues were dissected out and collected from the sacrificed animals from each group. The regenerated tissue samples were washed in ice-cold saline fixed in 10% buffered formalin for histopathological studies.

Incision wound model [15]

Creation of wound

Animals were anesthetized by ketamine (100 mg/kg, i.p.) before and during creation of the wounds. The dorsal furs of the animals were shaved with an electric clipper and longitudinal paravertebral incisions of 6 cm in length were made through the skin and cutaneous muscle on the back. After complete hemostasis, the wounds were closed using

interrupted sutures placed at equidistance points about 1 cm apart. The wounds were left undressed. The extracts were applied topically from 0 day to 9th post-wounding day. The sutures were removed on the 8th post wound day, and the treatments were continued up to 10th day. The wound breaking strength was estimated on 10th day.

Determination of breaking strength [16]
The wound breaking strength was estimated by continuous, constant water flow technique. The granuloma tissue was held with a tissue holding forceps attached to a pulley.

Wound stripes of equal size (width) were then cut using a knife in which two blades were fixed at a fixed distance. Both ends of each strip were fixed with the help of a pair of steel clips. One clip allowed hanging on a stand and a polyethylene bottle was then allowed to fill with water gradually till the wound strip was broken at the site of wound. The amount of water required to break the wound was noted and expressed as breaking strength of wound in grams. The amount of weight required to break the wound is considered as a direct measure of the breaking strength of the wound. The breaking strength of the wounds of all the treatment group animals was compared with that of the wounded control group animals.

Collection of blood sample and granuloma tissue
At the end of the experimental period, animals were sacrificed by CO_2 inhalation euthanasia. On the 10th post wounding day, granulation tissue formed on the implanted tube was carefully dissected out along with the tube. The wet weights of the excised granulation tissue were measured. The tubular granulation tissues were cut along its length to obtain a sheet of granulation tissue. After taking the wet weight of the piece of the granulation excised, the tissues collected were dried in an oven at 60°C for 24 hrs to get a constant dry weight expressed as mg/100 g body weight. The dried tissues were then used for the determination of hydroxyproline content and hexosamine. The granuloma tissue samples were washed in ice-cold saline and fixed in 10% buffered formalin for histopathological evaluation.

Dead space wound model [17]
In this model, the physical and mechanical changes in the granuloma tissue were studied.

Creation of wound
Under anesthesia (thiopentone sodium 40 mg/kg, i.p.) dead space wounds were inflicted by implanting subcutaneously a 2.5 cm × 0.5 cm polypropylene tube in the lumber region of dorsal side in anesthetized rats. The day of the wound creation is considered as day zero. Animals received the above specified treatments from 0 day to 9th post wounding day.

Collection of blood sample and granuloma tissue
At the end of the experimental period, animals were sacrificed by CO_2 inhalation euthanasia. On the 10th post wounding day, granulation tissue formed on the implanted tube was carefully dissected out along with the tube. The wet weights of the excised granulation tissue were measured. The tubular granulation tissues were cut along its length to obtain a sheet of granulation tissue. After taking the wet weight of the piece of the granulation excised, the tissues collected were dried in an oven at 60°C for 24 hrs to get a constant dry weight expressed as mg/100 g body weight. The dried tissues were then used for the determination of hydroxyproline content and hexosamine.

Statistical analysis
The results were expressed as mean±standard error mean. The statistical significance was assessed using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test and $p<0.001$ was considered significant.

RESULTS AND DISCUSSION

Since ancient times, herbs have been commonly used to treat various ailments including wounds. In spite of the various challenges encountered in the medicinal plant-based drug discovery, natural products isolated from plants still remain an essential component in search of new medicines. Proper utilization of these resources and tools in bioprospecting employing modern analytical tool and researchers will certainly help in discovering novel lead molecules and medicines from plants. The screening of plant extracts for their therapeutic potential has been of great interest to researcher's dealing with discovery of new drugs. Indian flora is one of the most extensive floras in the world and can provide rich source for these researchers. A number of reports are available dealing with the antibacterial, anti-inflammatory and wound healing activity of plant extracts sourced from India.

In this study, one of the Asteraceae member commonly available in and around Tiruchirapalli is screened for wound healing potentials employing *in vivo* models, as till date, there are no studies available dealing with the scientific assessment of the wound healing property claimed in traditional medicine for the leaf extract of *C. punctatum* Cass.

In majority of patients, normal healing established through tissue integrity quickly and effectively. However, at times this healing is delayed and the ability to accelerate the wound healing becomes a highly desirable one [18].

Skin wounds could happen through several causes such as physical injuries resulting in opening and breaking of the skin [19]. The most common symptoms of wounds are bleeding, loss of feeling or function below the wound site, heat and redness around the wound, painful or throbbing sensation, swelling of tissue in the area, and pus like drainage [20].

Wound healing is a very complex, multifactor sequence of events involving several cellular and biochemical processes. The various processes of wound healing will help to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin. Initially, wound healing involves acute inflammatory phase followed by the synthesis of collagen and other extracellular macromolecules, which are later removed to form a scar [21]. The use of single model for wound healing study is inadequate, and no reference standard exists that can collectively represent the various phases of wound healing. Drugs, which influence one phase, may not necessarily influence another. Hence, in this study, different models such as dead space (for collagenation phase), incision and excision wounds (for wound contraction and epithelization phases) have been employed to assess the efficacy of the selected drug in various phases, which run concurrently, but independent of each other.

It is important to note that throughout the period of wound healing study, the extract did not cause irritation or pain to the animals as the experimental animals neither showed any signs of restlessness nor scratching or biting of wound site when the extracts were applied.

Effect on excision wound

Effect of ethanol and aqueous extract of C. punctatum on rate of wound contraction in excision wound model

The results obtained on excision wound model experiment after administration of both ethanol and aqueous extract at different concentrations (5%, 10% and 20% w/w) are given in Table 1 and graphically represented in Fig. 1. The mean percentage of wound contraction was calculated on 3rd, 6th, 9th, 12th and 15th day. A better healing pattern with complete wound closure was observed in test drug-treated rats within 15 days, whereas it took about 21 days in control rats with different concentration of ointment (Plate 1). The study revealed that both ethanol extract ointment and aqueous extract ointment treated groups showed good wound healing which may be attributed to the individual or combined action of phytoconstituents present in the extract such as alkaloids, flavonoids, saponins, and terpenoids [22,23]. The wound healing property of *C. punctatum* extracts is presumably because of its constituent's ability to promote cell division and thereby facilitate the healing of wound. The wounds treated with the aqueous extract showed a higher rate of contraction during the treatment period

Table 1: Effect of ethanol and aqueous extract of *Centratherum punctatum* on wound contraction and epithelization on excision wound models

Groups	Wound contraction (%)					Period of epitheli-zation (days)
	3rd day	6th day	9th day	12th day	15th day	
Group I	11.16±9.96	45.18±12.52	68.35±8.04	80.43±8.68	87.12±6.40	21
Group II	10.24±8.02*	44.95±8.65*	82.26±3.13*	91.67±2.96*	95.59±1.08*	18
Group III	11.74±11.00[a]	45.67±8.56[a]	73.49±4.20	87.66±3.61[a]	92.17±4.60[a]	17
Group IV	14.44±8.50[b]	46.00±17.13[b]	64.92±5.51	82.66±1.87	92.26±1.41	18
Group V	23.04±33.70[c]	47.13±7.57[c]	82.17±9.74[c]	71.46±5.25	82.04±3.76	19
Group VI	11.90±11.29[a]	45.95±14.51[a]	82.31±6.63[a]	87.37±10.51	92.555±5.406	16
Group VII	10.98±5.692[b]	46.03±6.58[b]	82.67±3.99[b]	92.58±1.68[b]	94.563±2.579[b]	16
Group VIII	11.79±5.58[c]	45.54±11.55[c]	82.96±3.41[c]	90.51±4.85	93.746±2.943	16

Results are expressed as mean±standard error mean from 6 animals in each group. *p<0.05 when experimental groups compared with Group I, [a]p<0.05 when experimental groups compared with Group II, [b]p<0.01 when experimental groups compared with Group II, [c]p<0.001 when experimental groups compared with Group II

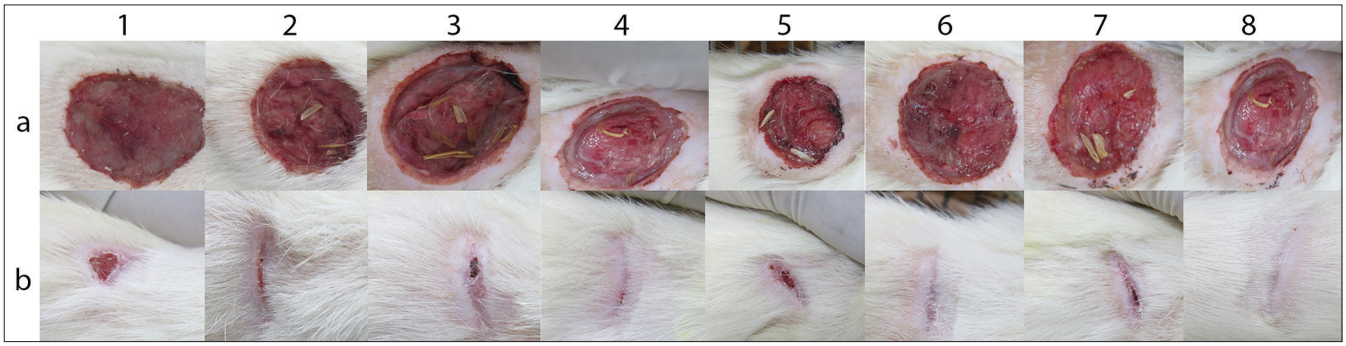

Plate 1: (a) Effect of ethanol and aqueous extract of *Centratherum punctatum* on the rate of wound contraction on day 0, (1) disease control, (2) standard drug, (3) 5% ethanol ointment, (4) 5% aqueous ointment, (5) 10% ethanol ointment, (6) 10% aqueous ointment, (7) 20% ethanol ointment, (8) 20% aqueous ointment, (b) effect of ethanol and aqueous extract of *Centratherum punctatum* on the rate of wound contraction on day 18, (1) disease control, (2) standard drug, (3) 5% ethanol ointment, (4) 5% aqueous ointment, (5) 10% ethanol ointment, (6) 10% aqueous ointment, (7) 20% ethanol ointment, (8) 20% aqueous ointment

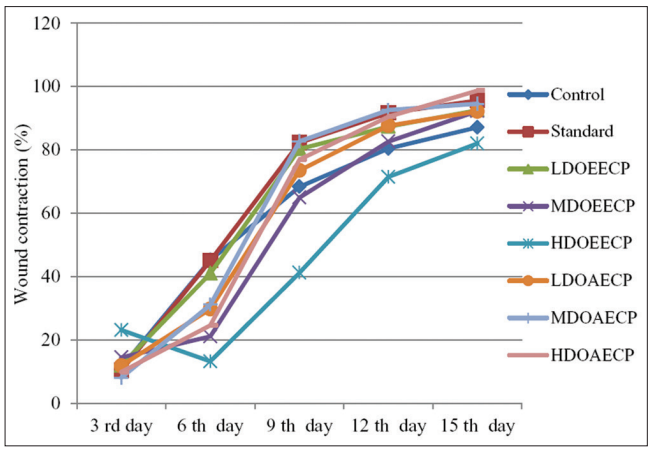

Fig. 1: Comparison on the effect of ethanol and aqueous extract of *Centratherum punctatum* on excision wound models. LDOEECP: 5% ointment of ethanol extract of *C. punctatum*, MDOEECP: 10% ointment of ethanol extract of *C. punctatum*, HDOEECP: 20% ointment of ethanol extract of *C. punctatum*, LDOAECP: 5% ointment of aqueous extract of *C. punctatum*, HDOAECP: 10% ointment of aqueous extract of *C. punctatum*, MDOAECP: 20% ointment of aqueous extract of *C. punctatum*

as compared to diseased control group and ethanol extract. Group VIII showed rapid tissue repair as compared to control and standard drug treated animals. The faster wound contraction observed in the aqueous extract of *C. punctatum* treated experimental animals may be probably due to stimulatory effect of the plant extract on interleukin-8, an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes, and junctional intracellular communication in fibroblasts, and induced an increased gap maturation of granulation tissue. This is in agreement with previous reports as observed by Moyer *et al.* [24]. The significant increase in wound contraction observed in Group VIII animals indicates rapid granulation, collagenation, and collagen maturation [25]. The test drug demonstrated highly efficient wound healing activity throughout the experiment displaying a significant reduction in wound area as compared to not only the normal control but also the positive control. Hence, we can infer that the test drug not only is actively promoting faster wound contraction but is also acting as a potent agent in aiding the process of tissue granulation and remodeling of the connective tissue during the first and second weeks of the healing process.

Effect of ethanol and aqueous extract of C. punctatum on epithelialization

The epithelization of wound was faster in the alcoholic extract treated animals as compared to control and standard groups. The data of the results obtained on epithelialization was tabulated in Table 1 and presented in Fig. 2. Studies on the epithelization time also suggest that in the alcoholic extract treated animals epithelization is faster as compared to control and standard group. This means that ethanol extract of *C. punctatum* without enhancing the activity of the myofibroblasts and proliferation of epithelial cells might have enhanced the migration. This kind of differential actions of a drug on the healing is not surprising, as these two phases run independent to each other.

Effect of ethanol and aqueous extract of C. punctatum on wound surface microbial load count

Table 2 shows the result of antimicrobial efficacy of *C. punctatum*. No bacteria were isolated in swabs taken from experimental animal agar cultures treated with test drug. On day three, it was observed that the wound surface was still wet and attracted microorganisms but as

the healing progresses there was decrease in microbial population. The aqueous extract gel was more potent than the ethanol extract in reducing the microbial load of the wound surface.

Incision wound model

Effect of ethanol and aqueous extract of C. punctatum on breaking strength

Table 3 and Fig. 3 show the effects of the ethanolic and alcoholic extract of *C. punctatum* on breaking strength in rats inflicted with incision wound. The test drug was applied topically at different concentrations like 5%, 10% and 20% w/w for 10 days. Promotion of wound healing activity is also well gazed by the breaking strength provided by the selected drug in the incision wound. In general, wound healing agents possess properties to enhance the deposition of collagen content, which provides strength to the tissues and forms cross-linkages between collagen fibers. Collagen is known to play an important role in the process of wound contraction and gain in tensile strength. Breaking strength depends on the increase in the collagen content.

From the data obtained in this study, it was observed that the mean breaking strength of granulation tissue in the control group was 477±102 g. The breaking strength in standard treated group was 789.268±171.018 g. A significant increase in the wound breaking strength (955.268±173.801 g) was observed in aqueous extract of *C. punctatum* treated animals as compared with the controls and ointment of ethanol extract. The breaking strength observed in the animal treated with 10% and 15% w/w of aqueous extract ointment is highly significant as compared to control. From the data of the results, it is inferred that the wounds treated with the test formulations showed increase in breaking strength as compared to untreated control group and standard group.

In incision wound models, increase in breaking strength is indicative of improved collagenation. The increase in tensile strength noticed in the treated animal wounds may be due to an increase in collagen concentration and stabilization of the fibers which facilitated wound healing. Increased breaking strength observed in the aqueous extract treated animals depicted that ointment of aqueous extract promoted better wound healing activity.

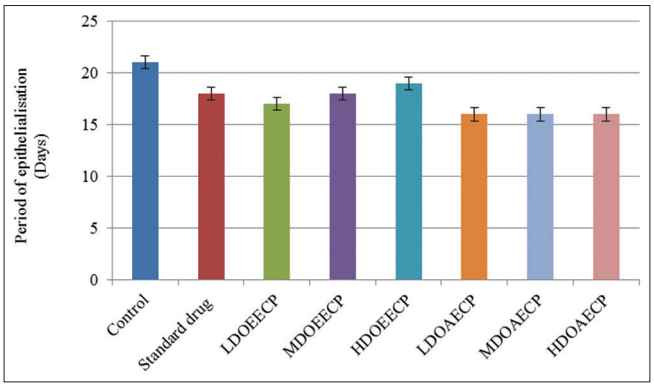

Fig. 2: Effect of ethanol and aqueous extract of *Centratherum punctatum* on epithelialization

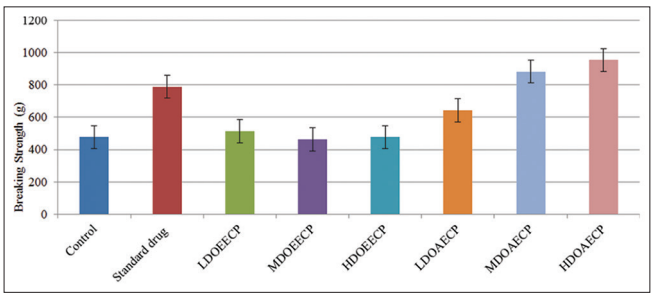

Fig. 3: Effect of ethanol and aqueous extract of *Centratherum punctatum* on breaking strength

Wound healing efficacy of the test drug might be due to the regulation of collagen expression [26] and increase in breaking strength. This is in agreement with observations reported by earlier workers [27]. Similarly, enhanced healing activity may also be attributed to increased collagen formation and angiogenesis as observed by Trabucchi *et al*., Shukla *et al*., Shukla *et al*. [28-30] in their wound healing efficacy studies. Collagen plays a central role in the healing of wounds and it is the principal component of connective tissue and provides a structural framework for the regenerating tissue [31]. Angiogenesis in granulation tissues improves circulation to the wound site by providing oxygen and nutrients that are essential for the healing process [32] such as re-epithelization, epithelial cell proliferation, and angiogenesis [33].

Effect of ethanol and aqueous extract of C. punctatum on hydroxyproline and hexosamine of granuloma tissue

The levels of hydroxyproline and hexosamine in granuloma tissue treated with ethanol and aqueous extracts of *C. punctatum* were represented in Table 4 and graphically in Figs. 4 and 5.

Dead space wound

Effect of ethanol extract and aqueous extract of C. punctatum on dead space wound model

Table 5 and Figs. 6 and 7 show the effects of the ethanolic and aqueous extract of *C. punctatum* on granuloma weight on dead space wound. The test drug was applied topically at different concentrations such as 5%, 10% and 20% w/w for 10 days. Wound has three phases; inflammatory, proliferative and maturational and is dependent on the type and extent of damage, the general state of the host's health and the ability of the tissue to repair. The inflammatory phase is characterized by

Table 2: Effect of ethanol extract and aqueous extract of *Centratherum punctatum* on wound surface microbial load

Groups	Bacterial isolated on day				
	3rd	6th	9th	12th	15th
Group I	++	+++	++	+	+
Group II	+++	+++	++	++	++
Group III	+++	+++	++	++	++
Group IV	+++	+++	+++	++	++
Group V	++	++	++	++	+
Group VI	+++	++	++	+	−
Group VII	++	+	++	+	−
Group VIII	+	+	+	−	−

+Ve indicates presence of growth, −ve indicates absence of growth

Table 3: Effect of ethanol and aqueous extract of *Centratherum punctatum* on breaking strength of granuloma tissue in incision wound

Groups	Breaking strength of granulation tissue (g) mean±SE
Group I	477±102
Group II	789.26±171.01*
Group III	514.02±119.18[a]
Group IV	462.69±88.44[b]
Group V	477.24±56.13[c]
Group VI	642.8±129.3[a]
Group VII	882.3±127.7[b]
Group VIII	955.26±173.80[c]

Results are expressed as mean±standard error mean from 6 animals in each group. *p<0.05 when experimental groups compared with Group I, [a]p<0.05 when experimental groups compared with Group II, [b]p<0.01 when experimental groups compared with Group II, [c]p<0.001 when experimental groups compared with Group II

hemostasis and inflammation, followed by epithelization, angiogenesis, and collagen deposition in the proliferative phase. In the maturational phase, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue.

A significant (0.56 mg/kg) increase was observed in the weight of the granulation tissue in the animals treated with the aqueous extract at a concentration of 20%. The increase in dry granulation tissue weight in the test drug-treated animals suggests higher protein content. The aqueous extract of *C. punctatum* demonstrated a significant increase in the hydroxyproline content of the granulation tissue indicating increased collagen turnover. The increased weight of the granulation tissue also indicated the presence of higher protein content. This is confirmed by the increase in collagen content in granuloma excised from the wound. Among these treated animals, the response was better in ointment of aqueous extract treated animals. This may be attributed to the enhanced collagen maturation due to increased cross-linking of collagen fibers. This is supported by the data that revealed high hydroxyproline content.

Granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema, and new small blood vessels. Collagen contains large molecules of glycine, proline, and hydroxyproline [34]. Collagen, the major component which strengthens and supports extracellular tissue is composed of the amino acid - hydroxyproline, which has been used as a biochemical marker for tissue collagen [35].

Effect of ethanol and aqueous extract of C. punctatum on hydroxyproline and hexosamine levels of granuloma tissue

The effects of ethanol and aqueous extract of *C. punctatum* on granuloma tissues of experimental animals were represented in Table 6 and represented graphically in Figs. 8 and 9.

Table 4: Effect of ethanol and aqueous extract of *Centratherum punctatum* on hydroxyproline and hexosamine of granuloma tissue

Groups	Hydroxyproline (mg/g)	Hexosamine (mg/g)
Group I	31.97±6.70	3.31±0.69
Group II	65.88±2.92*	6.58±0.95*
Group III	44.81±6.32[a]	2.86±0.45[a]
Group IV	45.59±10.27[b]	3.97±0.85[b]
Group V	60.96±9.79[c]	4.11±0.55[c]
Group VI	49.37±5.42[a]	3.77±0.38[a]
Group VII	58.37±4.12[b]	4.05±0.86[b]
Group VIII	76.10±7.81[c]	5.58±2.81[c]

Results are expressed as mean±standard error mean from 6 animals in each group. *p<0.05 when experimental groups compared with Group I, [a]p<0.05 when experimental groups compared with Group II, [b]p<0.01 when experimental groups compared with Group II, [c]p<0.001 when experimental groups compared with Group II

Table 5: Effect of ethanol extract and aqueous extract of *Centratherum punctatum* on granuloma weight on dead space wound

Groups	Wet weight (mg)	Dry weight (mg)
Group I	0.44±0.06	0.23±0.05
Group II	0.24±0.18*	0.14±0.02*
Group III	0.32±0.074[a]	0.18±0.01[a]
Group IV	0.34±0.06[b]	0.20±0.010[b]
Group V	0.39±0.13[c]	0.20±0.04[c]
Group VI	0.41±0.08[a]	0.21±0.02[a]
Group VII	0.52±0.21[b]	0.21±0.02[b]
Group VIII	0.56±0.16[c]	0.25±0.00[c]

Results are expressed as mean±standard error mean from 6 animals in each group. *p<0.05 when experimental groups compared with Group I, [a]p<0.05 when experimental groups compared with Group II, [b]p<0.01 when experimental groups compared with Group II, [c]p<0.001 when experimental groups compared with Group II

Hydroxyproline

Soon after an injury, a rapid protein synthesis occurs in the wound area. Collagen is the principal extracellular protein in the granulation tissue of the healing wound. Collagen plays a key role in the hemostasis in addition to, providing an integrity and strength to the tissue matrix [36]. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of this hydroxyproline, therefore, has been used as an index of collagen turnover. Collagen plays a central role in the healing of wounds and it is a principal component of connective tissue and provides

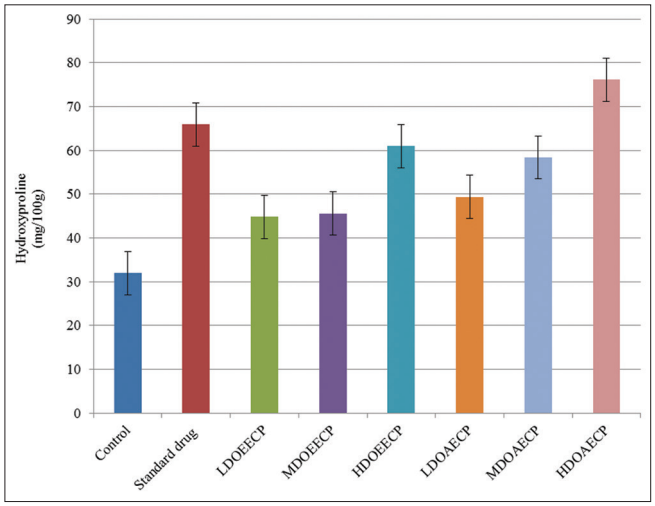

Fig. 4: Effect of ethanol and aqueous extract of *Centratherum punctatum* on hydroxyproline level of granuloma tissue in incision wound

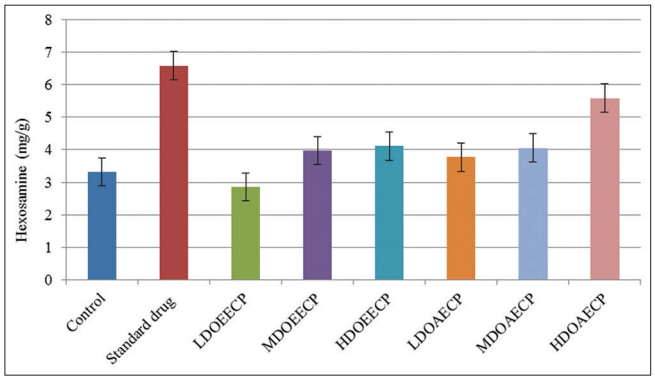

Fig. 5: Effect of ethanol and aqueous extract of *Centratherum punctatum* on hexosamine level of granuloma tissue in incision wound

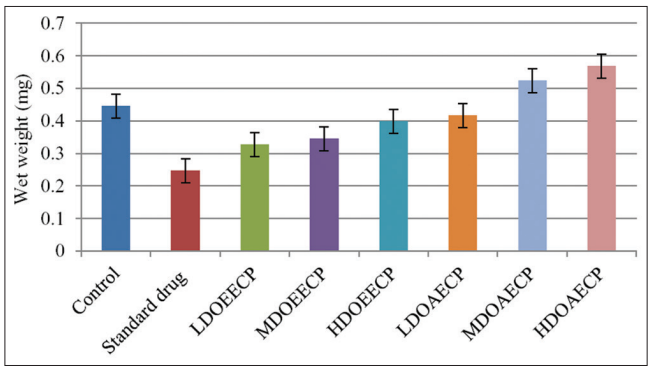

Fig. 6: Effect of ethanol extract and aqueous extract of *Centratherum punctatum* on wet granuloma weight

a structural framework for the regenerating tissue [37]. A wound healing mechanism termed as angiogenesis improves the circulation to the wound site by facilitating the supply of oxygen and nutrients required for the wound healing process which involves re-epithelization, angiogenesis and epithelial cell proliferation as suggested in the earlier reports [32].

Higher concentration of hydroxyproline in the antioxidant study indicates faster rate of wound healing. Table 6 records the concentration of hydroxyproline in the tissue of animals, which were treated with different extracts and formulation of the selected plants for 10 days. Concentration of hydroxyproline was highest in the group of animals treated with ointment of aqueous extract as compared to the standard and ointment of ethanol extract. The increased hydroxyproline content in the dead space wounds indicates faster collagen turnover leading to rapid healing process with concurrent increase in the breaking strength of the treated wounds.

Table 6: Effect of ethanol and aqueous extract of *Centratherum punctatum* on hydroxyproline content of granuloma tissue

Groups	Hydroxyproline (mg/g)	Hexosamine (mg/g)
Group I	31.47±6.81	3.31±0.69
Group II	65.05±4.06*	6.91±0.54*
Group III	44.31±6.52[a]	3.02±0.80[a]
Group IV	43.92±12.92[b]	4.04±0.83[b]
Group V	61.46±10.41[c]	4.28±0.69[c]
Group VI	49.70±5.71[a]	3.85±0.30[a]
Group VII	58.54±4.22[b]	4.03±0.85[b]
Group VIII	76.44±8.02[c]	5.75±2.75[c]

Results are expressed as mean±standard error mean from 6 animals in each group. *p<0.05 when experimental groups compared with Group I, [a]p<0.05 when experimental groups compared with Group II, [b]p<0.01 when experimental groups compared with Group II, [c]p<0.001 when experimental groups compared with Group II

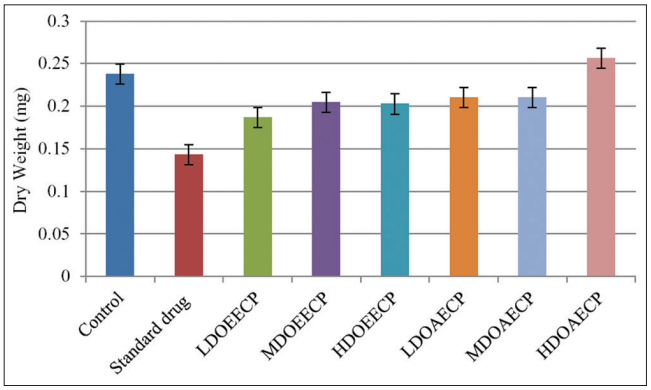

Fig. 7: Effect of ethanol extract and aqueous extract of *Centratherum punctatum* on dry granuloma weight

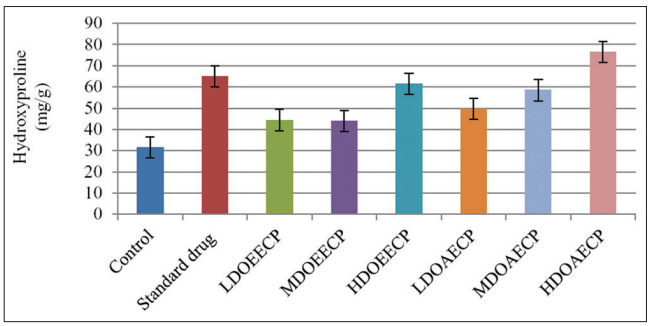

Fig. 8: Effect of ethanol and aqueous extract of *Centratherum punctatum* on hydroxyproline level of granuloma tissue on dead space wound

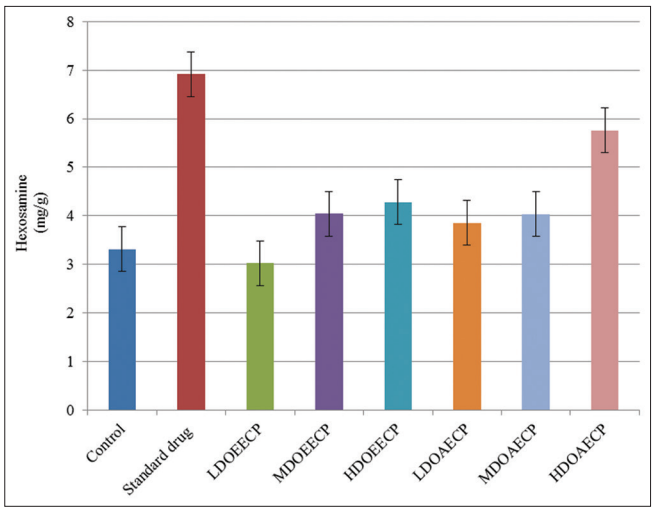

Fig. 9: Effect of ethanol and aqueous extract of *Centratherum punctatum* on hexosamine level of granuloma tissue on dead space wound

Hexosamine

Hexosamine is a matrix molecule, which act as ground substratum for the synthesis of new extracellular matrix. The glycosaminoglycans are known to stabilize the collagen fibers by enhancing electrostatic and ionic interactions with it and possibly control their ultimate alignment and characteristic size [38]. Hexosamine content increases in the early stages of wound healing, which indicate that the fibroblasts are actively synthesized ground substances (mucopolysaccharides) on which the collagen can be laid on [39]. In this study, hexosamine concentration which is one of the components of glycosaminoglycans significantly increased in the animals treated with ointment of aqueous extract as compared with control indicating better stabilization of collagen fibers in animals treated with aqueous extract ointment.

Histopathological studies

Histopathological features of the tissue of the test drug-treated animals obtained on 18th day in excision wound and on 10th day in incision and dead space wound were observed. Tissues of the test drug-treated animals revealed the presence of well-organized bands of rich collagen, more fibroblasts and few inflammatory cells as compared to controls which showed inflammatory cells, scanty collagen fibers and fibroblasts. Granuloma tissues depicted normal architecture in the test drug administered animals thereby suggested the nontoxic nature of the test drug. This is in agreement with the data obtained by Habibipour *et al.* [40] where they also have reported that in the treated group a large amount of fibroblast proliferation, collagen synthesis, and new vascularization resulting in an increased tensile strength and accelerated wound healing.

In the present work, histopathological study revealed interesting features like increased collagen deposition in treated groups as compared to control. Besides also revealed better remodeling of the wound tissues, which may be due to the chemotactic effect of the test drug and this effect might have attracted inflammatory cells toward the wound site and might have helped in remodeling of skin. This is in agreement with the earlier observations [41].

Increased cellular proliferation may be due to the mitogenic activity of the test drug which might have contributed significantly to healing process. Early dermal and epidermal regeneration in treated rats also confirmed that the test drug had a positive effect toward cellular proliferation, granular tissue formation, and epithelialization. This is in line with the findings of studies carried out by Karodi *et al.* [42].

Histology of the wound tissue of the control animals showed the presence of acute inflammatory cells, fibroblastic connective tissue

and very little number of blood vessels, lesser epithelialization and lesser collagen formation indicating incomplete healing of the wound in control animals and the standard drug treated group animals showed increased collagen deposition and better wound healing.

SUMMARY AND CONCLUSION

Aqueous extract of *C. punctatum* possessed a definite pro-healing action. This is demonstrated by a significant increase in the rate of wound contraction and by enhanced epithelization. There was a significant decrease observed in the epithelization period. The drug extract also facilitated the rate of wound contraction significantly at the dose levels administered.

The promotion of wound healing activity is also well gazed by the tensile strength observed in the incision wound. Significant increase was also observed in skin breaking strength and hydroxyproline content which was a reflection of increased collagen levels that was further supported by histopathological studies and gain in granuloma breaking strength.

Histopathological study of granuloma tissues of animals treated with ethanol extract and aqueous extract revealed a high level of fibrosis as well as well-formed collagen fibers. This also further suggested nontoxic nature and wound healing efficacy of the selected drug.

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Research Article

HIERARCHICAL NANOFEATURES PROMOTE MICROBIAL ADHESION IN TROPICAL GRASS: A GREEN APPROACH TO FOOD PRESERVATION

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ABSTRACT

Objective: Lunar eclipse occurs when the Moon passes directly behind the Earth into its shadow. During solar and lunar eclipse, when the natural bactericidal radiations are not available to the earth surface, there is an increase in the microorganisms' count and mutation. Fermentable food items are known to get spoiled during eclipse, consumption of which causes problems in the digestive system. Hence, it is not advisable to consume food during eclipse. To prevent food being spoilt, Indian people use Darbha grass (*Desmostachya bipinnata L.*) in the food container. It is a tropical grass used as an inevitable material in traditional practices and rituals of the Indian Hindus. This particular grass has hierarchical nanoscale morphology, that is, distinct nano/micro scale features on the surface, which removes excess bacterial growth from fermentable food items and preserves them. This study demonstrates the potential and mechanism of Darbha grass in preserving ferment-able food, also the present investigation suggests that the grass could be used in designing biomimetic antifouling surfaces inspired by Darbha grass, which could find applications in health care and food preservation.

Methods: Collection of plant materials: Dried leaves of *D. bipinnata L.* Darbha grass were collected from different places. Sample preparation: Freshly fermented curd and rice batter was taken in stainless steel vessels each and covered with Darbha grass. Platting for colony-forming unit (CFU) count: Loop-full of the samples was diluted and plated on the Petri plates and was incubated at to check for CFU and morphology of different colonies.

Results: Darbha, due to its hierarchical nanofeatures captures the microbial overgrowth by biofilm formation, which is further verified in the results obtained in this work where the accelerated microbial growth during the eclipse is checked by the Darbha and even there seems to be no morphological changes in the samples with Darbha.

Conclusion: Darbha thus shows a promising role in arresting the excessive growth of microbes and helps in food preservation.

Keywords: Lunar, Eclipse, Darbha, Grass, Food, Preservation.

INTRODUCTION

Microbial spoilage of food is an area of global concern, causing serious foodborne intoxications and resulting in high economic losses for the food producing sector [1].

Food spoilage is a metabolic process that causes foods to become undesirable or unacceptable for human consumption due to changes in sensory characteristics. Spoiled foods may be safe to eat, i.e., they may not cause illness because there are no pathogens or a toxin present, but changes in texture, smell, taste, or appearance cause them to be rejected [2].

Lot of chemical preservatives are used to prevent spoilage. Although they are essential for food storage, they can give rise to certain health problems. They can cause different allergies and conditions such as hyperactivity and attention deficit disorder, asthma, hay fever and certain reactions such as rashes, vomiting, headache, tight chest, hives and worsening of eczema in the some people. Although they are essential for food storage, the cumulative effect' of their hazards as "slow poisons" will increase the risk of becoming a host to disease or "premature death" [3].

Sulfites are common preservatives used in various fruits that may produce side effects in the form of headaches, palpitations, allergies, and even cancer. Nitrates and nitrites: These additives are used as curing agents in meat products, it gets converted into nitrous acid when consumed and is suspected of causing stomach cancer. Benzoates are used in foods as antimicrobial preservatives and have been suspected to cause allergies, asthma and skin rashes. Sorbates/sorbic acid added to foods as antimicrobial preservatives, may also cause allergies [4].

Hence, there is an urgent need for green preservation, a natural agent which can provide protection against microbes without altering the food quality.

In ancient Hindu texts, during lunar eclipse, it is advised to stay indoors and to keep the fermentable food container covered with Darbha grass. Few speculations were made to understand the reason behind this practice. One of the hypotheses was that there are harmful radiations during the eclipse [5], while the other was an increase in the microorganisms' count and mutation during this time [6]. Fermentable food items are known to get spoiled during eclipse, consumption of which causes problems in the digestive system. Hence, it is not advisable to consume food during eclipse. As an alternative, Darbha (*Desmostachya bipinnata L.*) grass has been traditionally dropped in food during solar and lunar eclipse and the food becomes consumable after the eclipse once the grass is discarded. However, the direct connection between eclipse, food spoilage and usage of Darbha has not been scientifically explored so far.

Darbha (*D. bipinnata L.*) is a tropical grass which has been used by the ancient Aryans in their cultural rituals and is considered very sacred. The reason for choosing this grass among several other grass species is supported by the existence of distinct nano/micro scale features on the grass surface as we have reported earlier [7].

Lunar eclipse occurs when the Moon passes directly behind the Earth into its umbra (shadow). This can occur only when the sun, earth and moon are aligned exactly, with the Earth in the middle. The absolute surface temperature of the moon decreases steadily during the phases of total eclipse [8].

In the mission report of Apollo 15 submitted by NASA, it is stated that during the lunar eclipse, sun shield temperature of the central station dropped from 60°C to 97°C with accompanying rates of change of temperatures up to 126°C/h [9]. These changes in the cosmic environment lead to certain changes on the earth as in one observation, during the lunar eclipse, it is mentioned that the temperature steadily decreases from 26.4°C to 23.3°C and relative humidity increased from 55% to 67% [10]. These changes alter the microbial life on earth in a very subtle but definite way leading to some mutations that can even be permanent. Microorganisms are known to mutate and adapt to the change in its environment to survive the stress [11]. In an article, it is mentioned that in harsh environmental conditions, the number of fungal colonies of *Candida albicans* remarkably increased suggesting active multiplication with acquisition of greater virulence [12]. Morphological changes also contribute to increased pathogenic potential of the fungus. This transition has also been recorded by molecular studies [13]. Environmental stimuli such as aerobic/anaerobic conditions and different substrate concentrations have been reported to change the morphology and growth patterns of yeast [14]. In another study conducted during an solar eclipse, bacterial colonies showed difference in colony morphology, and antibiotic sensitivity pattern [6].

Thus, eclipse is known to alter the nature and population of microorganisms in the environment. On the other hand, Darbha grass is known to remove bacteria using its nanoscale surface features as reported earlier [7]. In this context, this study demonstrates the potential and mechanism of Darbha grass in preserving fermentable food especially during eclipse.

METHODS

Cow's curd and rice batter (Prepared by grinding rice and white lentils in 3:1 ratio in water to make doughy paste) were studied as model fermentable food in this work. The effects of lunar eclipse on these fermentable foods were observed and the effect rendered on this process by the addition of Darbha grass was also observed.

The studies were conducted on the lunar eclipse which occurred on 4th April 2015 at Shanmugha Arts, Science, Technology and Research Academy (Coordinates 10.7275° N, 79.0207° E). The set of experiments were conducted before, during and after the eclipse (duration of study 2 pm to 8 pm) to understand the phenomenon occurring during this timeline.

Collection of plant materials

Dried leaves of *D. bipinnata* L. Darbha grass were collected from two different places (Trichy, Tamil Nadu, India; and SASTRA University campus, Thanjavur, Tamil Nadu, India).

Sample preparation

Freshly fermented (24 hrs) curd and rice batter (unsalted, prepared with rice and white lentils in 3:1 ratio) was taken in stainless steel vessels each and covered with Darbha grass.

Platting for colony-forming unit (CFU) count

Loop-full of the samples was diluted in 10 ml distilled water and plated on the Petri plates with nutrient agar (HiMedia Pvt. Ltd., India) and were incubated at 37°C for 24-48 hrs to check for CFU and morphology of different colonies.

Controls

- Set 1: Control experiments without grass and open plate analysis in which the sterile nutrient agar plates were exposed to open air for 1 minute to check the difference in the air borne microbes before and during eclipse was also carried out.
- Set 2: Samples exposed to eclipse without the grass were platted on nutrient agar.
- Set 3: Consortium of microbes taken from curd was inoculated in nutrient broth and microbial growth was checked for both broth with Darbai and without Darbai.

RESULTS AND DISCUSSION

The following experiment was conducted to understand the effect of eclipse on the count and nature of air-borne microorganisms. For this purpose, sterile nutrient agar plates were exposed to open air at different time points (before the eclipse and during the penumbral and total eclipse) on the day of lunar eclipse in 4th April 2015. The plates after incubating for 24 hrs at 37°C are shown in Fig. 1. The count of air borne microorganisms seems to be increased during the lunar eclipse, also there appears to be a morphological change in these colonies. The increase in microbial count could be attributed to the decrease in the intensity of blue radiation (which has inherent disinfecting property) reaching the earth during eclipse [15]. On the other hand, morphology changes in the microbial colonies could be due to the change in temperature during lunar eclipse [16]. The phenotype switching has been previously reported as a response to the change in environment to prove the mutation and evolution in bacteria under different conditions [17] and few of these mutations are permanent in nature even when the conditions are brought back to normal. Thus it is understood that even during a short duration (4 hrs) of lunar eclipse, there is a significant increase in microbial count in air.

After confirming the increasing microbial growth in air during eclipse using nutrient agar plates, it would be interesting to understand the effect of eclipse on microbial population in fermentable food items. To understand this aspect, curd and batter were exposed to air, aliquots of the samples were taken at different time points of the lunar eclipse and then cultured in sterile agar plates. To avoid variations in exposure and culturing conditions, all the plates were exposed for the same duration to air and cultured under same conditions. Furthermore, the sample dilutions were kept the same. Fig. 2 shows a significant progressive increase in the count of the microbes in plates from curd and batter as the eclipse advances. Moreover, morphological changes are observed among the microbes on the plates. This observation could be compared with the count and morphological changes in air-borne microbes as discussed in Fig. 1.

Natural fermentation of batter, involves the process of converting carbohydrates to acid, alcohol and gas due to microbial action. Rice/flour batter fermentation is usually aided by yeast. Fermentation of curd involves converting lactose present in the milk into lactic acid using strains such as *Streptococcus lactis*, *Streptococcus cremoris, Thermophilus, Lactobacillus bulgaricus, Lactobacillus acidophilus,* and *Lactobacillus plantarum.* During the fermentation process, the microbes ferment glucose by way of glycolysis to pyruvate, which is decarboxylated to acetaldehyde and carbon dioxide (CO_2) gas is released [18]. During the lunar eclipse it is found that the fermentation process is accelerated (as seen in the picture provided in the supplementary data). Increase in the CO_2 levels affects the bacteria as it goes into a reproductive overdrive [19]. This could be the reason for the overgrowth of the bacterial colonies during eclipse. Having understood the effect of lunar eclipse on microbial population in air and fermentable food like curd and batter, the next objective of the study was to verify the disinfectant ability of Darbha grass in preventing the over-fermentation of the food items. To verify this aspect, Darbha grass was dropped in the curd/batter samples and microbial analysis was performed for aliquots drawn from the samples at different time points during lunar eclipse. Accordingly, Fig. 3 shows the effect of Darbha on the curd and batter throughout the eclipse period and how it controls the microbial proliferation. Darbha not only controls the microbial proliferation it is also providing an environment due to which there are no morphological changes observed as well. This is in accordance with our earlier report that Darbha can disinfect liquid samples by attracting to its surface nanofeatures [7].

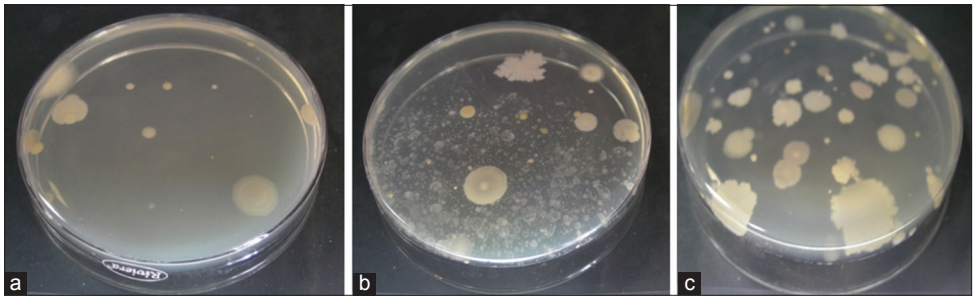

Fig. 1: Plates of nutrient agar exposed to air: (a) 2 hrs before lunar eclipse, (b) penumbral lunar eclipse, (c) during total eclipse

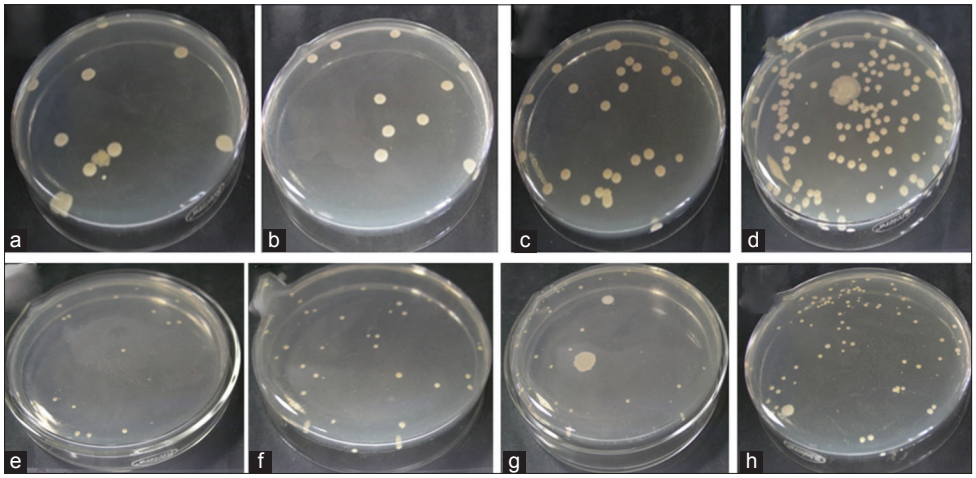

Fig. 2: Plates from curd exposed to eclipse (a) 2 hrs before, (b) penumbral, (c) partial, (d) complete eclipse; plates from batter exposed toeclipse – (e) 2 hrs before, (f) penumbral, (g) partial, (h) complete eclipse

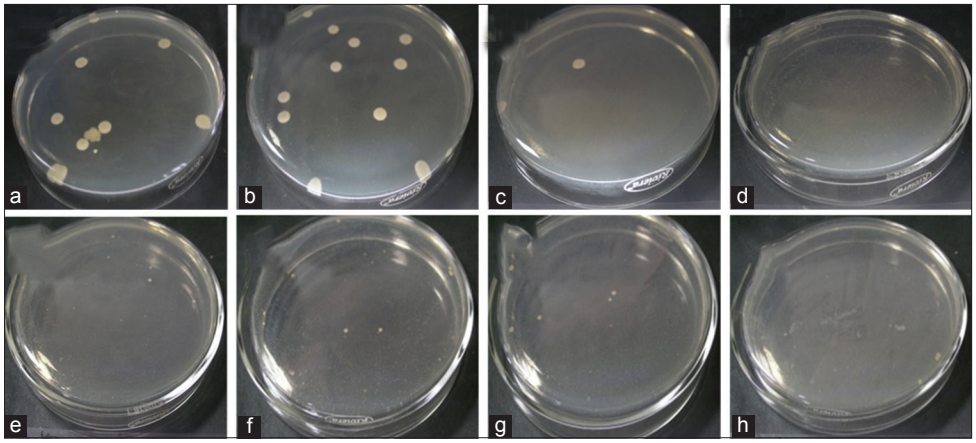

Fig. 3: Plates from curd with Darbha grass exposed to eclipse – (a) 2 hrs before, (b) penumbral, (c) partial, (d) complete eclipse; plates from batter with Darbha grass exposed to eclipse – (e) 2 hrs before, (f) penumbral, (g) partial, (h) complete eclipse

Microbes have a tendency to form biofilms on a solid surface. A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface [20]. In a previous work, it has been shown that the adhesion of bacterial cells on titanium surfaces is promoted by the presence of nanoscale topographical features [21]. In another work, the correlation between bacterial adhesion and the spatial organization of nanofeatures of different shape and sizes has also been studied [22]. It is also found that the bacterial adhesion and biofilm formation depend on nanoroughness in a nonmonotonous way [23] and a biofilm saturation is reached on the nonavailability of free surface for adhesion and it is found that the number of bacteria on surfaces was dramatically higher than in the surrounding medium [24]. Thus, Darbha grass with hierarchical nanofeatures also has the potential to remove bacteria from liquid samples by forming biofilms.

In the above experiments, aliquots drawn from curd and batter samples were platted because it is impossible to assess turbidity due to microbial growth directly in the curd/batter samples due to their inherent turbidity. Hence, as a control experiment to confirm the effect of lunar eclipse on the growth of consortium of microbes cultured from the curd, loopful of curd was inoculated in nutrient broth and growth during lunar eclipse was followed. Another set was repeated in the presence of Darbha grass and microbial growth was followed using turbidity method. Optical density (OD) at 600 nm was checked and the result was plotted against the time-point of eclipse. Interestingly and in accordance with Figs. 3 and 4 shows drastic decrease in turbidity in the culture flask containing Darbha grass. Furthermore, significant reduction in the growth is observed in the OD graph shown in the right panel of Fig. 4.

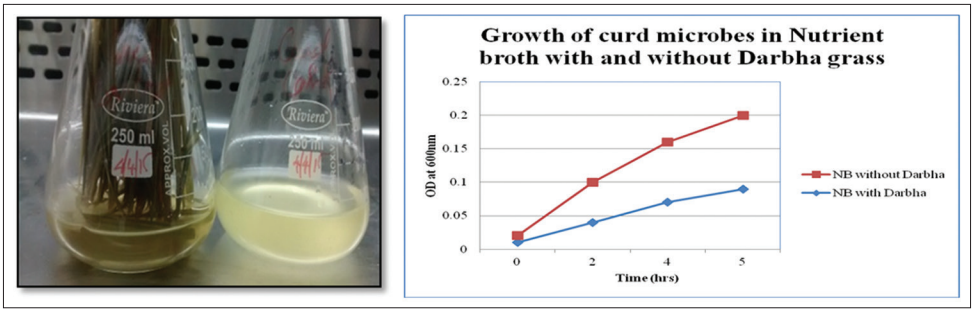

Fig. 4: Growth of microbes harvested from curd in liquid culture medium during lunar eclipse in the presence (left) and absence (right) of Darbha grass. Graph in the right shows the growth of curd microbes in nutrient broth with and without Darbha grass

Darbha is supposed to be kept in the vessels throughout the eclipse and then discarded. It is mentioned in a work that the surface available for microorganisms was found to be a limiting factor for the biofilm's development [25]. Since this is also a surface phenomenon, the Darbha gets saturated in time and thus it is advised to discard after the eclipse is over.

In accordance with the work carried out by our group previously, Darbha, due to its hierarchical Nanofeatures captures the microbial overgrowth by biofilm formation, which is further verified in the results obtained in this work where the accelerated microbial growth during the eclipse is checked by the Darbha and even there seems to be no morphological changes in the samples with Darbha.

Darbha thus shows a promising role in arresting the excessive growth of microbes and helps in food preservation.

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Research Article

IMMUNOTHERAPEUTIC POTENTIAL OF *CRESCENTIA CUJETE. L* AIDS RECUPERATION OF ZEBRAFISH FROM BACTERIAL INFECTION

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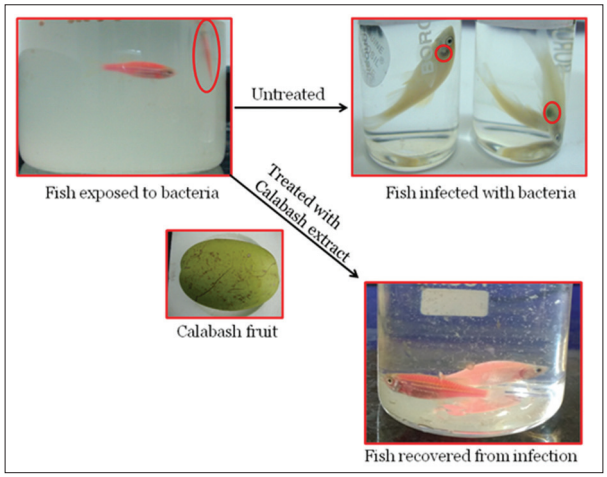
ABSTRACT

Background: The dry shell of *Crescentia cujete* (also called the 'Beggar's Bowl') has been proposed in ancient Indian texts to have the ability to prevent viral and bacterial contamination of food stored in it. We explored the immunotherapeutic potential of a tropical fruit, *Crescentia cujete* (Calabash) in preventing and treating bacterial infection.

Methods: Phytochemical analysis of the Calabash extract was done. Zebrafish and *E. coli* (MG1655) have been used as model fish and bacteria, respectively. The infected fish were exposed to Calabash extract. Neuro and hepatotoxicity studied were conducted using Ellman's degradation method and histopathology sections, respectively.

Results: Excellent recovery was observed as evident from visual observation, biochemical assays, and histopathological assays. Also fish pre-treated with Calabash extract were found to have better immunity towards bacterial attack.

Conclusion: The ability of *C. cujete* (calabash) to treat bacterial infection in fish has been demonstrated in this work.



Keywords: Calabash, *Crescentia,* Fruit, Immunity, Infection, Zebrafish.

INTRODUCTION

Bacterial infection caused through ornamental and edible fish species to humans is becoming very common and more hazardous owing to the evolution of newer bacterial species in the environment [1]. The contamination is generally expected to occur during handling, processing, and packaging of fish products. *Escherichia coli* and other coliforms along with *Staphylococcus* sp. are generally used as indices for bacterial contamination in fish and fish products [2]. *E. coli* is an enteric bacteria which causes gastroenteritis if ingested. For instance, tuna fish paste contaminated with *E. coli* caused an outbreak of diarrhea in Japan in the year 1998 [3]. In general, freshly caught fish is not expected to have any accumulation of such bacteria in their organs. However, fish fed with bacterial contaminated food and those grown in bacteria contaminated water can serve as vectors for the microorganisms and transfer them to humans on ingestion [4]. In addition to health hazards, bacterial infection in fish bred in fisheries also affects aquaculture economics to a considerable extent [5].

One of the most common treatment methods for fish infected by bacteria is antibiotic therapy. Intensive-dose of antibiotic specific to the bacterial strain responsible for infection is administered to the affected fish orally through injection [6,7]. Broad-spectrum antibiotics are also used to prevent bacterial infection and are administered to the fish through medicated food. However, about 30% of the antibiotic settles down with unconsumed food, while 80% of the medicated food consumed by the fish remains unabsorbed and gets excreted by the fish [8]. The unabsorbed antibiotics, in turn, pollute the water. To minimize the side effects and pollution caused by chemicals, antibiotics, and drugs, the focus has recently shifted to natural immunostimulants

which can improve the immune system of fish [9]. A considerable number of plant extracts have been tested as immunostimulants for fish including *Astragalus membranaceus, Scutellaria baicalensis, Ganoderma lucidum,* and *Lonicera japonica* [10].

In this context, we explore the effect of calabash pulp (*Crescentia cujete*). The dry shell of *C. cujete* (also called the "Beggar's Bowl") has been proposed in ancient texts to have the ability to prevent viral and bacterial contamination of food stored in it. This provided enough hygiene to those who used the shell to collect and store food. Calabash pulp has a high concentration of minerals like sodium (about 3.20% in the wet pulp) and vitamins, especially, thiamine (1.50 µg/g of wet pulp) [11]. Thiamine (vitamin B1) plays a major role in the immune system of humans, which justifies the use of calabash pulp in folk medicine [12]. Thiamine is also a major determinant for the immunity levels in fish. For example, thiamine deficiency has been identified as one of the major causes for the early mortality syndrome (EMS) found in most of the offsprings of salmonine from the Great Lake of New York [13]. Thiamine deficiency in the young salmonids was later found to be due to the consumption of forage containing thiaminase enzyme. Salmonines in the Great Lake and New York's Finger Lakes feed on a forage fish called alewife (*Alosa pseudoharengus*) which has been found to contain viable thiaminase-positive bacteria, *Paenibacillus thiaminolyticus*, in its viscera [14]. Thiaminase-containing bacteria are also known to cause diseases related to thiamine deficiency like beriberi in humans [12]. As a therapeutic measure, the affected offsprings of salmonines were treated with thiamine solution in the hatching stage and swim-up stage which reduces EMS in the offsprings [15]. In this context, the calabash pulp with high thiamine content could also be used as a natural medicine to treat fish infected with bacteria. In this work, zebrafish (*Danio rerio*) is chosen as a model to study the effect of bacterial infection and the curative properties of calabash pulp. Zebrafish is chosen for this study because it is a widely accepted model for studying host-pathogen interactions [16-18]. For instance, zebrafish has been used as a model to understand the toxicity of lipopolysaccharides secreted by *E. coli* 0111:B4 strain [19]. In another report, pathogenicity of extra-intestinal *E. coli* strains (MG1655 and W3110) has been assessed using zebrafish models [4].

METHODS

Calabash fruits were collected from Datta Peetham, Mysore, India. Adult zebrafish (*D. rerio*), irrespective of sex, of length 3-4 cm were purchased from local aquarium and maintained in tap water at room temperature. Commercially available feed was provided, and the tank was cleaned and water was replaced regularly. Fishes were allowed to acclimatize for 48 hrs before any experimental exposure. All the chemicals used for the experiments such as phytochemical screening, concentration of dissolved oxygen, and metabolic enzyme assays were purchased from Sigma-Aldrich, USA. *E. coli* strain MG1655 was from centre for DNA Fingerprinting and Diagnostics, Hyderabad.

Scanning electron microscopy (SEM)

The surface morphology of the dried fruit shell was imaged using (JEOL, JSM-6360, scanning electron microscope, Japan) Field emission SEM at 15kv. SEM uses electrons to image the objects instead of light. The specimen was scanned across the surface by an electron beam. The specimen emits different types of signal and detected by the detectors. These signals were amplified to form the image. Since the sample was nonmetal, it was sputter coated before imaging.

Phytochemical screening [20]

To find the phytochemical composition of the fruit pulp, a simple chemical test procedure was followed.

1. Flavones: To find the presence of flavones the following tests were done.
    i. Shinoda test: Magnesium turnings and few drops of concentrated hydrochloric acid was added to the fruit extract and heated
    ii. Alkaline reagent test: 10% NaOH was added to the fruit extract
    iii. Ferric chloride test: Ferric chloride was added to the extract
    iv. Lead acetate solution test: 10% Lead acetate solution was added to the fruit extract.
2. Reducing sugars: To confirm the presence of reducing sugars Fehling's test was performed.
3. Quinones: To find the presence of quinones the following procedure was followed: 10% NaOH was added to the extract.
4. Anthroquinones were determined by Borntrager's test
5. Carbohydrates: Molisch's test: Alpha-naphthol in alcohol and drops of concentrated sulfuric acid was added to the extract.
6. Phenolic compounds: Alcoholic ferric chloride was added to the extract.
7. Glycosides: Anthrone and few drops of concentrated sulfuric acid were added to the fruit extract and warmed for few minutes.
8. Saponins: Water was added to the extract and shaken well.
9. Tannins: Lead acetate was added to the fruit extract.
10. Steroids:
    i. Liebermann–Burchard test: Chloroform and acetic anhydride was added to the extract and then concentrated sulfuric acid was added in drops
    ii. Ferric chloride-acetic acid test: Chloroform was added to the extract, and then ferric chloride-acetic acid reagent is added followed by concentrated sulfuric acid.
11. Alkaloids:
    i. Dragendorff's: Acetic acid and dragendorff's reagent was added to the extract
    ii. Meyer's test: Diluted hydrochloric acid and Meyer's reagent was added to the extract.
12. Proteins: Million's test: Extract was added with Million's reagent.
13. Terpenoids: Small piece of tin and thionyl chloride was added to the extract.

Concentration of dissolved oxygen

To find the concentration of dissolved oxygen in different dilutions of fruit extract, the following method was followed. 250 ml of five different concentrations (×10, ×50, ×100, ×200 and ×300) of fruit extract were collected in separate beakers. 2 ml of 2 M manganese sulfate and 2 ml of alkali iodide-azide solution was added and mixed. After shaking and allowing sufficient time for all oxygen to react, the brown colored precipitate of manganese oxide was allowed to settle leaving clear liquid in the upper portion. Then, 2 ml of concentrated sulfuric acid was added and mixed until the suspension was completely dissolved and yellow color was uniform throughout the bottle. A volume of 203 ml was taken into conical flask and 1-2 ml of starch was added as an indicator, then titrated with 0.025 N sodium thiosulfate solution until the first disappearance of blue color. For all the dilutions, the method was repeated thrice [21].

Fish exposure and assay

To find the effect of fresh fruit pulp extract on zebrafish, the fishes were exposed to various dilutions (×10, ×50, ×75, ×100, ×150, ×200, ×250 and ×300) of the fruit extract. Five 500 ml beakers containing 250 ml of water along with the respective fruit pulp dilutions were taken. Six healthy fishes were added to each of the beakers. Fish feed was added in equal amounts in each of the beakers. Fruit extract and water (control) was changed every 24 hrs. Before studying the fruit extract, the tolerance of zebrafish to *E. coli* infection was verified first as follows. Three 500 mL beakers containing bacterial biomass of 3.5 mg, 7 mg, 35 mg (G1, G2, and G3) of 16 hrs *E. coli* culture in 250 ml tap water were taken. Exposed samples were changed with fresh 16 hrs culture every 24 hrs. Six healthy zebrafish were added to each of the beakers. Fish feed was added in equal amounts in each of the beakers. The immunity of the zebrafish exposed to the fruit extract toward *E. coli* attack was verified as follows. Three beakers containing ×100, ×200 and ×300 (P1, P2 and P3) diluted fruit extract in tap water with the volume of 250 ml each were taken. Six healthy zebrafishes were added to each of the beakers, and an equal amount of fish feed were given. The total exposure was for 15 days in which the fish from each sample were transferred to bacterial culture (3.5 mg *E. coli* in 250 ml tap water) 60 hrs before the assay. For this purpose, only 3.5 mg/250 ml bacterial concentration was chosen as 7 mg and 35 mg were highly toxic as inferred from direct

bacterial exposure studies explained above. To check whether zebrafish previously infected by *E. coli* are recovered by *C. cujete,* the following studies were performed. Three 500 mL beakers containing biomass of 3.5 mg, 7 mg, 35 mg (R1, R2 and R3) of 16 hrs *E. coli* culture in 250 ml tap water were taken. Six healthy zebrafish were added to each of the beakers. Fish feed was added in equal amounts in each of the beakers. Exposed samples were changed with fresh 16 hrs culture every 24 hrs. Fish present in R1, R2, and R3 samples were transferred to individual beakers containing 250 ml of ×300 diluted fruit extract each after the exposure duration of 60 hrs, 12 hrs and 20 minutes, respectively. Control experiments were done simultaneously by transferring another set of fish exposed to 3.5 mg, 7 mg and 35 mg *E. coli* biomass in 250 ml tap water to individual beakers containing 250 ml tap water each after exposure duration of 60 hrs, 12 hrs and 20 minutes, respectively. These samples were named as T1, T2 and T3, respectively. Exposure to fruit extract and tap water of recovered fish was continued as usual until the entire 15 days before assay. During the exposure water was changed and feed was added every 24 hrs. To verify whether the fruit pulp extract protects the zebrafish from bacterial attack the following procedure was followed. Three 500 ml beakers containing 250 ml of ×300 extract with added bacterial biomass of 3.5 mg, 7 mg and 35 mg were taken. Six healthy fishes were added to each of the beakers. Fish feed was added in equal amounts in each of the beakers. Exposed samples were changed with fresh 16 hrs culture and fresh ×300 fruit extract every 24 hrs. Only 2 days exposure was possible due to high toxicity levels.

Estimation of metabolic enzymes in fish tissue

The acetylcholinesterase activity was measured by Ellman's degradation method [22]. The substrate acetylcholine iodide is hydrolyzed in the presence of acetylcholinesterase to thiocholine. Thiocholine reacts with 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) solution to form a yellow colored compound 5-thio-2-nitrobenzoic acid whose optical density was measured by spectrophotometer at 400 nm. 150 µl of head homogenate in 100 mM sodium phosphate buffer, pH 7.5, was added to 500 µl of 100 mM sodium phosphate buffer, pH 7.5. To this mixture 25 µl of 10 mM DTNB solution was added, and the enzymatic reaction was started by adding 25 µl of 12.5 mM acetylcholine iodide and incubated at room temperature for 5 minutes until a yellow color was observed. Optical density of the samples was measured at 400 nm against a blank containing buffer instead of sample. The activity was expressed as µM acetylthiocholine hydrolyzed/minute. Carboxylesterase activity was measured by the method specified by [23]. α/β napthyl acetate is broken into α/β naphthol in the presence of α/β carboxylesterase which in turn combines with the diazonium dye solution resulting in the formation of a colored azo compound whose optical density can be measured by spectrophotometer. 50 µl of the viscera homogenate in 20 mM sodium phosphate buffer pH 7.0 was incubated with 500 µl of 250 mM α napthyl acetate for 30 minutes at room temperature. After incubation, 200 µl of freshly prepared 0.3% Fast Blue B in 3.3% sodium dodecyl sulfate (SDS) was added to stop the enzymatic reaction. The mixture was incubated for 30 minutes at room temperature until a dark blue color was observed. The optical density of the samples was measured at 430 nm against a blank containing buffer instead of the homogenate. The amount of α carboxylesterase was calculated using standard graphs and expressed as µM α napthol released/minute. 50 µl of the viscera homogenate in 20 mM sodium phosphate buffer pH 7.0 was incubated with 500 µl of 250 mM β napthyl acetate for 30 minutes at room temperature. After incubation, 200 µl of freshly prepared 0.3% Fast Blue B in 3.3% SDS was added to stop the enzymatic reaction. The mixture was incubated for 30 minutes at room temperature until a red color was observed. The optical density of the samples was measured at 588 nm against a blank containing buffer instead of the homogenate. The amount of β carboxylesterase was calculated using standard graphs and expressed as µM β napthol released/minute. The samples were fixed in 10% neutral buffered formalin (NBF) for 48 hrs. Following this, the tissues were processed using automated tissue processor in series of alcohol followed by xylene and was impregnated with paraffin wax. The processed tissues were embedded in paraffin wax and 3-4 micron thick sections were taken using a rotatory microtome. The sections were stained using routine hematoxylin-eosin procedure in an automated linear stainer. The stained sections were mounted using DPX mountant and coverslips were placed over the section. The sections were read with the light microscope for the presence of histological changes, and the images were taken using Trinocular Nikon Eclipse Ci H600L microscope.

Histopathological sectioning and analysis

Tissues were preserved in 10% NBF and processed for histopathological examination. After proper fixation of 48 hrs, tissues were cut into thinner section (2-3 mm thick), processed using automatic tissue processor (Leica Tissue Processor TP 1020), embedded in paraffin wax (Leica EG 1150 H and C Embedding machine) and cut into 3-4 µm sections (Leica RM 2125 RTS) and stained by routine haematoxylin and eosin staining technique using a Linear automatic stainer (Leica ST 4040). Histopathology was carried out in all the aforementioned organs/tissues of all animals in the control and high-dose groups [24,25].

RESULTS AND DISCUSSION

Fig. 1 shows the photographs of *C. cujete* fruit with and without the outer shell. The pulp contains fine seeds distributed all over. The dry shell of *C. cujete* (also called the "Beggar's Bowl") has been proposed in ancient texts to have the ability to prevent viral and bacterial contamination of food stored in it. This provided enough hygiene to beggars who used the shell to collect and store food. Fig. 2 shows the scanning electron micrographs of the inner and the outer surface of the dry shell as well that of the inner skin that covers the inner surface. The SEM images reveal interesting microstructure on the shell surface as well as the inner skin. More specifically, the outer surface of the shell (Fig. 2a) consists of organized "microplates" which probably render the surface hydrophobic. The inner surface, on the other hand, consists of "microcups" (Fig. 2b) covered by an inner skin consisting of high aspect ratio "microfibers" each of about 1 µm diameter (Fig. 2c). In comparison with bacterial adhesion behavior on nano/micropatterned surfaces that has been reported in the literature [26,27], the dry shell of *C. cujete* with micropatterned surface, also promotes microbial adhesion. Accordingly, SEM images were recorded after incubating the shell and inner skin of the fruit in *E. coli* culture followed by fixation in glutaraldehyde solution (Fig. 2d-f). The inner skin (Fig. 2f) with microfibers is found to have excellent bacterial adhesion property which probably ensures the preservation of any food item stored in "Beggar's Bowl." On the other hand, the inner shell beneath the skin and the outer shell do not show considerable bacterial adhesion property (Fig. 2d and e). The leaf extract of calabash tree has also been reported to have antibacterial property [28]. The extract of *C. cujete* fruit has been used in folk medicine to treat inflammation, trauma, diarrhea and fever [29].

After understanding the ability of calabash shell to remove bacteria from liquid medium, the properties of the fruit extract were studied as described below. To assess the biocompatibility of the fruit extract, zebrafish were

Fig. 1: Photographs of calabash fruit with outer shell (left) and without outer shell exposing fresh pulp (right)

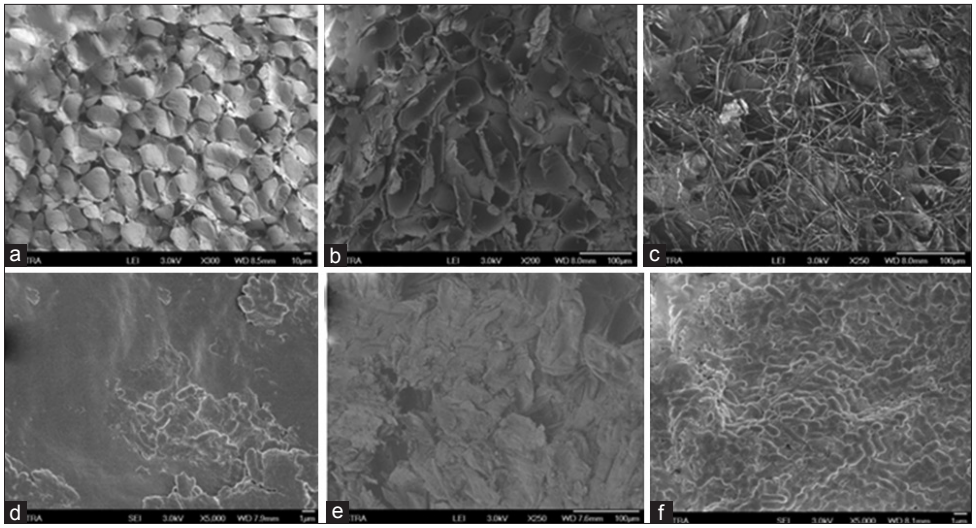


Fig. 2: Scanning electron micrographs of (a) the outer exposed part of the fruit shell; (b) inner part of the shell after peeling off the skin; (c) skin peeled off from the inner surface of the shell; (d) after incubating the inner skin in *E. coli* culture; (e) after incubating the outer shell in *E. coli* culture showing no considerable bacterial adhesion property; (f) the inner skin with microfibers have excellent bacterial adhesion property, which probably ensures the preservation of any food item stored in "Beggar's Bowl"

exposed to different concentrations of the extract. The concentrations are expressed as volume of water added to freshly ground wet pulp. Eight different dilutions, *viz*., ×10, ×50, ×75, ×100, ×150, ×200, ×250 and ×300 were studied by adding 6 healthy zebrafish to 250 ml of the diluted extract kept in a 500 ml glass beaker. Three replicates were performed for each dilution, each containing 6 fish in 250 ml diluted extract. The fish kept in ×10, ×50, ×75 diluted extracts were swimming on the surface and died within 24 hrs of exposure. To understand the reason behind such mortality in concentrated extracts, dissolved oxygen concentration was measured in the extracts as per the procedure explained in the methods section. The results summarized in Table 1 indicate that at high concentrations of the fruit extract, the dissolved oxygen level is below the optimum level prescribed for zebrafish (~7.8 mg/L@28.0°C) [30]. Hence, the mortality of zebrafish at higher concentrations of the fruit extract is attributed to the lower levels of dissolved oxygen. In the other dilutions, the fish were exposed for 15 days by changing the extract with freshly diluted extract every 24 hrs. In all the dilutions from ×100 to ×300, the fish were recognizing the feed and were behaving normally. This indicates that the calabash fruit does not alter the nature of water considerably unlike certain fruits reported earlier [31]. After 15 days exposure, the fish were sacrificed and biochemical analysis was performed for the activity of the enzymes, Acetylcholinesterase and α- as well as β- carboxyl esterases in the brain and liver, respectively (Fig. 3). Acetylcholinesterase is a common indicator for toxicity assays on fish as most of the toxins target the enzyme and inhibit its activity. For example, *Zanthoxylum armatum* DC., a piscicidal plant attacks the fish by inhibiting the enzyme, acetylcholinesterase [32]. On the other hand, α- and β-carboxylesterases present mainly in the liver participate in the phase I metabolism of toxins. In the case of calabash fruit used in this study, no such toxic effects were observed as the acetylcholinesterase activity of fish exposed to the extract was similar to that in the controls, i.e., fish kept in normal water (parameters of water used in this study as control are provided in the supporting information). Similarly, fish exposed to the fruit extract showed no significant decrease or increase in the activity of α- and β-carboxylesterases. Thus, the calabash fruit is considered to be harmless to the zebrafish. For further studies, ×300 dilution is chosen as the optimum concentration as the higher concentrations turned turbid in 20 hrs indicating microbial growth probably due to the inherent sugar content in the pulp.

Exposure of fish to bacteria: Visual observations

The next objective was to study the effect of the fruit extract on the health and behavior of infected zebrafish. The study was performed using three different methods. In one method, to verify whether the fruit extract protects zebrafish from bacterial attack, the fish were directly exposed to ×300 diluted extract containing three different concentrations of *E. coli* (MG1655) added to them, *viz*., 3.5 mg, 7 mg and 35 mg of bacterial biomass in 250 ml extract and the samples were labeled as S1, S2 and S3, respectively. For this set, fish kept in 3.5 mg, 7 mg and 35 mg of bacterial biomass added to 250 ml of water is used as controls and were labeled as G1, G2 and G3, respectively. In another method, to verify whether the calabash fruit can act as an immune-stimulant to improve the immunity of zebrafish and make them resistant/more tolerant to bacterial attack, the fish were first exposed to fruit extracts of different dilution *viz*., ×100, ×200, ×300 and then exposed to 3.5 mg of bacterial biomass per 250 ml of water. These samples were labeled as P1, P2 and P3, respectively. For this set, G1 is used as the control. In the third method, to verify whether the fruit extract aids in faster and/or more effective recuperation of fish infected with bacteria, the fish were exposed first to 3.5 mg, 7 mg and 35 mg of bacterial biomass added to 250 ml of water and then recovered in the fruit extract (300X dilution) and are labeled as R1, R2 and R3, respectively. For this set, fish kept in 3.5 mg, 7 mg and 35 mg of bacterial biomass added to 250 ml of water followed by recovery in sterile water was used as control and were labeled as T1, T2 and T3, respectively.

Table 1: Concentrations of dissolved oxygen for various dilutions of fruit extract. The dissolved oxygen concentration below 5 mg/L causes stress to the fishes and the level should not exceed 110 mg/L as it may result in respiratory disorders like gas bubble disease

Dilutions	Concentration of dissolved oxygen (mg/L)
×10	3.7
×50	6.4
×100	7.7
×200	8.3
×300	10.1

The zebrafish exposed to 3.5 mg (G1), 7 mg (G2) and 35 mg (G3) of bacterial biomass added to 250 ml of water each showed agitated behavior. All fishes in G3 did not recognize the feed and suffered a violent death in 20 minutes of exposure. In G2, the fishes recognized the feed immediately after exposure and survived for 24 hrs, after which mortality was observed. Whereas in G1, all fishes recognized the feed immediately after addition and survived for 72 hrs. For performing assays on metabolic enzyme activity, G1 fishes were sacrificed after

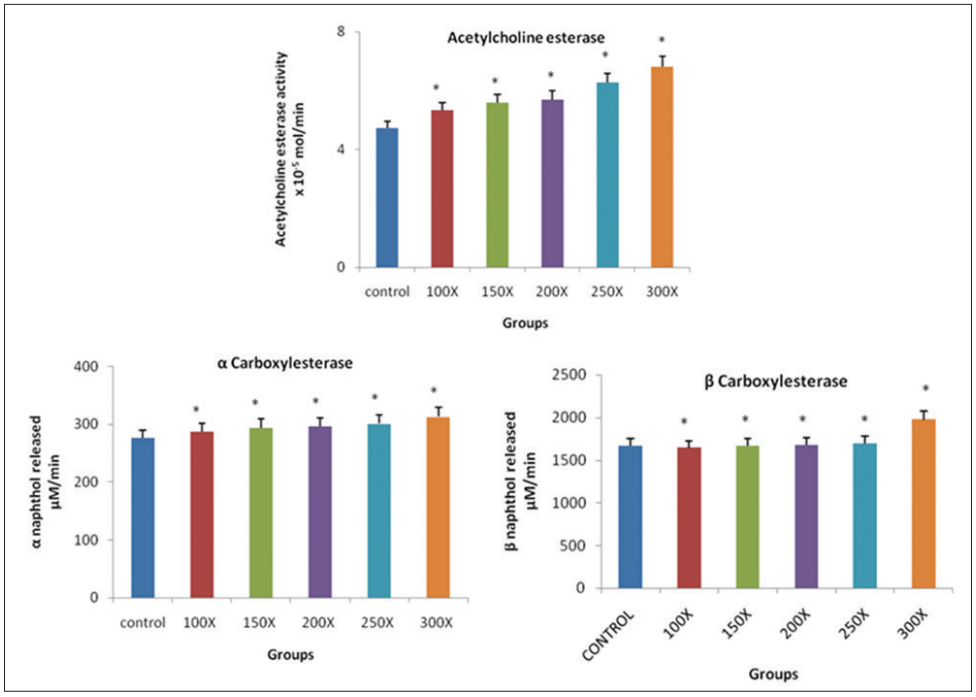


Fig. 3: Effect of different dilutions (×100, ×150, ×200, ×250, ×300) of *Crescentia cujette* L. fruit extract on the enzyme activity of zebrafish after 15 days exposure under ambient conditions (control refers to zebra fish exposed to normal water)

60 hrs of bacterial exposure, G2 after 12 hrs of exposure and G3 after 10 minutes of exposure. Recuperation studies were performed by transferring one set of fishes exposed to 3.5 mg, 7 mg and 35 mg of bacterial biomass in 250 ml water (exposed for 60 hrs, 12 hrs and 10 minutes, respectively) to sterile water (T1, T2, T3) and another set of fishes exposed to 3.5 mg, 7 mg and 35 mg of bacterial biomass in 250 ml water (exposed for 60 hrs, 12 hrs and 10 minutes, respectively) to ×300 diluted calabash fruit extract (R1, R2, R3). The infected fish were then exposed to the extract for 15 days, by changing into fresh extract every 24 hrs. Severe respiration problem was observed in fishes exposed to bacterial culture, which died with a sudden twitch when taken out of the bacterial culture while transferring to the extract. In general, zebrafish are capable of surviving up to 5 minutes after taking out of water and make several twitches. However, the fishes exposed to bacterial culture in this study were unable to withstand even 20 seconds out of water. This clearly shows the effect of bacteria on the respiratory activity and hence the functioning of gills of the zebrafish. Pathogens generally enter the fish through gills, skin and gastrointestinal tract [33,34]. In another report, gill-breathing fish were found to be more susceptible to toxins in water than air-breathing fish [32]. In comparison to these reports, the effect of bacterial toxicity on respiratory activity of zebrafish is obvious in the present case. Precautions were taken to prevent mortality of the fishes during transfer. The infected fish transferred to sterile water remained agitated and did not recognize the feed in all the three samples, *viz*., T1, T2 and T3. In samples T1 and T2 they started recognizing the feed after 24 hrs and 36 hrs, respectively, whereas in T3, the fish did not seem to have considerable appetite for 3 days. Mortality was observed in sample T2 after 10 days and in sample T3 after 7 days. Only two out of six fish were alive in T3 and three out of six fish were alive in T2. A dark green spot was observed in the abdomen of dead fish of sample T2 and near the gills of sample T3. In T1, all fish were alive over a period of 15-day of recovery in sterile water, and there was no green pigmentation anywhere in the body of the fish. Another set of fish exposed to bacterial culture, in the same manner, explained above were transferred to the fruit extract (×300 dilution) and were labeled as samples R1, R2 and R3. Surprisingly, fish transferred to extract were far less agitated and those in samples R1 and R2 recognized the feed immediately. Thus a significant improvement in fish behavior is observed between fish recovered in the fruit extract and those recovered in sterile water. Also in sample R3, stepwise recovery of one of the fish with respiratory problem in the extract was observed (probably due to bacterial attack on the gills) while the other two fish were healthy and all of them started recognizing the feed in 24 hrs.

After realizing the positive effect of the fruit extract on recuperation of infected zebrafish, the possibility of using the extract as immune-stimulant was verified as follows. In this part of study, the fish was first exposed to the fruit extract for 13 days and then transferred to the bacterial culture containing 3.5 mg (P1), 7 mg (P2) and 35 mg (P3) bacterial biomass per 250 ml of water. Fish transferred from normal water to bacterial culture (G1, G2, G3) were used as controls for this set of experiments. The fish pre-treated with fruit extract were less agitated after transferring to 3.5 mg/250 ml and 7 mg/250 ml bacterial culture compared to untreated fish, G1 and G2. However, the behavior of P3 and G3 fish seemed to be similar. In addition, the extract-treated fish, P1 and P2, exhibited lower mortality than the untreated fish indicating the positive role of the fruit extract. Interestingly, respiration problem observed in G1 and G2 was absent in P1 and P2. On the contrary, simultaneous exposure of zebrafish to 250 ml of fruit extract (×300 diluted) containing 3.5 mg (S1), 7 mg (S2) and 35 mg (S3) bacterial biomass yielded only negative results. In this set, all the fish remained agitated and did not recognize the feed. Fish in all the three samples, S1, S2 and S3 died within 24 hrs. This could be due to the sudden exposure of fish to two foreign substances, *viz*., the fruit extract and the bacteria simultaneously which did not allow them to adapt themselves. These results show that the fruit extract is more appropriate for treating infected fish and as a preventive "medicine."

Exposure of fish to bacteria: Biochemical assays

Biochemical assays were performed to assess the activity of metabolic enzymes, *viz*., acetylcholinesterase, α- and β-carboxylesterase in the brain and liver tissues of sacrificed fish, respectively. In all the three assays, fish recovered (recuperation) in fruit extract after bacterial exposure showed activity close to the values obtained for control samples, especially when the bacterial concentration was 3.5 mg and 7 mg/250 ml water, *viz*., R1 and R2. This supports the visual observation on the feeding behavior and physical activity of the fish explained in the previous section. More specifically, acetylcholinesterase activity increases from R1 to R3

and T1 to T3, respectively, with increasing concentration of bacteria compared to the control samples G indicating increasing levels of neurotoxicity. The direct relationship observed between neurotoxicity and concentration of bacteria implies bacterial attack in the brain tissue of the fish. However, no trend is observed in the case of enzymes present in the liver tissue of the sacrificed fish, *viz*., α- and β-carboxylesterase in samples R1, R2, R3, T1, T2 and T3. On comparing, the enzyme activities of fish recovered in fruit extract with those recovered in water; it is difficult to arrive at a conclusion based on the toxicity graphs presented in Fig. 4 since the trend is not clear. Difficulty could be arising due to the enzymes inherently present in the bacteria *E. coli* MG1655 which themselves could be interfering with the biochemical assays done on the enzymes present in the fish [35,36]. To verify this aspect, enzyme activity assays were performed on viable bacterial pellets prepared using centrifugation of the liquid bacterial culture. In accordance with literature reports, the bacterial pellet showed significant activity for all the three enzymes *viz.,* acetylcholinesterase, α- and β-carboxylesterase (supporting information). Thus, the biochemical assays performed on sacrificed fish tissues could have been affected by similar enzymes present in the bacteria accumulated in the tissues. Nevertheless, fish recovered in fruit extract were healthier than those recovered in water as evident from their feeding behavior, respiration and physical activity discussed in the previous section.

Biochemical assays were also performed on multiple replicates of fish pre-treated with calabash extracts of different dilutions, *viz*., ×100, ×200 and ×300, followed by exposure to *E. coli* MG1655 strain (3.5 mg per 250 ml of water) (samples P1, P2 and P3). For these pre-treated samples, fish transferred from water to bacteria-contaminated water with different levels of bacteria, *viz*., 3.5, 7 and 35 mg per 250 ml water, were used as controls (samples G1, G2 and G3). In both the sets, *viz*., G1, G2, G3 and P1, P2, P3, there was no significant difference or trend in acetylcholinesterase activity with respect to bacterial concentration unlike that observed in the case of recuperation studies discussed above. However, the activity of α-carboxyl esterase enzyme increased with bacterial concentration in the case of control samples, G1, G2 and G3. Furthermre, the fruit extract samples P1, P2 and P3, show almost equal activity, indicating that the concentration of fruit extract does not influence the toxicity. Whereas, biochemical assays performed on fish simultaneously exposed to the fruit extract and bacteria indicated high levels of neuro- as well as hepatotoxicity in accordance with the observations discussed in the above section.

On comparing toxicity levels in fish samples pre-treated with fruit extract followed by bacterial exposure with those post-treated (recuperated) with fruit extract following bacterial extract, the latter approach was found to be effective based on the toxicity graphs as well as visual observations recorded during the exposure. Especially, comparison of samples G1 and R1 in all the three toxicity graphs indicates that calabash extract helps in reducing the neurotoxicity exerted by the bacteria on zebrafish when the infected fish (G1) are post-treated with the extract (R1) though no significant advantage was observed on hepatotoxicity. Whereas, bacterial exposure of fish already pre-treated with the fruit extract (P1, P2, P3) is found to cause

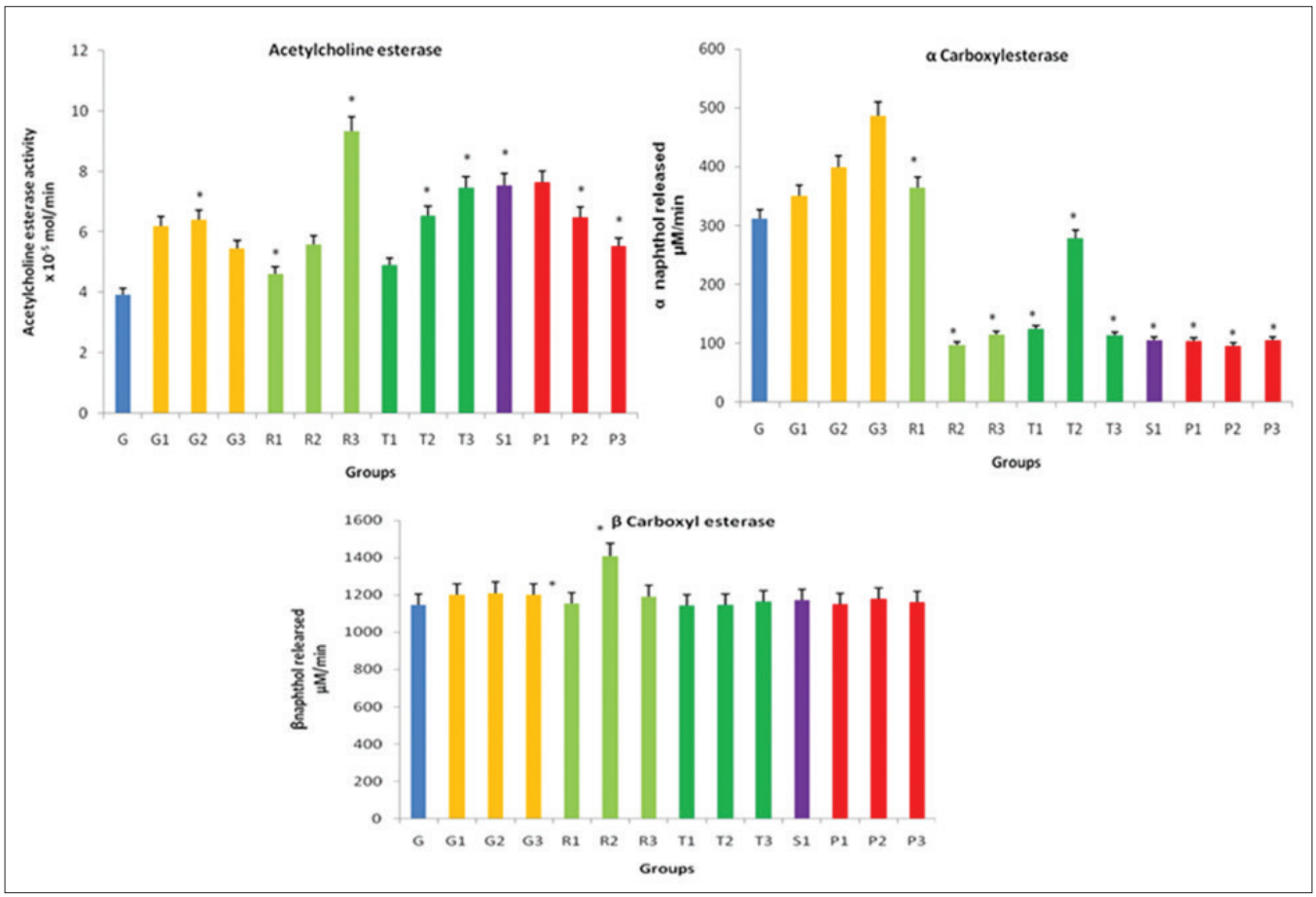


Fig. 4: Assay of metabolic enzyme activity in zebrafish after 15 days exposure to (G) sterile tap water control, (G1, G2, G3) sterile tap water followed by exposure to water containing 3.5 mg, 7 mg and 35 mg *Escherichia coli* per 250 ml, respectively, (P1, P2, P3) ×300 diluted fruit extract followed by exposure to tap water containing 3.5 mg, 7 mg and 35 mg *E. coli* per 250 ml, respectively, (T1, T2, T3) tap water containing 3.5 mg, 7 mg and 35 mg *E. coli* per 250 ml, respectively, followed by recovery in sterile tap water, (R1, R2, R3) tap water containing 3.5 mg, 7 mg and 35 mg *E. coli* per 250 ml, respectively, followed by recovery in ×300 diluted fruit extract, (S1) 250 ml of ×300 diluted fruit extract containing 3.5 mg *E. coli* biomass

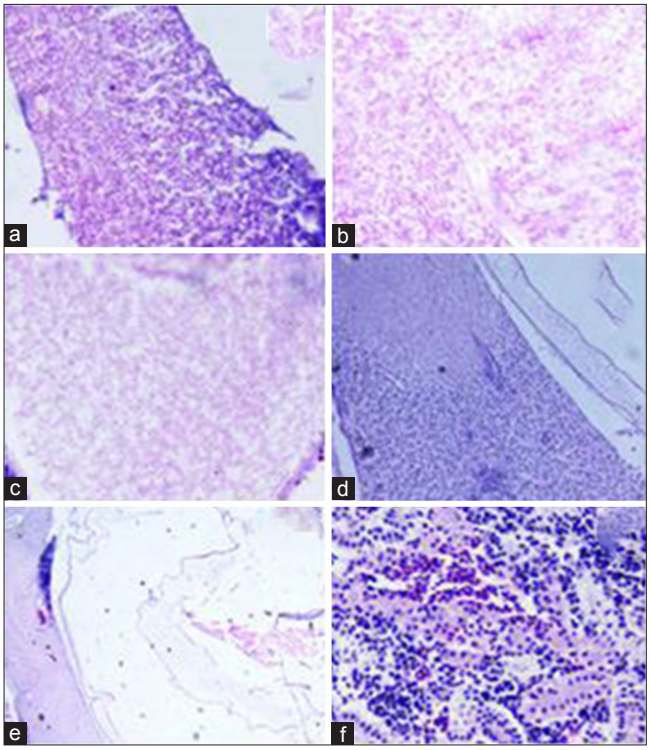


Fig. 5: Histological sections of organs of zebrafish exposed to fruit extract: Liver of samples (a) R1; (b) T1; (c) R2; (d) R3; swim bladder of T2 (e); kidney of T1 (f)

even more toxic effect on the fish as compared to the damage caused by direct bacterial exposure itself. The fruit extract, though non-toxic, is a non-natural environment for the fish, which could, in turn, affect their immune system. Natural and artificial environmental changes are known to induce stress in fish and affect their immune system [37]. Hence, fish previously exposed to calabash fruit extract in the present study are expected to have altered immune mechanism due to stress already existing to the non-natural environment provided by the extract. It should also be noted that the toxicity caused by bacterial infection in the fish is almost independent of the concentration of fruit extract used for pre-treatment of the fish. This further augments the interpretation that the toxic effect is not due to the fruit extract itself but is an indirect stress effect on the native immune system of the fish.

Histopathological examination

All the fish samples were fixed in formalin for 2 days before sectioning. The sections were then stained and examined under a light microscope. The stained sections revealed several interesting insights into the level of tissue damage and accumulation of microbes in the fish organs. Infection of zebrafish with *E. coli* was found to cause significant damage to the liver tissue. More specifically, in fish exposed to 3.5 mg bacterial biomass per 250 ml of water (G1), there was liver cell hyperplasia and necrosis due to microbes present in the liver. In sample G2 (7 mg/250 ml bacterial biomass), the kidneys appeared to be inflamed and necrosis was observed. In sample G3 (35 mg/250 ml bacterial biomass) severe inflammation was observed in the spleen, kidney, and liver. The observed results are in accordance with liver inflammation reported in zebrafish arising due to bacterial infection [38]. In accordance with the toxicity assay results discussed in the previous section, fish pre-treated with the fruit extract are even more affected by bacterial infection than those grown in water. Specifically, liver inflammation and necrosis were observed in the case of fish samples, P1 and P2, whereas kidney inflammation and microbial accumulation in swim bladder were observed in addition to liver necrosis in sample P3. Microbial accumulation in organs of fish in bacterial contaminated water bodies has been a controversial research topic. For example, *E. coli* has been found to be accumulated in the blowhole, anus, and skin of Belukha whales (*Delphinapterus leucas*) at the Mystic Marine Life Aquarium (Mystic, CT). Nevertheless, another report states that *E. coli* accumulation in fish organs occurs mainly through intake of bacteria-contaminated food by the fish and not directly from bacteria-contaminated water [39]. However, this study clearly shows the presence of viable bacteria in different organs of zebrafish such as swim bladder and gills.

On the other hand, the above pathologic effects were not observed in the case of fish recovered in the fruit extract after bacterial exposure. For instance, in R1 and R2 samples, the liver as well as the gills and kidney were found to be normal. However, in sample R3 though the intestine was normal, lot of mucus threads were observed in addition to mild necrosis in the liver (Fig. 5), which could be attributed to the higher dose of bacterial biomass added (35 mg/250 ml water). However, the situation was opposite in the case of fish recovered in water after bacterial exposure, T1, T2 and T3. In sample T1, there was severe liver necrosis, intestinal inflammation, and lot of infiltrating cells were observed in the kidney. In sample T2, there was liver and kidney inflammation and necrosis in addition to lot of microbes accumulated in the swim bladder. A bright green spot indicative of bacterial infection was also observed in the abdomen of fish sample T1 and near the gills of fish sample T2 (photo in supporting information). In T3, there was excessive liver inflammation and loss of cells. Thus it is clearly evident that zebrafish can be effectively recovered from bacterial infection using calabash pulp which could be attributed to its high thiamine content.

Thus, the ability of *C. cujete* (calabash) in treating zebrafish infected by bacteria has been demonstrated in this work. The fruit extract is found to be biocompatible as evident from similar behavior of zebrafish in normal water and water containing dissolved fruit extract. The fish suffered from severe loss of appetite, agitation, and troubled breathing after exposing to water contaminated with *E. coli*. The infected fish were found to successfully recover in calabash extract but not in sterile water. Furthermore, fish pre-exposed to fruit extract were found to have better immunity toward bacterial infection than those grown in normal water. The results were supported by biochemical assays, histopathological studies and visual observation of fish behavior. In essence, the results indicate that calabash fruit extract can be used as a preventive/recuperative medicine to treat fish with bacterial infection in various water bodies.

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Research Article

ANTICANCER EFFICACY OF A POLYHERBAL SIDDHA FORMULATION AGAINST A549-HUMAN LUNG CARCINOMA CELLS

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ABSTRACT

Objective: One of the strong myths about siddha medicine among the lay public and experts in the field of medicine is that chronic and often debilitating diseases such as cancer, diabetes, and arthritis are always managed by siddha drugs having high metals, minerals, and arsenical compounds. On the contrary, even a single herb exhibits a high level of anticancer efficacy. Our aim and Objective is to evaluate the Invitro Anticancer efficacy of a Polyherbal formulation against A549 – Human Lung Carcinoma cells and to prove its potentency.

Method: We have evaluated scientifically a poly herbal formulation for its efficacy in lung carcinoma through In vitro cell line studies. Initially SOP was carried out and subjected to in-vitro anticancer cell viability through MTT Assay Method followed by Cyto-toxicity profile were collected.

Results: The results are encouraging even at the lower concentration level. At the concentration of 6.25 µg/ml, the test drug kills nearly about 40% of cancer cells.

Conclusion: Review of Siddha literature on the ingredients of the selected poly herbal formulation gives us a clear insight of its safety, efficacy, and cost-effectiveness. This is only a preliminary study and needs further in depth studies to explore the anticancerous properties of the selected poly herbal formulation.

Keywords: Lung cancer, Siddha oncology, A549 lung carcinoma cell lines, Siddha anticancer herbs.

INTRODUCTION

Cancers are one of the leading causes of death among humans worldwide and their relative importance, and their awareness is also gradually increasing. Since an increasing proportion of cancer patients are acquiring resistance to current chemotherapeutic agents, it is necessary to search for new novel compounds that provide suitable specific antiproliferative affects which can be developed as anticancer agents.

Lung cancer is ranked as first in causing death followed by breast and colorectal cancer worldwide. About 58% of lung cancer cases occurred in less developed countries. The major attributable cause of lung cancer is to smoking habit. It contributes about 80-90% of lung cancer deaths among women and men, respectively. The extreme dangerous exposure to secondhand smoke incidence and mortality also increasing among nonsmokers every year. It is also caused by occupational exposures, including asbestos, uranium, etc. The combination of asbestos exposure and smoking greatly increases the risk of developing lung cancer.

India is sitting on a gold mine of well recorded and traditionally well-practiced knowledge of herbal medicines such as siddha and Ayurveda. The medicinal plants [1], besides having natural therapeutic values against various diseases are also highly efficacious and less produce adverse effects. The poly coded herbal formulation is mainly composed of herbs, which are formulated by the expert committee of Walters siddha research center (WSRC), Tirunelveli [2,3]. The same poly coded formula was formulated based on rich anticancer and cytotoxic activity of the ingredients and prepared following standard operative procedure [1,4,5]. Prepared formulation was subjected to *in vitro* cancer cell viability assay in human lung cancer cell lines.

METHODS

Plant material

The plant was identified and authenticated by the botanical experts from WSRC, Tirunelveli. The fresh leaves were separated from the plant and allowed to dry on shade. Finally, powdered leaves were sieved by traditional process *"Vasthirakayam"* [1,6,7]

Preparation of leaf extracts

By using soxhlet extractor, the compounds were extracted at a temperature 45°C. The extracts were concentrated and dried using rotary evaporator and were stored in a refrigerator at 4°C.

Cell lines and culture conditions

A549 (lung carcinoma) cells were initially procured from National Centre for Cell Sciences, Pune, India, and maintained in Dulbecco's modified Eagles medium (DMEM) (Gibco, Invitrogen).

Cell lines were cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% fetal bovine serum, L-glutamine, sodium bicarbonate, and antibiotic solution containing: Penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

Cancer cell viability studies

Cancer cell viability activity of poly herbal extracted was studied. The viability of cells was evaluated by direct observation of cells through inverted phase contrast microscope and followed by modified tertrozalium salt (MTT) assay method. [8]

Cells seeding in 96 well plate

About 2 days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100 µl cell suspension (5×10^4 cells/well) was seeded in 96 well tissue culture plate, and incubated at 37°C in a humidified 5% CO_2 incubator.

Preparation of plant extracts and compound stock

About 1 mg of each plant extract or compound was added to 1ml of DMEM and dissolved completely by cyclomixer. After that, the extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility [9].

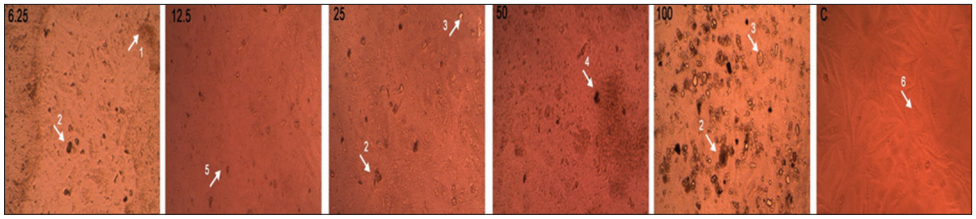

Fig. 1: Phase contrast images of morphological changes of cells. (1) Dead cells clumbed, (2) apoptotic bodies, (3) cell shrinkage, (4) membrane bleeding, (5) condensed nuclei, (6) control cell

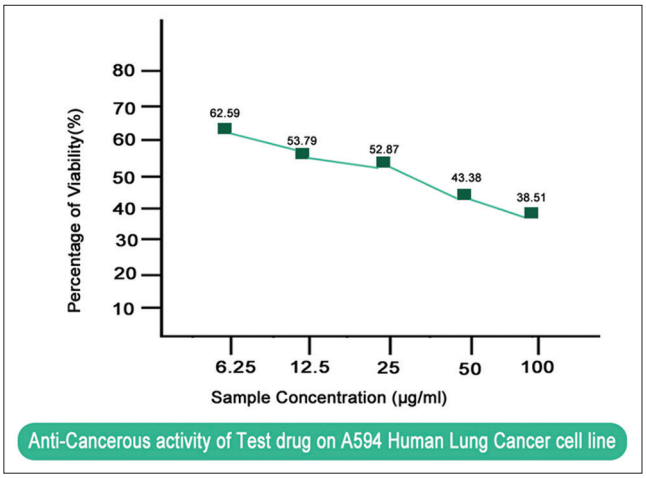

Fig. 2: Graphical representation of % viability of cancer cells

Table 1: Cancer cell viability of test drug in different concentrations

Serial number	Sample concentration (µg/ml)	Average OD at 540 nm	Percentage viability
1	Control	0.4764	
2	6.25	0.2982	62.59446
3	12.5	0.2563	53.79933
4	25	0.2519	52.87573
5	50	0.2067	43.38791
6	100	0.1835	38.51805

LD 50 value - 39.78 µg/ml

Cytotoxicity evaluation

After 24 hrs the growth medium was removed, freshly prepared each plant extracts in 5% DMEM were 5 times serially diluted by two-fold dilution (100 µg, 50 µg, 25 µg, 12.5 µg, and 6.25 µg in 100 µl of 5% MEM) and each concentration of 100 µl was added in triplicates to the respective wells and incubated at 37°C in a humidified 5% CO_2 incubator [10].

Cytotoxicity assay by direct microscopic observations

The entire plate was observed at an interval of each 24 hrs; up to 72 hrs in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro 5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity (Fig. 1).

Cytotoxicity assay by MTT method

About 15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml phosphate-buffered saline until completely dissolved and sterilized by filter sterilization. After 24 hrs of incubation period, the sample content in wells was removed and 30 µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5% CO_2 incubator for 4 hrs. After the incubation period, the supernatant was removed and 100 µl of MTT solubilization solution dimethyl sulfoxide ([DMSO] was added, and the wells were mixed gently by pipetting up and down to solubilize the formazan crystals. The absorbance values were measured using microplate reader at a wavelength of 570 nm.

The percentage of growth inhibition was calculated using the formula:

(% Viability = (OD [doctor of optometry] of test/OD of control)×100)

RESULTS AND DISCUSSION

The morphology changes like granulations and vacuolization of the cells were noted carefully through microscope. After the procedure, it was subjected to MTT assay to calculate the cytotoxicity (Table 1). Traditional siddha medicine has received increasing attention over the last few decades due to their potential as novel cancer preventive and therapeutic agents. In parallel, there is increasing evidence for the potential of plant-derived compounds as inhibitors of various stages of tumorogenesis, associated inflammatory processes, and oxidant scavenging.[11-14]

In the five different concentrations, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml, the test drug killed significant number of cancer cells and their percentage viability was at the rate of 62.59, 53.79, 52.87, 43.38, and 38.51, respectively (Table 1). However, the minimum viability of 38.51% was achieved at the higher concentration. The least concentration is 6.25 g/ml, where half of the proliferating cells were. From the data, it is observed that the test drug inhibits cell proliferation even at the lower concentration and gradually increases inhibition at higher dose levels (Fig. 2).

CONCLUSION

From the above results, it is evident that traditional siddha medicine can be useful in the management of cancer, besides being safe, cost-effective, and non-invasive. More researches on the herbal based siddha formulations could be taken up to explore and evaluate them scientifically for the betterment of cancer population.

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Research Article

EVALUATION OF ANTICANCER ACTIVITY OF CLASSICAL SIDDHA HERBOMINERAL PREPARATION SIVA GURU KULIGAI BY *IN VITRO* ASSAY

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ABSTRACT

Objective: The main objective of this work is to assess the anti - cancer activity of Siva Guru Kuligai.

Method: The anticancer activity of test drug was evaluated in HeLa cell line by MTT assay.

Result: The percentage of inhibition was found to be increasing with increasing concentration. The highest percentage of inhibition (94.02%) was obtained at the concentration of 1000 µg/ml.

Conclusion: The results suggested that the SGK significantly inhibited the proliferation of human cervical cancer HeLa cells.

Keywords: Siddha drug, Siva guru kuligai, *In vitro* HeLa cell line, Anticancer activity.

BACKGROUND

The truth is that throughout life we seem to swim in a sea of carcinogens and it is more by good fortune than by good management that some of us escape to die from other causes.

- William Boyd

As Boyd said, in this modern world, the air we breathe the food we eat and our life styles are all carcinogenic and are ultimately leading us by one way or other to dreadful disease cancer.

Everyone in this world, rich or poor, men or woman, young or old, and even animals are prone to get affected by this disease even when the vaccines against this disease were administered previously as a prophylactic measure. There is no medicine available in the world that would completely cure this disease.

Currently available treatments for cancer, viz., radiation, chemotherapy, surgery are all of very limited value. The surgical procedures are useful only in very early stage and it is miserable that the disease is often detected in advanced stages. The radiation technique and chemotherapy are useful only to prolong the survival period.

In Siddha system of medicine, there are medicines with the indication to be used to cure cancer.

The author Abdulla Shakibu in his work said that the mercurial Siddha drug Pooram is compared to Sanjeevi as it cures many in-curable diseases such as kiranthi (syphilis), allgul putru (uterine cancer), allgul ranam (uterine ulcer), and chronic nonhealing ulcers.

As per the reference, there is a herbomineral drug called Siva guru kuligai (SGK) containing Pooram which is prescribed in Siddha classical literature to treat uterine cancer within 7 days [1].

The author selected and traditionally prepared the test drug SGK and evaluated its anticancer activity through *in vitro* method.

METHODS

Rasa karpooram (mercury subchloride)

In siddha system of medicine Rasa karpooram (mercury subchloride) is used as an important medicine to cure many incurable diseases for many decades. It has not found a place in the list of 64 padanas but is considered one among them by the medical practitioners. It is prepared by the combination of rasam (mercury) and salt.

Method of preparation

"தானென்ற கற்பூரமொன்று சொல்வேன்
சாதகமாய்சூதம்ரெண்டு தூக்கி
வானென்ற சட்டிக்குள்மூன்று படியுப்பை
வளமாக பொடித்திட்டு நடுவே கேளு
தேனென்ற செங்கல்தூள்கால்படி தானிட்டு
திரமாகக்குளித்ததிலே சூதம்விட்டு
ஏனென்ற மறுசட்டி கவிழ்த்து மூடி
இயல்பாக வெழுசீலை மண்ணும்செய்யே
மண்செய்து தொண்ணூறு கடிகையப்பா
வாகாக மூத்தீயுமெரித்து மைந்தா
மண்செய்த மேல்சட்டிக்குள்ளே கேளு
வாகாக உரைத்திருக்கும்வெள்ளைமெத
மண்செய்த நற்பூரங்குழாயில்வைத்து
வாகாக பணவிடை தான்தூக்கிwக்கொண்டு

- Agasthiyar paripuranam 400[2]

Sulfur 67.2 g is melted in mud pot and mercury 336 g is added to it and ground well and there forms a black color powder (kajali). Brick stone powder is placed up to half of the level of a pot culinary salt (NaCl) 650 g is placed over it. Mercury sulfur camphor (kajali) is placed over the salt and sealed with mud paste cloth. It is burnt for 12 hrs with kadackini after it is cooled and the mercury subchloride (Hg_2Cl_2) found deposited on the upper pot is collected.

This method of preparation is selected from old classical siddha literature Agasthiyar paripuranam - 400, is in usage for more than 1000 years in siddha system of medicine. The same scientific formulation is in use to prepare mercury sub chloride in chemical labs, too. That is:

$HgSO_4 + 2NaCl + Hg \rightarrow Hg_2Cl_2 + Na_2SO_4$

Hg_2Cl_2 - Subliming mixture of mercuric sulfate, mercury, and sodium chloride heated in iron pot[4].

Purification of rasa karpooram

The poultice made of betel leaf (*Piper betel*) and pepper (*Piper nigrum*) each 8.75 g is taken and dissolved in 1.3 L of water. Calomel 35 g is tied in a cloth and immersed in the liquid from the cross bar and heated. After the water is reduced to ¾ of its volume, the calomel is taken out; washed with water and dried to get it in purified form[5].

Purified rasa karpooram is then traditionally prepared as SGK based on classical siddha literature to evaluate the anticancer activity by *in vitro* method.

Determination of apoptotic properties

Chemicals

3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and trypsin were obtained from Sigma Aldrich Co., St. Louis, USA. Ethylenediaminetetraacetic acid (EDTA), glucose and antibiotics from Hi Media Laboratories Ltd., Mumbai. Dimethyl sulfoxide and propanol from E. Merck Ltd., Mumbai, India, 4',6-diamidino-2-phenylindole (DAPI) from Hi Media, Mumbai.

Cell lines and culture medium

HeLa cell lines were procured from the National Centre for Cell Sciences, Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtiter plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of test samples

For cytotoxicity studies, weighed test drug was separately dissolved in DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT assay

Principle

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt MTT into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used [3].

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM containing 10% FBS. Later to each well of the 96 well microtiter plate, polymer were placed in triplicates, later 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 4 hrs, to each well 150 µl of DMEM supplemented with 2% FBS was added to the wells. The plate was then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 hrs interval. After 72 hrs, the drug solutions in the wells were discarded and plate was washed with PBS and then media containing MTT (5 mg/ml) was added each well. The plates were gently shaken and incubated for 3 hrs at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The solubilized formazan was then transferred into clean plate and absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \right)$$

Determination of apoptotic property by DAPI staining

To characterize cell-specific apoptotic process in HeLa cells, analysis of chromatin condensation and nuclear fragmentation was done by DAPI staining using fluorescence microscopy. After treatment of 70-80% confluent HELA cells with varying concentration of sample 1 for 24 hrs, cells were quickly washed with ice-cold PBS and fixed in ice-chilled acetone: Methanol (1:1) mixture for 10 minutes at 4°C in the dark. The cells were washed twice with ice-cold PBS and then incubated for 30 minutes with the DNA-specific fluorochrome, DAPI. The excess DAPI was removed with ice-cold PBS wash, and the cells were observed and photographed using fluorescence microscope at ×40 magnification.

RESULTS AND DISCUSSIONS

Result

Inappropriate apoptosis (either too little or too much) is a factor in many human ailment including neurodegenerative diseases, ischemic damage, autoimmune disorders, and many types of cancer. The ability to modulate the life or death of a cell is studied to evaluate scientifically therapeutic potential of the test drug against HeLa cell line.

Cytotoxicity activity

In this study, cytotoxicity study was carried out for a herbomineral drug SGK against HeLa cell line. The extract was screened for its cytotoxicity against HeLa cell lines at different concentrations to determine the CTC_{50} (50% growth inhibition) by MTT assay. Results are tabulated in Table 1 and graphically represented in Fig. 1. The percentage growth inhibition was found to be increasing with different concentration of test drug SGK. As the concentration increased from 62.5 to 1000 µg/ml, percentage of inhibition increased from 6.32% to 94.02%. The CTC_{50}

Table 1: Cytotoxic effect of SGK against HeLa cell line

S.No.	Name of test sample	Test concentrated (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	SGK	62.5	6.32±1.3	164.77±1.6
		125	40.12±0.2	
		250	66.84±1.0	
		500	93.22±0.1	
		1000	94.02±0.2	

SGK: Siva guru kuligai

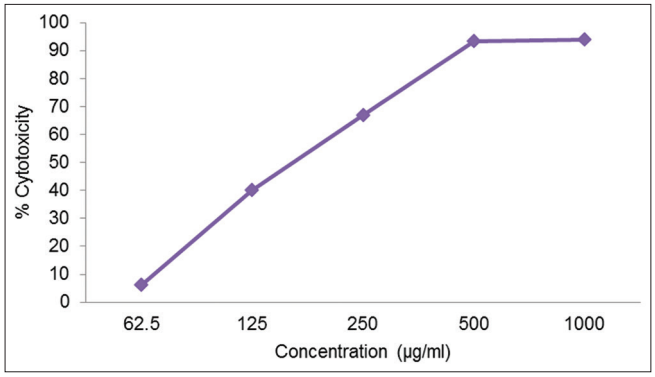

Fig. 1: Graphical representation of cytotoxic effect of Siva guru kuligai against HeLa cell line

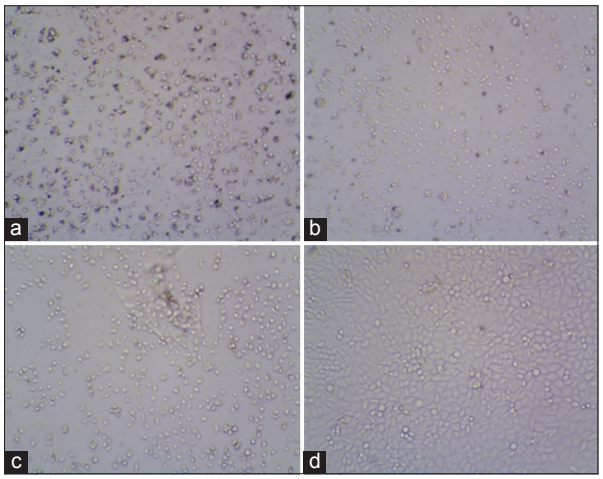

Fig. 2: Histopathological observation: (a) 125 mg, (b) 250 mg, (c) 500 mg, (d) 1000 mg

value on HeLa cell line was 164.77 µg/ml. The highest percentage of inhibition (94.02%) was obtained at the concentration of 1000 µg/ml (Fig. 2). The results suggested that the SGK inhibited the proliferation of human cervical cancer HeLa cells. Further studies are needed to explore the mechanism of action.

CONCLUSION

Present work is a preliminary study to evaluate the cytotoxic efficacy of the Siddha herbomineral preparation against the HeLa cell line.

Present management of cancer with a holistic approach, devoid of any side effects is the major challenge for medical workers. This work highlights SGK as novel anticancer drug that could provide a cost-effective and holistic remedy for the treatment of cervical cancer.

In future, the drug is to be validated scientifically to prove its efficacy and nontoxic nature. Mechanism of drug action needs also to be evaluated.

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Research Article

SUB-ACUTE TOXICITY STUDIES ON A CLASSICAL SIDDHA DRUG KANTHAKAATHI CHOORANAM

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ABSTRACT

Objective: The main objective of this work is to assess the safety of the drug Kanthakaathi chooranam (KKC) by sub-acute toxicity studies.

Methods: A total of 40 Wistar rats were randomly divided into four groups. A control and three experimental groups were fed with distilled water and test drug at different concentrations for 28 consecutive days.

Conclusion: The results of the sub-acute toxicity studies revealed that the drug "KKC" can be considered safe, as it did not cause either any lethality or adverse changes to the rats.

Keywords: Kanthakaathi chooranam, OECD 407, Sub-acute toxicity, Lethality.

BACKGROUND

The Siddha system of medicine is one of the ancient systems. The unique nature of this system is its continuous service to humanity for more than 5000 years in combating diseases [1]. The Siddha medicines contain many minerals and metals. If the medicine is prepared by unpurified raw drugs or given in excess dosage, it is sure to cause some serious adverse effects to the body [2]. Hence, preparation of the internal medicines requires high skilled work called *suddhi* (purification) [3]. Hence, the purification process of siddha medicine needs to be standardized [4]. For the global acceptance of siddha system, certain basic studies like toxicological studies are necessary [5]. Hence, the author has taken the herbomineral preparation Kanthakaathi chooranam (KKC) which contains sulfur as one of the raw drugs [6]. The medicine KKC is used in the treatment of cervical cancer, fistula, and parkinsonism since many decades without proper scientific validation [7,8].

From the data based on the study (OECD 407 Guidelines), it was observed that the administration of KKC at a dose of 300-900 mg/kg to the rats did not produce drug-related toxicity and mortality for 28 days, which will be discussed in detail in this study.

METHODS

The objective of this "sub-acute toxicity study of KKC on Wistar rats was to assess the toxicological profile of the selected preparation when administered as a single dose daily. Animals were observed for 28 days after the drug administration. The study was conducted on 5 male and 5 female Wistar rats for each group [9]. Four groups were selected.
Group I: Control (distilled water)
Group II: Low dose (300 mg/kg)
Group III: Middle dose (600 mg/kg)
Group IV: High dose (900 mg/kg).

Test guideline followed

OECD 407 guidelines - sub-acute toxic class method (repeated dose 28-day oral toxicity study in rodents).

Test system details

The study was conducted on 5 male and 5 female Wistar rats in each group. These animals were selected for oral studies as per OECD guidelines and are also easily procurable. 8-12 week-old male and female rats were selected after physical and behavioral examination. The body weight of the animals was between 80 g and 120 g. The body weight range was fallen within ±20% of the mean body weight at the time of randomization and grouping. The rats were housed in standard laboratory condition in polypropylene cages, provided with food and water *ad libitum* in the animal at M/s. Sree Venkateswara Enterprises Pvt. Ltd., Bengaluru. The experimental protocol was approved by the Institutional Animal Ethical Committee as per the Guidance of Committee for the purpose of control and supervision of experiments on animals, Ministry of Environment and Forest, Government of India.

Acclimatization

The animals were selected after veterinary examination by the veterinarian. All the selected animals were kept under acclimatization for a week.

Randomization and grouping

About 1 day before the initiation of treatment (day 0 - last day of acclimatization), the selected animals were randomly grouped into three different groups containing minimum 5 male and 5 female animals per group.

Numbering and identification

The animals were marked on the body with picric acid solution prepared in water. The marking within the cage was as below:

Numbering and identification

Group number	Animal marking
Control (distilled water)	H, B, T, HB, NM (male)
	H, B, T, HB, NM (female)
Low dose of Kanthakaathi	H, B, T, HB, NM (male)
chooranam (300 mg/kg)	H, B, T, HB, NM (female)
Middle dose of Kanthakaathi	H, B, T, HB, NM (male)
chooranam (600 mg/kg)	H, B, T, HB, NM (female)
High dose of Kanthakaathi	H, B, T, HB, NM (male)
Chooranam (900 mg/kg)	H, B, T, HB, NM (female)

Husbandry

Housing

The Wistar rats were housed in standard polypropylene cages with stainless steel top grill. Paddy husk was used as bedding. The paddy

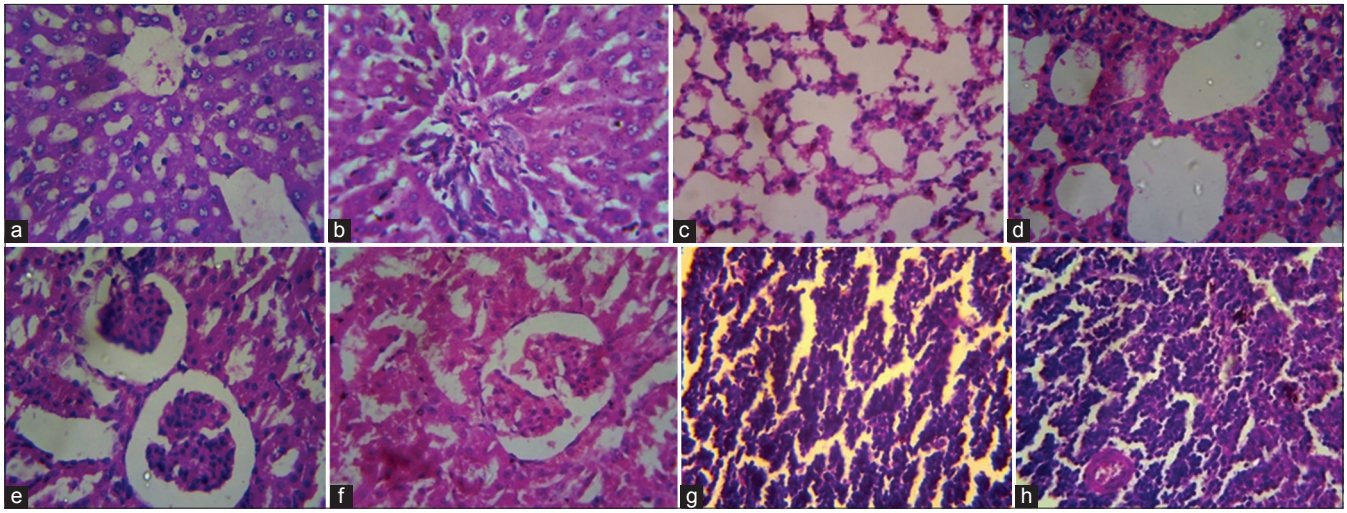


Fig. 1: Histopathological observations. (a) ×40 shows normal hepatocytes and central veinliver (control), (b) ×40 shows normal portal tract liver (high dose - 900 mg/kg), (c) ×40 shows mild normal alveoli lungs (control), (d) ×40 shows normal alveoli lungs (high dose - 900 mg/kg), (e) ×40 shows normal glomeruli kidney (control), (f) ×40 shows normal glomeruli kidney (high dose - 900 mg/kg), (g) ×40 shows normal white pulp and red pulp spleen (control), (h) ×40 shows normal white pulp and red pulp spleen (high dose - 900 mg/kg)

husk was changed at least twice a week. From the week before initiation of the treatment, each cage contained a maximum of 10 rats of the different sex and treatment group.

Environmental conditions
The animals were kept in a clean environment with 12 hrs light and 12 hrs dark cycles. The air was conditioned at 22±3°C and the relative humidity was maintained between 30% and 70% with 100% exhaust facility. The cages corresponding to each experimental group were distributed on racks in such a manner that external factors, such as environmental conditions, were balanced as far as possible.

Feed and feeding schedule
Feed was purchased from "Sai Durga Animal Feed," Bengaluru. Feed was provided *ad libitum* throughout the study period, except overnight fasting (18-20 hrs) before dose administration. After the substance has been administered, food was withheld for further 3-4 hrs.

Water
The water was offered *ad libitum* in bottles. Water was periodically analyzed to detect the presence of possible contaminants.

Doses
The doses for the study were selected based on literature search and range finding study. Following the period of fasting, the animals were weighed and then extract was administered orally as single dose using a needle fitted on to a disposable syringe of approximate size.

Dose preparation
KKC was added in distilled water and completely dissolved for oral administration. The dose was prepared of a required concentration before dosing by dissolving KKC in distilled water. It was mixed well. The preparation for different doses was varied in concentrations to allow a constant dosage volume.

Administration
The test drug was administered orally to each rat as single dose using a needle fitted on to a disposable syringe of appropriate size. The concentration was adjusted according to its body weight. The volume was not exceeding 10 ml/kg body weight. Variability in test volume was minimized by adjusting the concentration to ensure a constant volume at all dose levels.

Clinical signs of toxicity
All the rats were observed at least twice daily with the purpose of recording any symptoms of ill-health or behavioral changes. Clinical signs of toxicity were observed daily for 28 days.

RESULTS AND DISCUSSION

Table 1 shows the effect of sub-acute dose (28 days) of KKC on organ weight (physical parameter) in grams.

Organ weight
Group means relative organ weights (% of body weight) are recorded in Table 1. Comparison of organ weights of treated animals with respective control animals on day 29 was found to be comparably similar.

Table 2 shows effect of sub-acute dose (28 days) of KKC on biochemical parameters.

Biochemical investigations
Results of biochemical investigations conducted on the 29th day are recorded in Table 2, which revealed significant changes in the values of hepatic serum enzymes studied when compared with those of respective control. However, the increase or decrease in the values obtained was within normal biological and laboratory limits.

Histopathology
Histopathological examination of vital organs revealed normal architecture (Fig. 1) of the test drug-administered groups as compared to control group.

Clinical signs
All test drug administered animals in this study were free of toxic clinical signs throughout the dosing period of 28-day.

Mortality
All animals in control and in all the treated dose groups survived throughout the dosing period of 28-day.

CONCLUSION

The results of the sub-acute toxicity studies revealed that the drug "KKC" can be considered safe, as it did not cause either any lethality or adverse changes with general behavior of rats and also there were no observable detrimental effects in the doses (300-900 mg/kg body weight) administered over a period of 28-day. Our results have

Table 1: Effect of sub-acute dose (28 days) of KKC on organ weight (physical parameter) in grams

Group	Control	KKC - low dose	KKC - middle dose	KKC - high dose
Brain	1.289±0.1234	1.337±0.08963	1.532±0.114	1.212±0.06309
Heart	0.823±0.02989	0.8145±0.04234	0.8245±0.0515	0.808±0.05421
Liver	6.345±0.2363	6.764±0.2393	6.733±0.281	6.678±0.5239
Lungs	1.355±0.1081	1.088±0.1403	1.25±0.1554	1.202±0.1145
Kidney				
L	0.8382±0.02416	0.8557±0.03146	0.8683±0.02933	0.8102±0.02697
R	0.8147±0.02458	0.8428±0.02684	0.8273±0.04038	0.8157±0.03272
Testis	2.914±0.1975	2.826±0.1422	2.727±0.1444	2.913±0.202
Uterus	0.981±0.0877	1.043±0.0886	1.018±0.1147	1.09±0.06846

KKC: Kanthakaathi chooranam

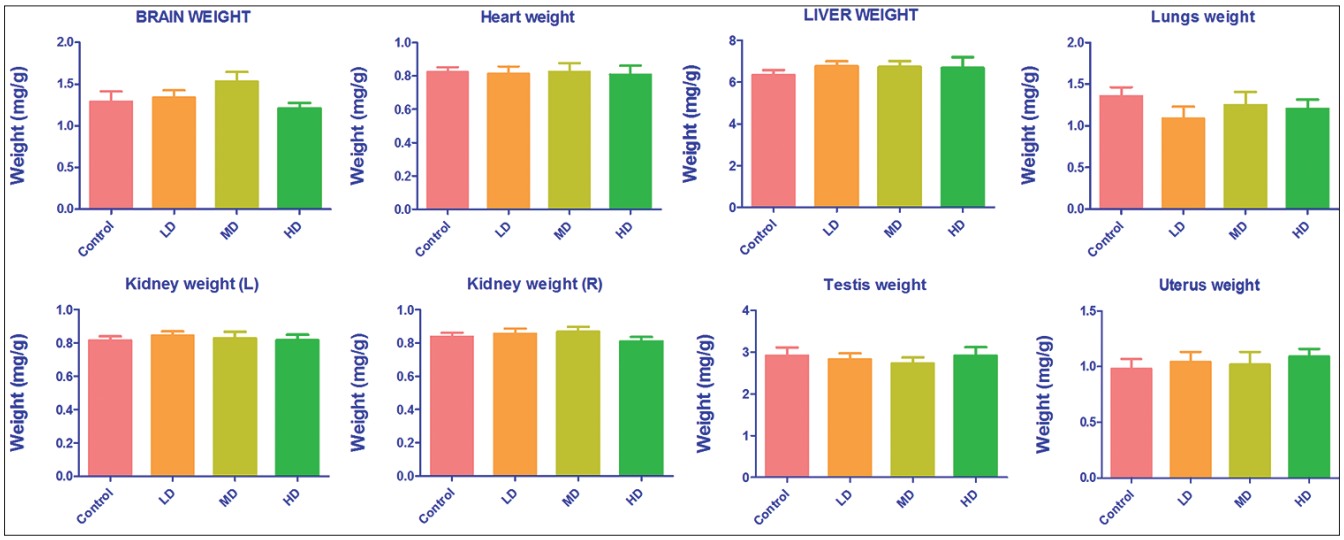
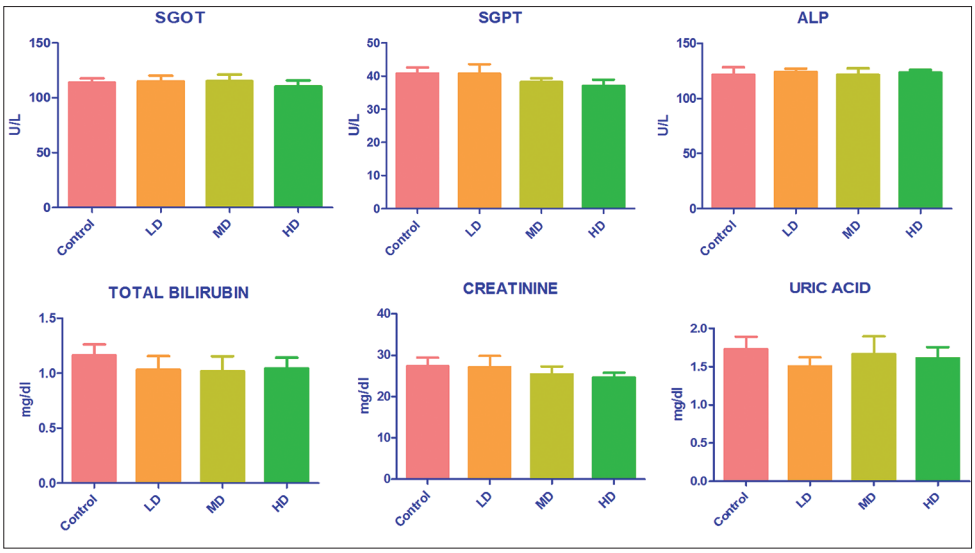


Table 2: Effect of sub-acute dose (28 days) of KKC on biochemical parameters

Group	Control	KKC - low dose	KKC - middle dose	KKC - high dose
SGOT (units/min/liter/mg protein)	113.9±3.8	114.9±5.297	115.2±6.013	110.3±5.544
SGPT (units/min/liter/mg protein)	40.75±1.875	40.68±2.912	38.18±1.176	37±1.934
ALP (units/min/liter/mg protein)	121.5±6.745	124±3.061	121.3±6.018	123.3±2.53
Total bilirubin (mg/dl)	1.165±0.09646	1.033±0.1222	1.018±0.1358	1.045±0.09542
Creatinine (mg/dl)	27.35±2.11	27.18±2.678	25.43±1.857	24.65±1.092
Uric acid (mg/dl)	1.728±0.1619	1.505±0.1174	1.663±0.2308	1.608±0.1452

KKC: Kanthakaathi chooranam, SGOT: Serum glutamic-oxaloacetic transaminase, SGPT: Serum glutamate-pyruvate transaminase, ALP: Alkaline phosphatase



demonstrated that the drug, "KKC" is relatively safe when administered orally in rats.

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Research Article

STANDARDIZATION AND CHEMICAL EVALUATION STUDIES ON AN ANTIDIABETIC CODED FORMULATION "DIA5"

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ABSTRACT

Objective: A formulation of "Dia5" is prepared comprising five antidiabetic herbs mentioned in Siddha medicinal system that could be useful in the management of diabetes. The herbs used in the formulation are leaves of *Murraya koenigii*, fruits of *Terminalia chebula*, whole plant of *Phyllanthus amarus* and aerial portions of *Aegle marmelos* and *Tinospora cordifolia*.This study was evaluated to view the presence of herbal drugs added in antidiabetic formulation through high performance thin layer chromatography (HPTLC) technique. Quality control studies of formulated drug and antidiabetic activity related components of tannins, bitters, and alkaloids were done by standard methods.

Methods: Physicochemical parameters were determined using standard methods. The content of bitters, tannins, and alkaloids was estimated according to the standard protocols. CAMAG HPTLC system was used for HPTLC analysis using different mobile phases.

Results: Physicochemical standards such as pH, loss on drying, total ash, acid insoluble ash, alcohol soluble extractive and water soluble extractive were determined. Total content of tannins, bitters, and alkaloids was found to be 5.790% (w/w), 3.436% (w/w), and 0.1814% (w/w), respectively, in the formulation. The herbs added in "Dia5" were confirmed through HPTLC technique.

Conclusion: In conclusion, the Siddha formulation was not adulterated or substituted by any other species. The HPTLC profile will help in authentication of the herbs in the formulated drug. This study also helps to assure the quality and acceptability of the developed herbal antidiabetic formulation.

Keywords: Antidiabetic formulation, High performance thin layer chromatography, Bitters.

INTRODUCTION

Medicinal plants have played important role and have also significantly contributed toward human health care. In spite of great advancement made in modern medicines, herbal formulations have reached extensive acceptability as therapeutic agents for the treatment of various diseases. Herbal technology is a science, for converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is merged. Standardization is a process of evaluating the quality and purity of crude drug using various quality parameters involving morphological, microscopic, physical, chemical, and biological properties [1]. However, herbal medicines differ from that of the conventional drugs as there exist greatest lacuna in these formulations such as lack of standards and quality control parameters. Some modern methods need to be adopted for quality assessment of these herbal medicines.

Chemical fingerprint analysis approach is the most effective tool for quality control of herbal medicines because of its accuracy and reliability. High performance thin layer chromatography (HPTLC) fingerprinting is a process that determines the presence of active constituents in a herb and could be utilized to detect the adulterants or substitutes [2,3]. The herbal drug preparations were adjusted to a defined content of a constituent or a group of substances with known therapeutic activity respectively by adding excipients or by mixing herbal drugs or herbal drug preparations. Evaluation of a drug means confirmation of its identity and determination of its quality and purity and detection of its nature of adulteration [4]. The existence of the herbs/adulterants in the prepared formulation could be identified by chromatographic techniques.

Chromatography is one of the fast promising tools by which the quality of herbs can be evaluated [5,6]. Using this technique, the identification of various chemical markers of the herbal drugs can be easily done and it also helps to identify the herbal adulterant in a combination. TLC/HPTLC analytical method for analysis of herbal drugs is very popular as this technique is economical, rapid besides simultaneous screening of large number of herbal samples is also possible [7,8]. TLC or HPTLC profile of the herbal drug can be employed to find out the presence or absence of the certain chemicals/chemical markers [9]. HPTLC technique was particularly important for screening and selecting raw materials, and in control process. It is suggested that with the help of chromatographic fingerprints obtained, the authentication and identification of herbal medicines can be accurately conducted [10]. However, in any herbal medicine and its extract, there are hundreds of unknown components, and many of them are present in a low amount. Moreover, there usually exists variability within the same herbal materials. Hence, it is very important to obtain reliable chromatographic fingerprints that represent the presence of pharmacologically active and chemically characteristic components in the herbal medicine. HPTLC has been investigated for simultaneous assay of several components in a multi-component formulation [11].

This study is focused on the identification of antidiabetic components present in the individual herbs used in the antidiabetic herbal formulation (Dia5) using HPTLC technique and estimation of antidiabetic compounds such as tannin, bitter, alkaloid, and essential oil were also carried out using standard chemical methods. The formulation contains equal proportion of herbal ingredients such as *Terminalia chebula* (TC), *Tinospora cordifolia* (TI), *Aegle marmelos* (AM), *Phyllanthus amarus* (PA), and *Murraya koenigii* (MK).

TC (Gaertn.) Retz. is a member of the Combretaceae family, frequently used medicinal herb in Ayurveda, Unani, Siddha, and Homeopathy

system of medicine. Recently, this herb is of great interest to researchers across the globe because of its reported medicinal properties such as antioxidant, antibacterial, antifungal, antineoplastic, antiviral, antidiabetic, cardioprotective, and immunomodulatory [12]. TC is a well-known Rasayana drug which prevents aging and imparts longevity, immunity and body resistance against diseases and also used extensively in several Ayurvedic formulations prescribed for infectious diseases such as chronic ulcers, leucorrhoea, pyorrhea, and fungal infections of the skin. It contains tannins (20-40%), chebulagic acid, chebulinic acid, ellagic, gallic acid, and syringic acid [13].

TI (Willd) Miers ex Hook. family and Thoms. belongs to the family Menispermaceae which is known by the common names *Guduci* and *Giloy*. It is an herbaceous vine indigenous to the tropical areas of India, Myanmar, and Sri Lanka. In Ayurveda, basically, it is used for its general adaptogenic, rejuvenating, and immunomodulatory activity [14]. The plant constituents include tinosporaside, sesquiterpene, tinocordifolin, alkaloids, triterpenoids, amino acids, steroids, xanthones, flavonoids, phenolics, coumarins, iridoids, alkyl disulfides, inorganic ions and guanidines that are reported to have antidiabetic activity. The possible substitution of *Tinospora cordifolia* being used was *Tinospora malabarica* (lank). Hook and Thomas [15].

AM (L.) Correa belongs to the family Rutaceae commonly known as bael, Bengal quince, golden apple, Japanese bitter orange, stone apple, or wood apple, is a species of tree native to Bangladesh and India. AM possesses antidiabetic, antidiarrheal, antimicrobial, antiviral, radioprotective, anticancer, chemopreventive, antipyretic, ulcer healing, antigenotoxic, diuretic, antifertility, and anti-inflammatory properties [16] and play significant role in the prevention and treatment of many diseases. It contains coumarins, polysaccharides, beta-sitosterol, alkaloids such as marmeline and dictamine [17]. For AM, *Feronia limonia* (Linn) swingle was used as a substitute.

PA Schum. and Thonn. belonging to the family Euphorbiaceae has been found to be useful in traditional medicines and is beneficial in the management of several health problems such as diarrhea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders, scabies, and wounds. Its effect in excretory system is due to its antiurolithic property and is used in the treatment of kidney/gallstones, appendix inflammation and prostate problems [18]. It contains phyllanthin, hypophyllanthin, tannins, lignans, and alkaloids [19]. For PA possible substitute being used was *Parachartergus fraternus*, *Phyllanthus urinaria*, *Phyllanthus simplex,* and *Phyllanthus maderaspatenis.* Furthermore, assessment of bitterness value is a powerful tool to differentiate PA from other species [20].

MK (Linn) Spreng. is a tropical to subtropical tree belonging to the family Rutaceae. This crop is native to India and Srilanka. In Ayurvedic medicine, MK leaves are believed to have several medicinal properties such as antidiabetic, antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, and hepatoprotective properties [21]. Leaves contain essential oil [0.14-0.32% v/w] whose main constituents are mono and sesquiterpenes such as sabinene, beta-caryophyllene, alpha-pinene, and beta-phellandrene [22].

MATERIALS AND METHODS

Collection of materials

The herbal drugs were purchased from the local market of Thanjavur, Tamil Nadu.

Physicochemical standardization of the formulation

Physicochemical standards such as pH, total ash, acid insoluble ash, loss on drying at 105°C, water soluble and alcohol soluble extractive values were determined according to the standard procedures [23].

Estimation of tannins

About 2 g of the drug was digested with 50 ml of distilled water on a water bath for 30 minutes with frequent stirring [24]. Allowed to settle, the supernatant liquid was decanted carefully to a volumetric flask. The extraction was repeated until the supernatant turns colorless. Then, the extract was cooled and made up to a known volume with distilled water. Pipetted out 25 ml of the filtrate into a conical flask; 750 ml of distilled water was added followed by 25.0 ml of indigo sulfonic acid solution. The mixture was titrated against 0.1 M potassium permanganate solution. The appearance of golden yellow color as the endpoint. The blank was performed as same as above by omitting the sample. Each ml of 0.1 M potassium permanganate solution is equivalent to 0.004157 g calculated as tannic acid.

Estimation of bitter

About 5 g of the drug was made into slurry with celite 545 filter aid [25]. The mixed slurry was packed into the glass column. Alternatively, $CaCO_3$ was also packed to remove the extraneous matter. The slurry was eluted with ethyl acetate until free from bitter. The collected ethyl acetate eluate was evaporated over a water bath and the residue was weighed and calculated as bitter.

Estimation of alkaloids

The alkaloid content was estimated according to the method of Indian Pharmacopoeia [26]. About 5 g of the drug was soaked in 100 ml of ether alcohol mixture (4:1 ratio, v/v) along with 2 ml of dilute ammonia solution. The mixture was allowed to stand for 1 hr with frequent shaking and the contents were filtered through Whatman No. 41 into the separating funnel. 30 ml of 1N H_2SO_4 was added and shaken well. Allowed to separate, collected the acid layer. Further ether alcoholic mixture was extracted with 0.5 N alcoholic H_2SO_4 in the ratio of 3:1 v/v, shake and collect the acid layer. The extraction was repeated until the solution became colorless. The total acid layer was extracted with chloroform to remove the extraneous matter. Then, it was made alkaline with dilute ammonia solution. The alkalinized solution was extracted with chloroform until free from alkaloid. The collected chloroform layer was evaporated to dryness over a water bath. The dried residue was calculated as total alkaloids.

Extract preparation for HPTLC analysis

TC

About 2 g of powered drug was extracted twice with 25 mL of 2 M hydrochloric acid under reflux for 2 hr, cooled and filtered into a separating funnel. The filtrate was extracted with diethyl ether. The combined diethyl ether extract was concentrated to dryness. The dried residue was dissolved in methanol and used for TLC identity test. The solvent system used was chloroform:ethyl acetate:formic acid in the ratio of 5:4:1.6.

TI

About 2 g of powdered drug was extracted with n-hexane in a Soxhlet apparatus to defat the material. Further, the drug was extracted with methanol for 8-10 hrs. The solvent was removed under reduced pressure and the collected residue was dissolved in methanol and used for TLC profiling. The ratio of chloroform:methanol 9:1 was used as solvent system.

AM

About 2 g of powdered drug was refluxed with methanol for 15 minutes, cooled and filtered. Then, the filtrate was evaporated to dryness under reduced pressure and dissolved in methanol. Equal ratio of toluene:diethyl ether (1:1) saturated with 10% CH_3COOH was used as a mobile phase.

PA

About 2 g of powered drug in methanol (3×25 mL) was refluxed over a water bath. Then, the contents were filtered, concentrated, and made up to known volume with methanol. The ratio of toluene:ethyl acetate:formic acid:methanol (3:3:0.8:0.4) was used as mobile phase.

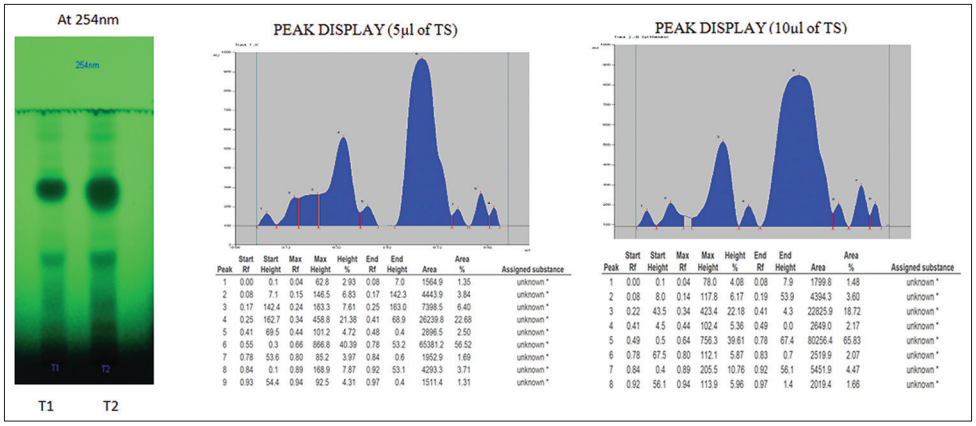

Fig. 1: Photo documentation and its chromatogram of *Terminalia chebula* under UV at 254 nm; T_1, T_2-5 µl, 10 µl of test solution

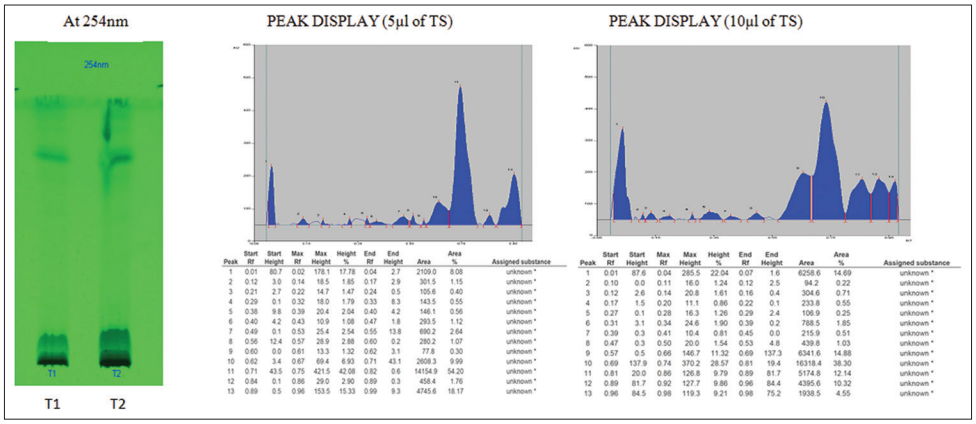

Fig. 2: Photo documentation and chromatogram of *Tinospora cordifolia under* UV at 254 nm; T_1, T_2-5 µl, 10 µl of test solution

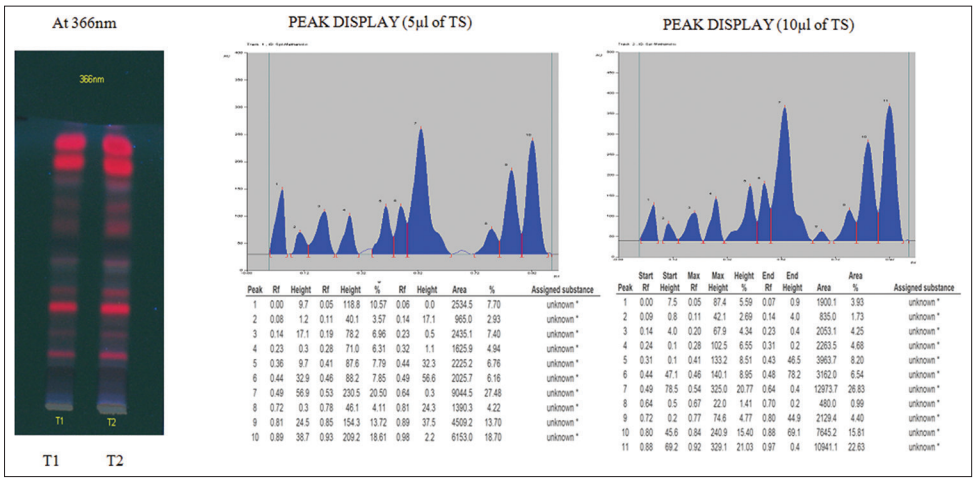

Fig. 3: Photo documentation and its chromatogram of *Aegle marmelos* under UV at 366 nm; T1, T2-5 µl, 10 µl of test solution

MK

About 2 g of the drug was mixed with equal volume of chloroform and hexane. The mixture was allowed to stand for 4 hrs with frequent shaking and filtered. The collected filtrate was evaporated over a water bath and the residue was dissolved in methanol. The ratio of hexane:chloroform:methanol (6.5:3.5:0.5) was used as a mobile phase.

HPTLC analysis

Instrumentation and chromatographic conditions

The HPTLC analysis was performed to obtain the fingerprinting profile of herbal drug added in the antidiabetic formulation. The prepared herbal extracts were spotted on a precoated silica gel $60F_{254}$ plate (10×10 cm) (E. Merck, Dermstadt, Germany) of thickness 0.2 mm, 7 mm wide band using Linomat 5 (CAMAG, Switzerland) automatic sample applicator. The plate was developed in a CAMAG glass twin trough chamber (10×10 cm) which was previously saturated with the mobile phase for 30 minutes at room temperature. After development, the plates were dried in hot air oven at 105°C and scanned densitometrically at particular wavelength using TLC Scanner 3 (CAMAG, Switzerland). The chromatogram was visualized and photographed under UV light at λ=254 and 366 nm using CAMAG Reprostar 3 (CAMAG, Switzerland) with win CATS software version of 1.3.4.

RESULTS

Developed antidiabetic herbal formulation "Dia5" was subjected to physicochemical characterization and the results were shown in Table 1. Ash value is useful in determining the authenticity and purity of drug and was found to be 6.003% (w/w). Loss on drying was found to be 5.510% (w/w). The less value of moisture content could prevent bacterial, fungal or yeast growth in the formulation. Extractive value indicates the amount of phytoconstituents present in the herbal drug. Water soluble extractive value was found to be higher than alcohol which indicated the presence of polar compounds.

The HPTLC finger printing profile of TC, TI, AM, PA, and MK was shown in Figs. 1-5. The spots at different R_f values with respective chromatogram indicates the number of active compounds present in the formulation.

The relevant antidiabetic compounds such as bitters, tannins, and alkaloids present in the drug were estimated and are presented in Table 2. The antidiabetic herbal formulation was rich in tannin content and was found to be 5.790%. The content of bitters and alkaloids was found to be 3.436% and 0.1814%w/w, respectively.

DISCUSSION

Chromatographic fingerprinting is mostly used in plant analysis for classification of plants, especially to differentiate related species, for stability testing and quality control. The fingerprint of impurity profile can clearly differentiate the genuine and counterfeit samples. Chromatographic techniques have great potential in the analysis of illegal Siddha preparation; they help not only in the detection of spurious materials but also can provide a complete image of the composition of the sample. However, several studies detected synthetic drugs as adulterant in herbal formulation representing the huge risks for public health [27,28].

HPTLC method is a very powerful tool for the identification of adulterants in herbal products based on the characteristic image produced [29].

Table 1: Physicochemical standardization of Siddha formulation

Parameters	Results
Appearance	Brown color fine powder
pH (1% w/v solution)	4.13
Total ash	6.003% w/w
Acid insoluble ash	0.5755% w/w
Loss on drying at 105°C	5.510% w/w
Water soluble extractive	31.23% w/w
Alcohol soluble extractive	16.235 w/w

Table 2: Estimation of antidiabetic compounds of formulation

Parameters	Results
Tannins	5.790% w/w
Bitters	3.436% w/w
Alkaloids	0.1814% w/w

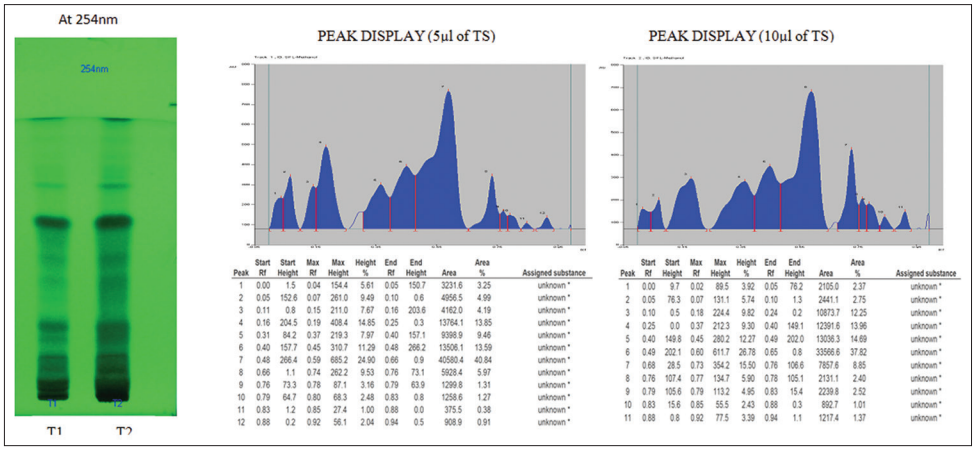

Fig. 4: Photo documentation and its chromatogram of *Phyllanthus amarus* under UV at 254 nm; T1, T2-5 µl, 10 µl of test solution

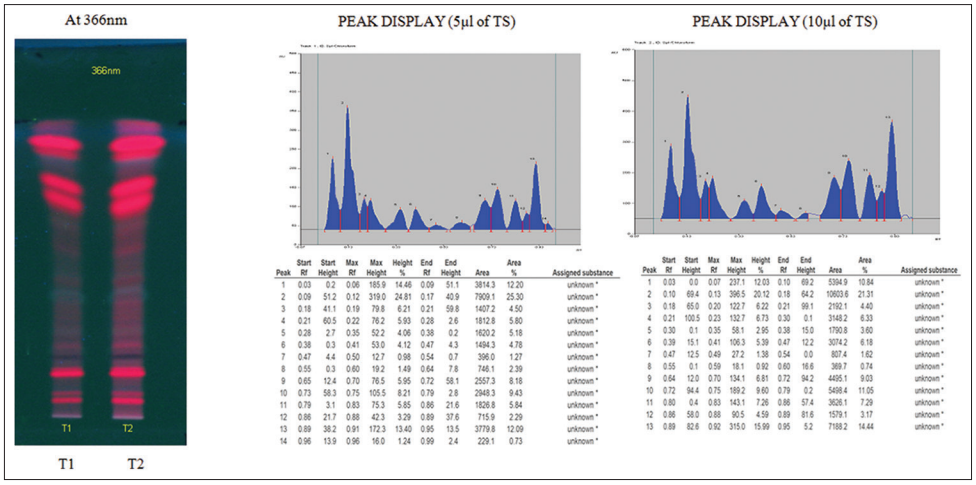

Fig. 5: Photo documentation and its chromatogram of *Murraya Koeingii* under UV at 366 nm; T1, T2-5 µl, 10 µl of test solution

Through HPTLC analysis, the fingerprinting profile of formulated drug was interrelated with pharmacopoeial TLC profile. Fig. 1 demonstrates that the test solution of TC shows a band at R_f value of 0.14, 0.34, 0.44, 0.80, 0.89, 0.94 which was compared with the TLC profile of TC [13]. The major spots were observed at R_f 0.34, 0.64 and 0.89. The end R_f value 0.49 correspond to gallic acid.

In Fig. 2, the test solution of TI shows a band at R_f value 0.28, 0.34, 0.41, 0.74 were compared with the TLC profile of TI [15]. The major spots at R_f 0.60, 0.74, 0.86, 0.92 were observed. A band (R_f 0.34) corresponding to tinosporaside is visible in test solution tracks. Fig. 3 indicated the various bands with relevant chromatogram of AM. The test solution of AM shows a band at R_f value 0.14, 0.28, 0.41, 0.54 were comparable with the authentic TLC profile of AM [17]. The major spots at R_f 0.41, 0.54, 0.84, 0.92 (all red) were observed. A dark blue fluorescent zone (R_f 0.28) corresponding to umbelliferone is visible in test solution tracks.

The test solution of PA shows a band at R_f value 0.18, 0.37, 0.43, 0.60, 0.73 were comparable with the authentic TLC profile of PA [30]. The major spots at R_f 0.18, 0.37, 0.43 and 0.60 were observed. A band (R_f 0.43) corresponding to gallic acid (black) is visible in test solution tracks (Fig. 4). The test solution of MK shows a band at R_f value 0.13, 0.23, 0.35, 0.41, 0.49, 0.59, 0.70, 0.75, 0.83, 0.92 were comparable with the authentic TLC profile of MK [22]. The major spots at R_f 0.13, 0.75, 0.92 was observed in the TLC chromatogram. A band (R_f 0.92) corresponding to α-pinene is visible in test solution tracks (Fig. 5).

We impart that the antidiabetic formulation "Dia5" has not been adulterated or substituted by any other drugs and also provide sufficient information about therapeutic efficacy of the herbal formulation. The antidiabetic Siddha formulation was estimated for tannins, bitters, and alkaloids and was found to be 5.790%, 3.436%, and 0.1814%w/w, respectively. The maximum level of tannins and bitters present in herbal formulation may strengthen the antidiabetic activity. These phytoconstituents will be helpful in the management of diabetes and will decrease the risk of other chronic disorders [31-33].

CONCLUSION

From our results, we concluded that the prepared formulation "Dia5" was properly mixed with genuine species. HPTLC analysis also strengthens the presence of added crude drugs in the formulation. This paper provides the importance of verifying the herbs present in the formulation.

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Research Article

IN VIVO MAST CELL STABILIZING ACTIVITY OF CLASSICAL SIDDHA HERBOMINERAL FORMULATION BKM

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ABSTRACT

Objective: The main objective of this work is to assess the mast cell stabilising activity of Bala Kabahari Mathirai (BKM).

Methods: the mast cell stabilising activity of BKM was tested in sensitised Wister rats.

Result: There is a significant reduction in the number of disrupted mast cells ($p<0.001$) when challenged with horse serum.

Conclusion: The results suggested that BKM significantly inhibited the pathological effects induced by the release of inflammatory mediators and shows significant mast cell stabilisation.

Keywords: Siddha herbomineral drug, Childhood asthma, BKM, Mast cell stabilizing activity.

BACKGROUND

According to Siddha concept, disease is caused when the normal equilibrium of three humors (Vatham, Pitham, and Kabam) is disturbed. The factors which affect this equilibrium are environment, climatic conditions, diet, and physical activities. The trial drug "Bala Kabahari Mathirai (BKM)" is chosen for its mast cell stabilizing activity on the basis of classical attributes of its respective ingredients according to the doctrine of *Suvai* (taste), *Gunam* (property), *Pirivu* (metabolic changes after digestion), *Veeriam* (potency), and *Seigai* (specific action which would pacify the vitiated humors).

Therefore I expect this gastric medicine to be safe and efficient in its action and bring quick recovery.

METHODS

Drug review
Name of the medicine: Balakabahari mathirai
Reference book - Agathiyar Vaithiya Pillai Tamil
Ingredients:

Name	Botanical name	Parts used	Quantity
Adhimadhuram	*Glycyrrhiza glabra*	Root	5.25 g
Koshtam	*Costus speciosus*	Root	5.25 g
Chukku	*Zingiber officinale*	Rhizome	5.25 g
Milagu	*Piper nigrum*	Seed	5.25 g
Arisi Thippili	*Piper longum*	Dried Fruit	5.25 g
Lavangapattai	*Cinnamomum verum*	Bark	5.25 g
Lavanga Pathiri	*Cinnamomum tamala*	Leaves	5.25 g
Sirunagapoo	*Mesua nagassarium*	Flower	5.25 g
Kadugurogini	*Picrorhiza scrophulariiflora*	Root	5.25 g
Sitrarathai	*Alpinia galanga*	Root	5.25 g
Akkarakaram	*Anacyclus pyrethrum*	Stem	5.25 g
Velicha pisin	*Gardenia resinifera*	Resin	5.25 g
Indhuppu	Sodium chloride impura	Salt	5.25 g
Thettran Vithai	*Strychnos potatorum*	Seed	5.25 g
Adathodai	*Adhatoda vasica*	Leaves	100 ml

Method of purification

All drugs will be purified as per classical Siddha texts.

Method of preparation

Raw drugs from item 1 to 13 are cleaned, purified, shade dried, roasted in a mild flame, and then powdered. They are sieved to get microfine powder and mixed homogeneously. Thettran vithai is made into small pieces and soaked in Adathodai leaf juice for 1 night. Then, all the above ingredients are grinded with Adathodai leaf juice for 9 hrs and made into fine tablets each weighing about 0.6 g (Thoothuvalai Pramanam). Finally, they are stored in cool and dry place.

Mast cell stabilizing activity of Siddha formulation of BKM

Introduction

Allergy is one of the most common diseases that affect humanity with diverse manifestations. The prevalence of allergy and asthma has risen in the recent years despite an improvement in the general health of the population [1]. Allergic diseases are responsible for significant morbidity and have severe economic impact [2]. Various epidemiological studies have identified the causes for an increase in the prevalence of upper and lower respiratory tract allergic diseases. Some of the postulated reasons are increasing environmental pollution [3] and increased predisposition of individuals producing excessive Ig_E through a major change in the gene pool, changing lifestyles, and an increasing awareness of the disorders [4]. Intensive research during the last several decades has highlighted the role of lymphocytes, immunoglobulins, mast cells, and various autacoids in the etiopathogenesis of allergic conditions. Inspite of the voluminous literature on the subject, the treatment of allergic diseases continues to be far from satisfactory. The available treatment options for upper and lower respiratory tract allergic diseases have major limitations owing to low efficacy, associated adverse events, and compliance issues [5].

AYUSH, an Indian system of medicine, has described several drugs from indigenous plant sources for use in the treatment of bronchial asthma and allergic disorders. In this study, the effects of Siddha formulation of BKM were studied on the active anaphylaxis and mast cell stabilization in rats and histamine-induced bronchospasm in guinea pigs.

Animals

Inbred Wistar rats (175-200 g) and guinea pigs (400-600 g) of either sex housed in standard conditions (temperature 22±2°C, relative humidity 60±5%, and 12 hr light/dark cycle) were used. They were fed with standard pellet diet and water *ad libitum*. The institutional animal ethics committee approved the experimental protocol. Histamine and horse serum were procured from sigma chemicals and toluidine blue from loba chemie, Mumbai. Elisa kit for Ig_E was supplied by Orion diagnostica, Espoo, Finland. All other chemicals and reagents were procured from Hi-Media Laboratories Limited, Mumbai.

Mast cell stabilizing activity

Treatment protocol

About 24 rats were divided into 5 groups of six animals in each group.

- Group I - served as control and received vehicle (water)
- Group II - (sensitized control group)
- Group III - served as the treatment control, which was treated with BKM at a dose of 200 mg/kg body weight, in oral route
- Group IV - served as the treatment control, which was treated with BKM at a dose of 400 mg/kg body weight, in oral route.

In Groups II-IV were sensitized by injecting 0.5 ml of horse serum subcutaneously along with 0.5 ml of triple antigen containing 20,000 million *Bordetella* pertussis organisms (Serum Institute of India Ltd., Pune), once a day for 14 days.

On day 14, the rats were sacrificed 2 hr after the treatment, and the intestinal mesentery was taken out for the study on mast cells. Mesenteries along with intestinal pieces were excised and kept in ringer Locke solution (NaCl 154, KCl 5.6, CaCl2 2.2, NaHCO3 6.0, and glucose 5.55 mM/L of distilled water) at 37°C. The mesenteric pieces were challenged with 5% horse serum for 10 minutes after, which the mast cells were stained with 1.0% toluidine blue and examined microscopically for the number of intact and degranulated mast cells [6].

Histamine-induced bronchospasm in guinea pigs

Bronchospasm was induced in guinea pigs by exposing them to 1% histamine aerosol under constant pressure (1 kg/cm^2) in an aerosol chamber (24 × 14 × 24 cm) made of perplex glass, of the three groups of six animals each.

- Group I - served as control
- Group II - served as the treatment control, which was treated with BKM at a dose of 200 mg/kg body weight, in oral route
- Group III - served as the treatment control, which was treated with BKM at a dose of 400 mg/kg body weight, in oral route.

The animals were exposed to 1% histamine aerosol under constant pressure (1 kg/cm^2) in an aerosol chamber on day 0 without any treatment. The end point, pre-convulsive dyspnea (PCD) was determined from the time of aerosol exposure to the onset of dyspnea leading to the appearance of convulsions [7]. As soon as PCD commenced, the animals were removed from the chamber and exposed to fresh air. This PCD was taken as day 0 value. On days 1 and 5, 2 hr after the administration of the drug, the time for the onset of PCD was recorded as on day 0.

Statistical analysis

The results of various studies were expressed as mean ± standard deviation of mean and analyzed statistically using one-way analysis of variance, followed by new Mann Keul's multiple range tests. $p<0.05$ was considered statistically significant. The analysis was performed using Graphpad Prism software package (version 4.0).

RESULTS AND DISCUSSION

Mast cell stabilizing the potential of BKM antigen challenge resulted in significant degranulation of the mesenteric mast cells. Pretreatment of sensitized animals with BKM at a dose of 200 mg/kg and 400 mg/kg, p o., for 2 weeks resulted in a significant reduction in the number of disrupted mast cells ($p<0.001$) when challenged with horse serum.

Table 1: Effect of BKM on mast cell stabilization in sensitized rats

Groups	Mast cells	
	Intact	Disrupted
Normal control	84.25±3.90	16.40±0.95
Sensitized rats	12.30±0.85	87.40±2.65
BKM 200 mg/kg	65.32±2.50*[a]	35.60±1.40*[a]
BKM 400 mg/kg	63.25±2.30*[a]	33.35±1.25*[a]

Values are expressed as mean±SEM. *[a]Significantly different from sensitized control at $p<0.01$, SEM: Standard deviation of mean

Effect on histamine-induced bronchospasm

BKM at a dose of 200 mg/kg and 400 mg/kg, p o., significantly prolonged the latent period of PCD ($p<0.001$) as compared to control, following exposure to histamine aerosols on day 5 (Table 1).

CONCLUSION

Experimental animal model of asthma is characterized by allergen-induced immediate airway constriction and late airway reactivity to a pharmacological vasoconstrictor such as histamine and leukotrienes. Histamine is a central mediator in the pathogenesis of allergic and inflammatory disorders. In this study, BKM prolonged the latent period of PCD in guinea pigs following histamine aerosol. This may be suggestive of an antihistaminic activity following treatment with BKM.

Antigen challenge, in sensitized animals, results in the degranulation of mast cells, which is an important feature of anaphylaxis. In this study, BKM showed marked protection against the mast cell degranulation following antigen challenge in sensitized animals. Mast cell stabilizing activity of BKM may be attributed to the presence of active constituents which are known for their mast cell stabilizing potential against antigen-antibody reaction and/or due to the suppression of IgE antibody production, which is responsible for degranulation mast cells [8].

This antianaphylactic and antihistaminic effect may be caused by the stabilization of the mast cell membrane, suppression of IgE, and inhibition of pathological effects induced by the release of inflammatory mediators in BKM treated animals [9]. All the above findings lend credence to the beneficial use of BKM in the treatment of asthma and related conditions.

However, further studies with other experimental models, especially to explore the role of cytokines are warranted to substantiate the antiasthmatic and antiallergic activity of BKM.

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Research Article

IN VITRO ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTIBACTERIAL ACTIVITIES OF THE LYOPHILIZED AQUEOUS EXTRACT OF FRUITS OF *LUFFA ACUTANGULA* L.

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ABSTRACT

Objective: This study was aimed at detecting the phytochemicals and ascertains the antioxidant, anti-inflammatory and antibacterial activities of the lyophilized aqueous extract of fruits of *Luffa acutangula* L. (*Cucurbitaceae)* using *in vitro* experimental models.

Methods: In this study, 10 different concentrations have been screened for their antioxidant and anti-inflammatory activities. Four different concentrations were determined for antibacterial activity by the disc diffusion method against *Bacillus cereus*, *Staphylococcus aureus, Campylobacter jejuni,* and *Salmonella enteritidis* strains. Total phenolic and flavonoid content of the extract was determined as the gallic acid and quercetin like compounds, respectively.

Results: The fruits of *L. acutangula* L. extract exhibited maximum activity of 91.86% and 89.93% at 1000 µg/ml in the lipid peroxidation and 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assays, respectively, which are comparable to that of standard butylated hydroxytoluene. High concentration (1000 µg/ml) of the extract revealed anti-inflammatory efficacy up to 98.66%, 99.49%, and 99.50% by inhibiting protease activity, albumin denaturation and membrane stabilization, which is equivalent to the action of the regular aspirin. Results showed that the extract possesses significant activity against all four bacterial strains, which is comparable to that of the standard gentamicin.

Conclusions: Due to the presence of bioactive compounds, the fruits of *L. acutangula* L. extract might possess antioxidant, anti-inflammatory and antibacterial activities.

Keywords: Antioxidant activity, Anti-inflammatory activity, Antibacterial activity, *Luffa acutangula* L., Lyophilized aqueous extract.

INTRODUCTION

Antioxidants play a central role in defusing free radical species which are formed from various biochemical reactions in normal system [1]. High amounts of free radical molecules cause oxidative stress in cells which results in destructing important macromolecules comprising DNA, lipids, and proteins. The mutilation of macromolecules leads to inflammation and several deteriorating disorders such as Parkinson's diseases, aging, diabetes, atherosclerosis, ischemic heart disease, immunosuppression, and reduced membrane fluidity [2,3]. These free radicals are the main culprits in lipid peroxidation. In various inflammatory ailments, there is extreme production of O_2–, OH radicals as well as nonfree radical's species (H_2O_2) and activation of phagocytes [4], which can harm severely tissues either by powerful direct oxidizing action or indirectly by reacting with hydrogen peroxide and –OH radical formed from O_2– and initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by producing mediators and chemotactic factors [5]. The free radical species are also known to activate matrix metalloproteinase damage [6]. Nonsteroidal anti-inflammatory medicines are presently used for the management of inflammation. These medicines are known to produce severe side effects in the body such as heart attacks and stroke [7]. Hence, robust limitations have been positioned on their application and there is a drift to substitute them with phytomedicine. Nutraceuticals having antioxidant, anti-inflammatory and antibacterial properties are nontoxic or may have minimum side effects than synthetic compounds. In this concern, our attempt is to search out a herbal nutraceutical to substitute synthetic ones. The aim of this study is to investigate the antioxidant, anti-inflammatory, and antibacterial activities of aqueous extract of fruits of *Luffa acutangula* L. on different *in vitro* models, a common herbal dietary supplement used by the Indians.

L. acutangula L. (*Cucurbitaceae*) (Peerku in Tamil) is a pan tropical climbing herb and cultivated throughout India and can grow in all types of soils in summer or in rainy season. Chemical constituents of *L. acutangula* L. mainly include carbohydrates, carotene [8], fat, protein [9], phytin, amino acids - alanine, arginine, cystine, glutamic acid, glycine, hydroxyproline, leucine, serine, tryptophan; pipecolic acid, flavonoids [10], saponins [11], lectin [12], and luffangulin, a novel ribosome inactivating peptide with an N-terminal sequence; cucurbitacin B, sapogenin, oleanolic acid [13]. The most common use of the ridge gourd fruit is cooked as a vegetable and contains good amount of fiber, vitamins and minerals including vitamin B2, vitamin C, carotene, niacin, calcium, phosphorus and iron. Reported biological activities are hepatoprotective [14,15], antidiabetic activity [13,16], central nervous system depressant [17], anticataleptic [18,19], analgesic [20], antiulcer [21], antimicrobial [22], and larvicidal [23]. The ethnobotanical survey of the hilly areas in Maharashtra revealed that very fine powder of fruits of *L. acutangula* L. is used as a snuff to protect jaundice [23]. It is also used traditionally in insect bites by tribes of Western Maharashtra. A powder of the fruit is used for rubbing on the swollen hemorrhoids. Kernel of the seeds is soft smooth and an efficient remedy for dysentery while the juice of roasted young fruit is applied to cure headache [22].

METHODS

All analytical grade chemicals and reagents used in this study were purchased from Sigma-Aldrich, Bengaluru. The glassware were made sterile by washing in dilute nitric acid thoroughly and then with double distilled water and dried in hot air oven.

Collection, authentication of plant materials and preparation of extract

Fruits of *L. acutangula* L. were collected from in and around Thanjavur and Mannargudi, Tamil Nadu. The selected plant drug was identified with the help of Flora of Presidency of Madras and authenticated by comparing with voucher specimen deposited at RAPINAT Herbarium, Department of Botany, St. Joseph's College, Tiruchirappalli. After proper identification and authentication, the collected material was cleaned, shade dried, and coarsely powdered. Aqueous extract was prepared by soaking the coarse powder (100 g) in distilled water (1 L) and kept in shaker for 48 hrs at room temperature. Then, the content was filtered through Whatman filter paper (Number 42) and the filtrate was collected, frozen, and lyophilized for further studies. All the parameters were taken in triplicate and data of the results obtained were presented.

Phytochemical analysis

Estimation of total flavonoids [24] and total phenols [25] was performed using standard procedures.

***In vitro* antioxidant activity**

Lipid peroxidation assay

Lipid peroxidation was initiated by adding 100 µl of aqueous extract, 15 mm ferrous sulfate and 3 ml of red blood cell (RBC) and vitamin C. After 30 minutes 100 µl of this reaction mixture was taken in a tube comprising 1.5 ml of 10% trichloroacetic acid. After 10 minutes, the tubes were centrifuged and supernatant was variegated with 1.5 ml of 0.67% thiobarbituric acid in 50% acetic acid. The mixture was heated in hot water bath. The intensity of pink colored complex developed was measured at 535 nm in a spectrophotometer. In this test, butylated hydroxytoluene (BHT) was used as positive control.

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method

A stock solution of 100 µg/ml aqueous extract as well as of standard BHT was prepared. Different concentrations were made of 1.95-1000 µg/ml from stock solution using water and 0.1 mM solution of DPPH in water in a volumetric flask which was completely kept away from light. Then 1 ml of all concentrations of test solutions and standards were mixed with 1 ml of DPPH solution. This solution was kept for 30 minutes in dark. Only water with DPPH was used us negative control. Absorbance of all the samples was observed using ultraviolet-spectrophotometer at 517 nm.

$$\%\text{Inhibition} = 1 \times \frac{A_{control} - A_{test}}{\text{Standard}} \times 100$$

***In vitro* anti-inflammatory activity**

Protease inhibitory activity

The reaction mixer consists of 0.5 ml trypsin and bovine serum albumin (8.000 armor units of enzyme activity), 1.0 ml 25 mMtris – HCl buffer (pH 7.4) and 1.0 ml aqueous extract (1.9-1000 µg/ml) and standard, aspirin (100 µg/ml). The mixtures were hatched at 37°C for 5 minutes. Then 1.0 ml of 0.87% (w/v) bovine serum albumin was added. The combinations were incubated for further 20 minutes. 2.0 ml of 70% perchloric acid was added to dismiss the reaction. Gloomy suspension was centrifuged. Absorbance of the supernatant was measured at 214 nm against water as a blank. Protein inhibitory activity (in %) is calculated as follows.

$$\text{Protein inhibitory activity } (\%) = 100 - \frac{(\text{ODof control-}\ \text{OD of test solution})}{(\text{ODof control})} \times 100$$

Inhibition of protein (albumin) denaturation

The reaction mixture (0.5 ml) comprised 450 µl 5% aqueous bovine serum albumin and 50 µl of aqueous extract (1.92-1000 µg/ml), pH was accustomed at 6.3 using 1 N HCl. The samples stood hatched at 37°C for 20 minutes followed by heating at 57°C for 3 minutes. This mixture was then carried to room temperature and 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity formed was measured at 660 nm, 50 µl distilled water was used in place of extracts for control test. The percentage inhibition of protein denaturation was calculated as follows:

$$\text{Percent inhibition} = 100 - \frac{(\text{ODof test} - \text{ODof product control})}{\text{OD of control}} \times 100$$

Membrane stabilization activity (heat-induced hemolysis)

Human blood (10 ml) was collected and moved to the heparinized centrifuged tubes. The tubes were centrifuged at 3000 rpm for 10 minutes and were washed thrice with equal capacity of normal saline. The volume of the blood was measured and altered as 10% v/v suspension with normal saline. The reaction mixture contained aqueous extract (1.92-1000 µg/ml) and standard aspirin (100 µg/ml) and 1 ml of 10% RBCs suspension. Instead of drug only saline was added to the control test tube. Tubes containing reaction mixture were incubated in a water bath at 56°C for 30 minutes. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 minutes and the absorbance of the supernatants was measured at 560 nm. Membrane stabilizing activity (%) was calculated by the following formula.

$$\text{Inhibition } (\%) = 100 - \frac{(\text{ODof control} - \text{ODof test})}{(\text{ODof control})} \times 100$$

Antibacterial activity

The aqueous fractions of the sample were screened at four different concentrations (125, 250, 500 and 1000 µg/disc) against bacterial pathogen using the disc diffusion method. Solution of known concentration of the test sample was prepared by liquefying dignified volumes of samples in calculated solvent volumes. Dehydrated and pasteurized filter paper discs (4 mm diameter) were then saturated with known quantities of the test materials using a micropipette. Discs having the test material were positioned on nutrient agar medium consistently owed with the pathogenic test microbes. Standard antibiotic discs (gentamycin, 30 µg/disc) and blank control discs (soaked with solvents) were used as positive (standard) and negative controls, respectively. These plates were then kept at 4°C for 1 hr diffusion of the test material. There was a steady change in concentration adjacent the discs. The plates were then incubated at 37°C for 24 hrs to permit organism growth. The test materials having antibacterial activity inhibited microorganism growth and a clear discrete zone of inhibition adjoining the discs was envisioned.

RESULTS AND DISCUSSION

Phytochemical analysis

Estimated amounts of total flavonoids and phenols were presented in Table 1.

From the results summarized in Table 1, we can easily conclude that the extract is rich in phenol and flavonoid. The total phenol content was 0.62 mg equivalent gallic acid/g of extract. The total flavonoid content was 0.45 mg equivalent quercetin/g of extract.

***In vitro* antioxidant activity**

An antioxidant with rich nutraceutical nature is very much beneficial to health in conjunction with good nutrition and regular exercise. There is a belief that antioxidants and antioxidant rich nutraceuticals provide an antiaging mechanism within the body. Antioxidants can certainly slow

Table 1: Estimation of total flavonoids and phenols

S. No.	Phytochemicals	Quantity (mg/g)
1	Total flavonoids	00.45±0.06
2	Total phenols	00.62±0.08

down the progression of aging. The fruits of *L. acutangula* L. extract exhibited maximum activity of 91.86% and 89.93% at 1000 µg/ml in lipid peroxidation and DPPH free radical scavenging assays, respectively, which are equivalent to that of standard BHT (Table 2). The activity was dose-dependent, which has been found to be amplified with rise in the concentration of the extract. Such radical scavengers might shield tissues from reactive oxygen species (ROS) and thus avoid oxidative impairment associated ailments. Such radical scavengers may well safeguard tissues from ROS and thus thwart oxidative impairment linked illnesses.

Inhibition of lipid peroxidation

Malondialdehyde is in many instances the most abundant individual aldehyde resulting due to lipid peroxidation [26]. In this study, plant extract inhibits the production of malondialdehyde from the lipid peroxidation. Phenol and flavonoid contents of extract could protect the cells from oxidative damage. Maximum inhibition 91.86% was observed at the concentration of 1000 µg/ml. BHT, a typical antioxidant exhibited maximum inhibition of 98.62% at the concentration of 100 µg/ml.

DPPH free radical scavenging activity

The DPPH free radical is a stable free radical, which has been widely used for estimating the free radical-scavenging activities of plant extract. The antioxidant properties of natural and synthetic antioxidants are believed to be responsible for their beneficial effects during treatment of inflammation disorders. Phenolic compound of the extract is possibly involved in their free radical reactions by reducing the stable DPPH radical to a yellowish colored diphenylpicrylhydrazine derivative. Maximum inhibition 89.93% was observed at the concentration of 1000 µg/ml. BHT, a standard antioxidant exposed the extreme inhibition 87.21% at the concentration of 100 µg/ml.

***In vitro* anti-inflammatory activity**

Inflammation, which is a functionally protective response, can be considered as a complex series of events that develop when the body is injured either by mechanical or chemical agents by a self-destructive process. In many inflammatory disorders, there is an excessive activation of phagocytes and production of free radicals which increase vascular permeability, protein denaturation and membrane alteration [27]. High concentration (1000 µg/ml) of the extract revealed anti-inflammatory efficacy up to 98.66%, 99.49%, and 99.50% by inhibiting protease activity, albumin denaturation and membrane stabilization, respectively, which is comparable to the activity of the standard aspirin (Table 3). The extract exhibited anti-inflammatory efficacy in a dose-dependent manner.

Inhibition of proteinase activity

Neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and a significant level of protection was provided by proteinase inhibitors. The extract exhibited significant antiproteinase activity. Maximum inhibition 98.66% was observed at the concentration of 1000 µg/ml. Aspirin, a normal anti-inflammatory drug revealed the maximum inhibition of 99.44% at the concentration of 100 µg/ml.

Table 2: Antioxidant activity of fruits of *L. acutangula* L.

S. No.	Concentration (µg)	Inhibition of lipid peroxidation (%)	DPPH free radical scavenging activity (%)
1	1000	91.86±0.49	89.93±0.35
2	500	88.85±1.55	85.84±1.83
3	250	81.34±0.07	81.53±4.03
4	125	76.07±0.49	79.31±0.70
5	62.5	64.66±2.25	78.10±0.85
6	31.25	56.39±0.98	77.43±0.56
7	15.6	55.64±2.68	76.55±1.20
8	7.81	48.12±0.63	76.22±1.69
9	3.90	44.36±0.84	62.83±1.48
10	1.95	41.35±1.34	60.29±0.63
11	BHT (100 µg/ml)	98.62±4.25	87.21±0.14

Values are mean±SD (n=3). SD: Standard deviation, BHT: Butylated hydroxytoluene

Inhibition of albumin denaturation

Inflammation is a typical defensive retort to tissue damage instigated by physical trauma, noxious chemical or microbial agents. Protein denaturation is a procedure in which proteins lose their tertiary structure and secondary structure due to peripheral stress or compound, such as strong acid or base, or due to a concentrated inorganic salt, or an organic solvent or heat. Most biological proteins miss their biological role once denatured. Denaturation of proteins is a well-documented source of inflammation and moreover one of the reasons of rheumatoid arthritis. Production of autoantigen in certain arthritic ailments might be because of denaturation of protein. The mechanism of denaturation possibly includes modification of electrostatic, hydrogen, hydrophobic and disulfide bonding [28]. As part of the investigation on the mechanism of the anti-inflammation activity, ability of extract protein denaturation was studied. It was effective in inhibiting heat-induced albumin denaturation. Maximum inhibition of 99.49% was observed at the concentration of 1000 µg/ml. Aspirin, a standard anti-inflammatory drug showed maximum inhibition of 99.51% at the concentration of 100 µg/ml.

Membrane stabilization

The erythrocyte membrane is similar to the lysosomal membrane and its steadiness infers that the extract might alleviate lysosomal membranes. Steadying of lysosomal is significant in restraining the inflammatory response by averting the discharge of lysosomal ingredients of triggered neutrophil, for example, bacterial enzymes and proteases that creates additional tissue swelling and impairment on extra cellular discharge. The lysosomal enzymes discharged through inflammation create several ailments. The extra cellular movements of these enzymes are supposed to be linked to severe or prolonged inflammation [29]. The nonsteroidal drugs act both by inhibiting these lysosomal enzymes and by alleviating the lysosomal membrane. These outcomes afford signal for membrane balance as an extra mechanism of their anti-inflammatory result. The extract repressed the warmth tempted hemolysis of RBCs to fluctuating degree as presented in Table 3. Maximum inhibition of 99.50% was detected at the concentration of 1000 µg/ml. Aspirin, a common anti-inflammatory medicine disclosed extreme inhibition of 99.52% at the concentration of 100 µg/ml. The extract might perhaps inhibit the release of lysosomal content of neutrophils at the spot of inflammation. These neutrophils lysosomal constituents comprise bactericidal enzymes and proteinases, which on extracellular release cause more tissue inflammation and impairment [30]. Current studies have shown that many flavonoids and related polyphenols contribute significantly to the anti-inflammatory activity of many plants. Due to the presence of these compounds, the fruits of *L. acutangula* L. extract might have shown anti-inflammatory activity.

Antibacterial activity

In this work, four different concentrations of the lyophilized aqueous extract have been evaluated for antibacterial activity against food poison causing bacterial pathogens employing disc diffusion method and the data of the results obtained were shown in Table 4. Existing data obtained on plant extracts showed higher antibacterial activities against Gram-positive bacteria than Gram-negative bacteria [31,32]. This has been endorsed to structural disparities witnessed in the bacterial cell envelope (including those of cytoplasmic membrane and cell wall components) between Gram-positive and Gram-negative bacteria [33]. Nonetheless, in the existing study, fruit extract inhibited both Gram-positive and Gram-negative pathogens equally.

Extract exerted better activity at 1000 µg/disc concentration against the tested microorganisms. The results suggested that antibacterial

Table 3: Anti-inflammatory activity of fruits of *L. acutangula* L.

S. No.	Concentration (µg)	Protease inhibition activity (%)	Inhibition of albumin denaturation efficacy (%)	Membrane stabilization efficacy (%)
1	1000	98.66±0.28	99.49±0.49	99.50±2.47
2	500	98.15±1.20	99.05±2.33	99.45±1.69
3	250	97.81±14.01	98.05±1.55	99.36±2.75
4	125	97.79±13.06	97.39±1.35	99.06±7.34
5	62.5	97.74±12.23	96.69±6.08	98.73±3.46
6	31.25	97.66±13.8	95.95±2.26	98.44±0.35
7	15.6	97.63±12.86	95.30±3.88	98.18±2.12
8	7.81	97.56±8.13	93.86±5.23	97.98±5.45
9	3.90	97.24±2.82	92.01±7.28	97.77±1.97
10	1.95	97.20±3.74	90.50±9.97	97.57±4.12
11	Aspirin (100 µg/ml)	99.44±2.89	99.51±3.59	99.52±2.51

Values are mean ± S.D. (n=3), SD: Standard deviation, *L. acutangula: Luffa acutangula*

Table 4: Antibacterial activity of fruits of *L. acutangula* L.

S. No.	Bacterial organisms screened	Zone of inhibition (mm in diameter)					
		Control	Standard*	A (125µg)	B (250µg)	C (500µg)	D (1000µg)
1	*Bacillus cereus*	-	9±0.03	5±0.05	6±0.06	8±0.08	9±0.03
2	*Staphylococcus aureus*	-	9±0.05	4±0.04	6±0.04	7±0.05	9±0.09
3	*Campylobacter jejuni*	-	9±0.04	6±0.09	7±0.03	8±0.07	9±0.07
4	*Salmonella enteritidis*	-	8±0.05	4±0.06	6±0.07	7±0.01	8±0.08

*Gentamicin (30 µg/disc); Values are mean±S.D. (n=3), SD: Standard deviation

activity of fruits of *L. acutangula* L. against test organisms increased in high concentration. The result of this study indicated that some major bioactive compounds present in the test drug might have inhibited the bacterial growth.

CONCLUSION

The data of the results presented in this study depicted that lyophilized aqueous extract of fruits of *L. acutangula* L. possess antioxidant, anti-inflammatory and antibacterial activities. Further, in depth studies in this plant drug can lead to the development of eco-friendly herbal antioxidant and anti-inflammatory drug.

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Research Article

IN VITRO ANTI-INFLAMMATORY ACTIVITY OF METHANOL EXTRACT OF *PEDALIUM MUREX*

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ABSTRACT

Methanol extract of whole plant of *Pedalium murex* (Family: Pedaliaceae) was assessed for anti-inflammatory activity by *in vitro* method like membrane stabilization at different concentrations. Diclofenac sodium was used as standard drug. The results showed that *P. murex* methanol extract at a concentration range of 100-300 µg/ml. Hypotonicity-induced hemolysis was significantly inhibited at the concentration range of 300 µg/ml. The results obtained in this study indicate that methanol extract of *P. murex* can be a potential source of anti-inflammatory agent.

Keywords: *Pedalium murex,* Anti-inflammatory, Human red blood cell.

INTRODUCTION

Recently, plant-derived substances have become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs [1].

Inflammation

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as the increase of vascular permeability, increase of protein denaturation, and membrane alteration. When cells in the body are damaged by microbes, physical agents or chemical agents, the injury occurs in the form stress. Inflammation of tissue is mainly resulted from stress response. It is a defensive response that is characterized by redness, pain, heat, and swelling, and loss of function in the injured area. Loss of function will occur depending on the site and extent of the injury. Since inflammation is one of the body's non-specific internal systems of defense, the response of a tissue to an accidental cut is similar to the response that results from other types of tissue damage, caused by burns such as heat, radiation, bacterial or viral invasion [2].

When tissue cells become injured they release kinins, prostaglandin, and histamine. These work collectively to cause increased vasodilation (widening of blood capillaries) and permeability of the capillaries. This leads to increased blood flow to the injured site. These substances also act as chemical messengers that attract some of the body's natural cells a mechanism known as chemotaxis. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. Several experimental protocols of inflammation are used for evaluating the potency of drugs. The management of inflammation-related diseases is a real issue in the rural community; the population in these areas uses many alternative drugs such as substances produced from medicinal plants. Hence, this investigation is aimed to screen the anti-inflammatory potential of whole plants of *Pedalium murex* Linn by employing *in vitro* assays.

METHODS

Collection, identification, and authentication of plant material

The plant species *P. murex* was collected from in and around Thirukollikadu, Thiruvarur District, Tamil Nadu, India. The plant was identified with the help of the Flora of presidency of Madras and authenticated by Dr. S. John Britto, RAPINAT Herbarium, St. Joseph College, Tiruchirappalli.

Preparation of plant powder

The plant was air dried under shade for 10-15 days. Then, the dried material was ground to fine powder using an electric grinder and stored in air tight bottles. The powder was used for further analysis.

Preparation of the aqueous extract

The plant materials (whole plant) were shade dried and coarsely powdered with an electrical blender. 200 g of *P. murex* was mixed with 1200 ml of water. Then, it was boiled until it was reduced to one-third and filtered. The filtrate was evaporated to dryness. A paste form of the extract obtained was subjected to *in vitro* assays.

Preparation of the methanol extracts

Methanol extracts were prepared according to the methodology of Indian Pharmacopoeia [3]. The coarse powder material was subjected to Soxhlet extraction separately and successively with 210 ml methanol and 90 ml distilled water. The extract was concentrated to dryness in flash evaporator under reduced pressure controlled at a temperature (40°C-50°C). The paste form of the extract was put in an air tight container and stored in refrigerator.

In vitro anti-inflammatory assay

Preparation of red blood cells (RBCs)

The blood was collected from healthy human volunteer who has not taken any nonsteroidal anti-inflammatory drugs for 2 weeks before the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 minutes and were washed 3 times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline.

Hypotonicity-induced hemolysis

Different concentration of extract (100-500 µg/ml), reference sample, and control were separately mixed with 1 ml of phosphate buffer, 2 ml of hyposaline, and 0.5 ml of human RBC (HRBC) suspension. Diclofenac sodium (100 µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37°C for 30 minutes and centrifuged at 3000 rpm.

Table 1: *In vitro* anti-inflammatory activity of *P. murex*

S.No.	Concentration (µg/ml)	Aqueous extract		Methanolic extract	
		Optical at density 560 nm	% of inhibition	Optical at density 560 nm	% of inhibition
1.	Control why the values in this control group are "0"	0.00	0.00	0.00	0.00
2.	100	0.20	93.33	0.06	99.94
3.	200	0.18	99.82	0.05	99.95
4.	300	0.13	95.66	0.04	99.96
5.	Diclofenac sodium	0.03	100	0.02	100

P. murex: *Pedalium murex*

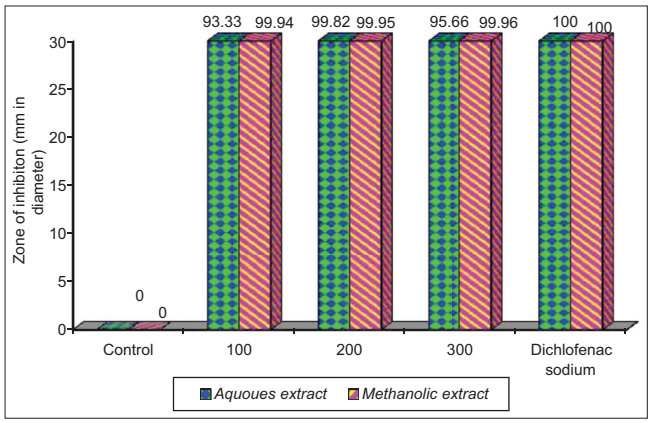

Fig. 1: *In vitro* anti-inflammatory activity of *Pedalium murex*

The supernatant liquid was decanted and the hemoglobin (Hb) content (Here you are analyzing the content of Hb, but only the denaturation of RBC membrane, so revise the sentences accordingly) was estimated by a spectrophotometer at 560 nm. The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100%. Percentage protection = 100 − (OD sample/OD control) × 100 it was carried out as per the method of Sadique *et al.* [4].

Only this RBC membrane stabilization assay is sufficient to investigate the anti-inflammatory activity of your plant sample? You could also give some other *in vitro* methods such as protein denaturation, serine protease enzyme inhibition, Cox enzyme inhibition, and cell line studies as experimental evidence.

RESULTS AND DISCUSSION

In vitro anti-inflammatory activity

The HRBC membrane stabilization has been used as a method to study the *in vitro* anti-inflammatory activity because the erythrocyte membrane is analogs to the lysosomal membrane (Shenoy *et al.*, 2010) [5] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage on extra cellular release. The lysosomal enzymes released during inflammation produce various disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The nonsteroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane [6].

Anti-inflammatory property of the methanolic extract was estimated by HRBC membrane stabilization method. This method showed the significant anti-inflammatory property at a concentration of 100 µg/ml when compared to that of standard drug diclofenac sodium (Fig. 1 and Table 1).

It is also essential to analyze the phytochemicals in the extract, which are responsible for the anti-inflammatory potential of this plant.

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Research Article

DETERMINATION OF QUERCETIN BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY METHOD IN *ACHYRANTHES ASPERA* (L.) PLANT EXTRACT

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ABSTRACT

Objective: *Achyranthes aspera* (L.) is a traditional medicinal plant used as pungent, antiphlegmatic, antiperiodic, diuretic, purgative and laxative, and also useful in edema, dropsy, piles, and eruptions of skin. Phytochemical analysis of the plant showed the presence of alkaloids, glycosides, proteins, phytosterols, phenol, and flavonoids. In this study, an attempt was made to quantify the flavonoid quercetin present in the whole plant extract.

Methods: Thin layer chromatography (TLC) was done to confirm the presence of quercetin and high performance TLC (HPTLC) method has been developed for quantification of quercetin in the ethanolic whole plant extract. TLC silica gel 60 F_{254} plate was used as stationary phase and the solvent system used is toluene: Ethyl acetate: Formic acid: Ethanol (5:4:1) as the mobile phase. Quantitative analysis was carried out in the absorbance at 254 and 366 nm using CAMAG REPROSAT3.

Results: The percentage amount of quercetin in *A. aspera* ethanolic extract was found to be 0.1152% w/w.

Conclusions: However, further detailed studies are required to determine the active components responsible for these effects and mechanism pathway.

Keywords: *Achyranthes aspera*, Quercetin, High performance thin layer chromatography.

INTRODUCTION

Plants have been used as medicines since thousands of years. People depend on plants for several purposes for wood, timber, nontimber forest products, food, medicine, etc., [1]. They have always been used as a rich source of biologically active drugs and have numerous medicinal uses and have served mankind for thousands of years [2]. Nowadays, they are used widely because of growing awareness among people regarding side effects and high cost of the allopathic medicines.

Medicinal plants are integral part of human society to combat diseases, since the dawn of civilization [3]. Medicinal plants are important sources of previously unknown chemical substances with potential therapeutic effect. The medicinal use of plants is an ancient tradition, much older than the contemporary sciences of medicine, pharmacology, and chemistry. The World Health Organization has estimated that over 75% of the world's population still relies on plant derived medicines, usually obtained from traditional healers, for their basic health care needs. Herbal medicines are in great demand in the developed as well as developing countries for primary health care because of their wide biological and medicinal activities, higher safety margins, and lesser cost.

Flavonoids are water soluble phytochemical possessing the antioxidant, anticancer, and anti-inflammatory activities. These prevent cells from oxidative damage and carcinogenesis. Flavonoids are also inhibit free radical generation [4]. Flavonoids occur virtually in all parts of the plant, the root, heart wood, sap wood, bark, leaf, fruit, and flower [5].

High performance thin layer chromatography (HPTLC) analysis

HPTLC is one of the sophisticated instrumental techniques for qualitative and quantitative analysis of the herbs and herbal drugs. HPTLC is a modern adaptation of TLC with better and advanced separation efficiency and detection limits.

Quantitative estimation of these compounds is important for current research and a variety of methods are required for this. TLC and HPTLC are the methods primarily used for separation, qualitative identification, and semi-quantitative visual analysis of the samples [6]. HPTLC is an important tool that can be used qualitatively as well as quantitatively for checking the purity and identity of crude drug and also for quality control of finished products [7]. Consequently, this study was focused on the quantitative estimation of the flavonoid in the herbal species *Achyranthes aspera* using HPTLC.

METHODS

Collection, identification, and authentication of plant material

The plant *A. aspera* was collected from in and around Koothanallur, Thiruvarur District, Tamil Nadu, India. The plant was identified with the help of the flora of presidency of Madras and authenticated by Dr. S John Britto, RAPINAT Herbarium and Centre for Molecular Systematics, St. Joseph's College, Tiruchirappalli (Voucher number of the specimen, AMTA 001) [8].

Preparation of plant powder

The plant was air dried under shade for 10-15 days. Then the dried material was ground to fine powder using an electric grinder and stored in air tight bottles. The powder material was used for further analysis.

Preparation of the ethanol extract

Ethanolic extract was prepared according to the methodology of Indian pharmacopoeia [9]. The coarse powder material was subjected to Soxhlet extraction separately and successively with 210 ml ethanol and 90 ml distilled water. These extracts were concentrated to dryness in flash evaporator under reduced pressure controlled at a temperature (40°C-50°C). The paste form of the extracts was put in an air tight container stored in refrigerator.

Chromatographic profiling

HPTLC

HPTLC was performed with a view to develop chemical standards. Flavonoids were analyzed by HPTLC according to the method proposed by Kritikar and Basu [10].

HPTLC instrumentation

In this work analysis of flavonoid was performed with the help of HPTLC instrument. The HPTLC system (CAMAG, Muttenz, Switzerland) consists of the following:

1. Linomat V sample applicator.
2. Photo documentation system CAMAG, Reprostar III.

Preparation of sample

Weighed about 103.2 mg of ethanol extract and dissolved the same in 10 ml of ethanol and used for TLC analysis.

Preparation of standard

20.3 mg of quercetin standard was dissolved in 10 ml of ethanol. From the stock pipetted out 10 ml and from the standard solution 0.5-3.5 µl was spotted in the range of 100-700 ng.

Preparation of stationary phase

TLC plate precoated with silica gel 60 F_{254}, obtained from Merck was used as stationary phase. The thickness of the plate was 0.2 mm.

Preparation of mobile phase

Toluene: Ethyl acetate: Formic acid (5:4:1).

Chamber used for mobile phase

CAMAG Twin Trough Chamber was used.

Chamber saturation

Chamber saturation was done for 18 hr.

Procedure

The samples and standard were prepared. The TLC plate was activated by heating at 120°C for about 30 minutes before use. Ethanol extract of standard and sample were applied with Linomat V applicator. The mobile phase used was toluene: Ethyl acetate: Formic acid (5:4:1). No prewashing of the plate was done. Chamber saturation time was 18 hr. The TLC plate was kept for development to a migration distance of 8 cm. The plate was dried in hot air oven at 108°C for 10 minutes and scanned at 254 nm. The RF and peak area of the spots were interpreted.

Development of chromatogram

After the application of sample, the chromatogram was developed in twin trough glass chamber 20×10 cm saturated with solvent vapors of toluene: Ethyl acetate: Formic acid (5:4:1). The linear ascending development was carried out and 15 ml of mobile phase was used per chromatography run.

Photodocumentation

The plate was photo documented under 254 nm and 366 nm light using CAMAG REPROSTAR 3, equipped with 12 bit CCD camera. A band RF value of 0.58 corresponding to Quercetin is visible in test solution tracks.

RESULTS AND DISCUSSION

HPTLC analysis

HPTLC fingerprint analysis and quantitative analysis of marker compound were carried out using modern analytical techniques. In the last few decades HPTLC has become an important tool for the qualitative, semi-qualitative and quantitative phytochemical analysis of herbal drugs and formulations. The major advantage of HPTLC is several samples can be analyzed simultaneously using a small quantity of marker compound and mobile phase with very less time [11].

In this investigation, the flavonoid compounds were analyzed by HPTLC method. A densitometry HPTLC analysis was performed for the progress of characteristic fingerprint sketch which may be used as markers for quality evaluation and standardization of the herbal drug.

Fingerprinting analysis of sample was done through HPTLC method and the selected solvent system toluene: Ethyl acetate: Formic acid (5:4:1) was suitable for quantitative analysis. HPTLC fingerprinting of the plants under study was presented to photo documentation under 254 nm and 366 nm along with RF values (Fig. 1). Fig. 2 showed the HPTLC chromatogram of ethanolic extracts of *A. aspera*. Fig. 3 showed several spots (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 µg of sample) with maximum RF values (0.57, 0.58, 0.57, 0.57, 0.57, 0.57, 0.57). Fig. 4 showed (20, 30 and 40 µg of sample) three spots with the following RF values: (0.60, 0.60, 0.60). For quantitative analysis through HPTLC techniques, optimization of solvent system was found to be toluene: Ethyl acetate: Formic acid (5:4:1). Quantitative analysis was performed through HPTLC techniques using quercetin as standard marker compound in the *A. aspera*. In the HPTLC fingerprinting of ethanolic extract of *A. aspera* gave a band with RF value of 0.60, which was corresponding to quercetin as visible in test solution track. HPTLC photograph of standard quercetin and ethanolic extract of *A. aspera* were presented in the Fig. 5.

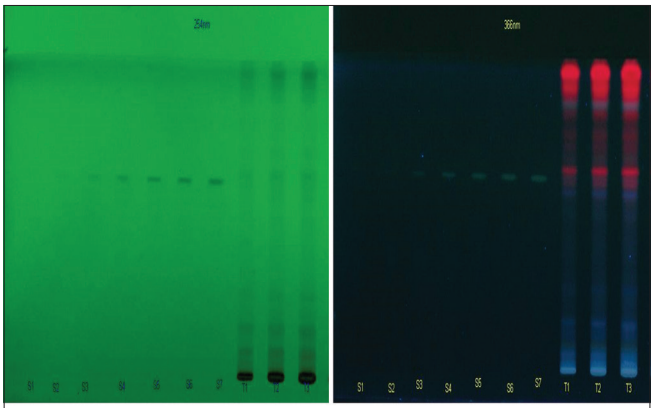


Fig. 1: Quantification of quercetin in *Achyranthes aspera* L. ethanolic extract photo documentation under UV. Thin layer chromatography details: T1-T6-0.5-3.0 µl of standard quercetin solution, T1-T4-10-25 µl of sample solution

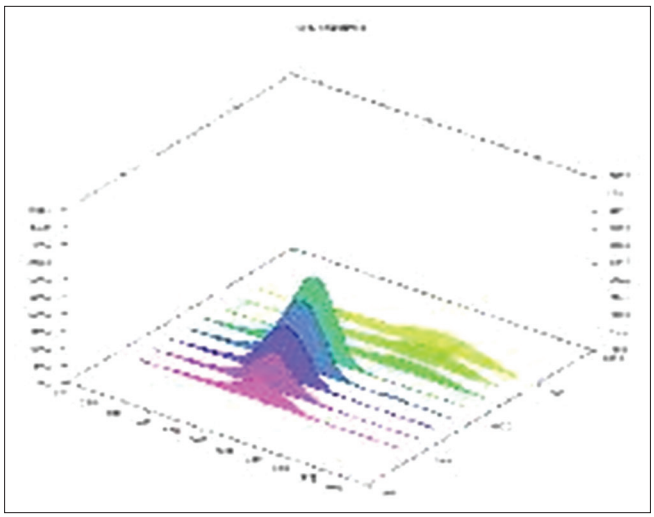
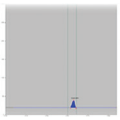


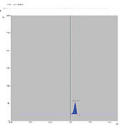
Fig. 2: High performance thin layer chromatography chromatogram of ethanolic extracts of *Achyranthes aspera* 3D DISPLAY @ 254 nm

Peak display (0.5µl of standard)



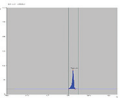
Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.53	0.4	0.57	106.9	100.00	0.60	1.0	1379.8	100.00	Quercetin

Peak display (1 µl of standard)



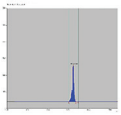
Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.54	0.2	0.58	31.6	100.00	0.60	0.1	477.9	100.00	Quercetin

Peak display (1.5 µl of standard)



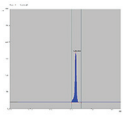
Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.53	0.0	0.57	63.3	100.00	0.60	0.2	835.4	100.00	Quercetin

Peak display (2 µl of standard)



Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.53	0.4	0.57	106.9	100.00	0.60	1.0	1379.8	100.00	Quercetin

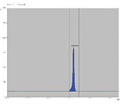
Peak display (2.5 µl of standard)



Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.53	0.5	0.57	146.4	100.00	0.59	0.3	1857.3	100.00	Quercetin

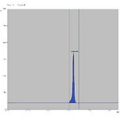
Fig. 3: High performance thin layer chromatography chromatogram of ethanolic extract of *Achyranthes aspera*

Peak display (3 µl of standard)



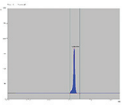
Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.53	0.7	0.57	193.1	100.00	0.59	0.6	2449.0	100.00	Quercetin

Peak display (3.5 µl of standard)



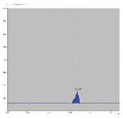
Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.54	5.9	0.57	227.9	100.00	0.59	0.3	2921.2	100.00	Quercetin

Peak display (20 µl of standard)



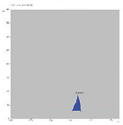
Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.54	0.1	0.60	31.5	100.00	0.63	0.3	837.3	100.00	Quercetin

Peak display (30 µl of standard)



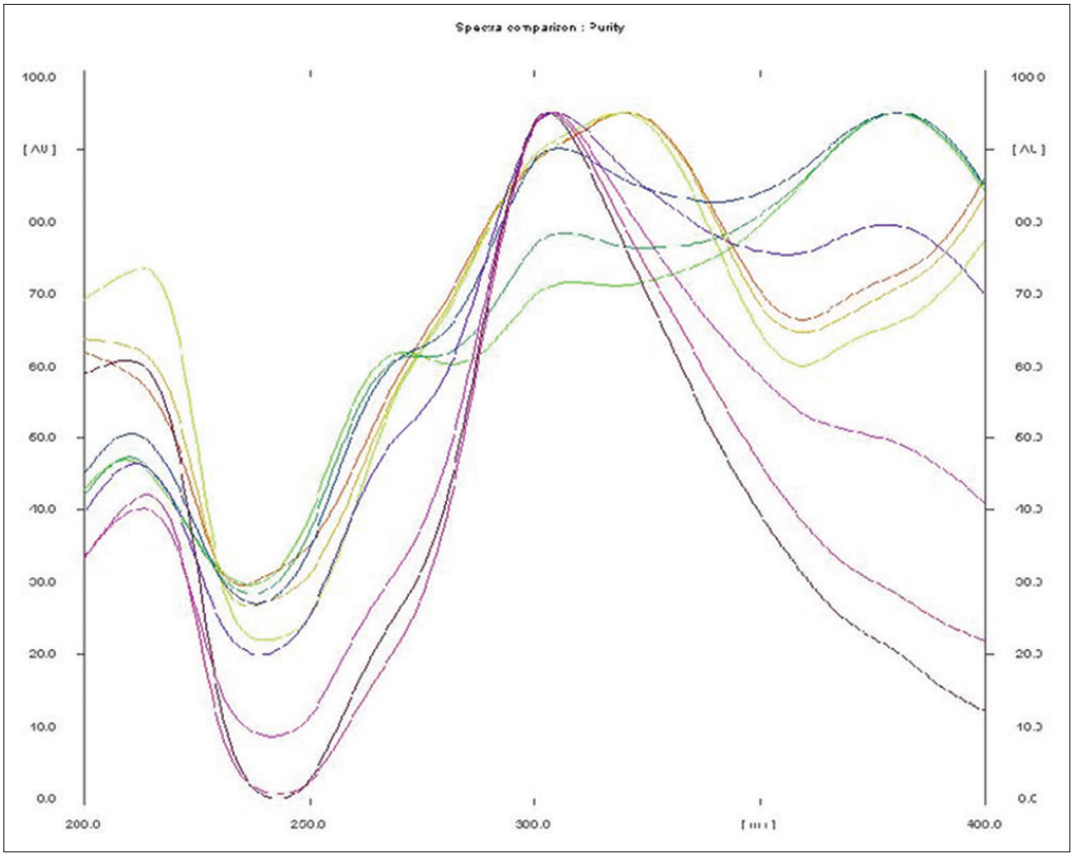
Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.54	0.3	0.60	45.2	100.00	0.36	0.1	1183.0	100.00	Quercetin

Peak display (40 µl of standard)



Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %	Assigned substance
1	0.54	0.1	0.60	55.1	100.00	0.63	0.2	1532.2	100.00	Quercetin

Fig. 4: High performance thin layer chromatography chromatogram of ethanolic extract of *Achyranthes aspera*



Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)	Sample D/remark
1	1							Not used
2	1							Not used
3	1	0.57	300.00 ng	63.12		835.40		
4	1	0.57	400.00 ng	106.88		1380.11		
5	1	0.57	500.00 ng	146.56		1864.40		
6	1							Not used
7	1							Not used
8	2	0.60		31.51	270.00 ng	837.25	298.40 ng	Spl-Ethanolic
9	3	0.60		45.17	270.00 ng	1182.98	365.60 ng	Spl-Ethanolic
10	4	0.60		55.08	278.00 ng	1532.16	433.47 ng	Spl-Ethanolic
11	5							Not used
12	6							Not used

Fig. 5: Spectral comparison for purity

The percentage amount of quercetin in *A. aspera* ethanolic extract was found to be 0.1152%. HPTLC method confirms the presence of flavonoid such as quercetin in ethanolic extract of *A. aspera*.

HPTLC fingerprinting is ideal which involves comparison between a standard and a sample. The chromatographic studies conducted with the ethanolic extract of *A. aspera* revealed appreciable amount of flavonoid quercetin content, which supports its traditional medicinal applications.

ACKNOWLEDGMENT

We would like to express our sincere and heartfelt thanks to our beloved correspondent Dr. V Dhivaharan, M.Sc., D E M., Ph.D., Department of Life Sciences, S T E T Women's College, Sundarakkottai, Mannargudi, for his encouragement and for providing adequate facilities to complete the project work successfully.

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Research Article

EVALUATION OF POMEGRANATE AND LEMON PEELS AS NATURAL SOURCE OF ANTIOXIDANTS

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ABSTRACT

Objectives: Industrially, peels of lemon (*Citrus limon* L.) and pomegranate (*Punica granatum* L.) are considered as agricultural waste by-products. In this study, emphasis was given to investigate the polyphenolic yield of lemon and pomegranate peels using solvent extraction, acid and alkali hydrolysis and attempts were also made to evaluate their antioxidant potential.

Methods: The peels were dried and powdered and then extracted in solvent (ethanol), acid hydrolysis and alkali hydrolysis at different timings. The total phenolic concentration and their antioxidant activity were evaluated using Folin-Ciocalteu reagent method and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, respectively.

Results: Higher yield of phenolic compounds was noted in lemon peel during acid hydrolysis (1300 mg gallic acid equivalents [GAE]/100 g) and in pomegranate peels (1465.50 mg GAE/100 g). The results of antioxidant assay revealed maximum activity for ethanolic extract of lemon peels (95.54%) when compared to acid hydrolysate of pomegranate peel (92.28%). The solvent extracts were purified by liquid-liquid partitioning and column chromatography, and the active fractions were subjected for estimating total phenolic content. Further, lipid peroxidation inhibition potential of lemon and pomegranate peels was investigated in butter sample.

Conclusion: The agro-food byproducts such as pomegranate and lemon peels could be explored as natural and inexpensive source of antioxidant agents for their application in food industries.

Keywords: Pomegranate peel, Lemon peel, Extract, Antioxidant, Polyphenols.

INTRODUCTION

Food spoilage means damage of original nutritional value, texture and flavor of the food and the food material becomes unfit for consumption. Food spoilage has been a major problem in food industry which occurs mainly due to oxidation of lipids and microbial contamination [1]. Lipid peroxidation occurs mostly in unsaturated fatty acids since it contains double bonds. Reactive oxygen species such as superoxide, hydroxyl and lipid peroxyl radicals cause lipid peroxidation and ultimately food spoilage. Synthetic antioxidant -such as butylated hydoxy toluene (BHT), butylated hydroxyl anisole (BHA), and propyl gallate -lhas been used to prevent food spoilage [2]. Natural antioxidants are preferred over synthetic antioxidants since the later produce some harmful effects on human beings. Natural antioxidant such as vitamin A, E, and C can be used to prevent food spoilage, but they are present in minor quantities in food, so they are not sufficient to preserve the food. As an alternative, plant phenolic compounds, which are strong antioxidants can be used as natural food preservatives. In this project, we are focusing on extraction of phenolic antioxidants from two locally available agro-food by-products such as pomegranate and lemon peels (Fig. 1) so that they can be used as natural food preservatives.

Pomegranate (*Punica granatum* L.), originated in the Middle East and India, is believed to have high medicinal value. It is widely cultivated in the Mediterranean regions of Southern Europe, the Middle East and Caucasus regions, Northern Africa, the Indian sub-continent, central parts of Asia [3]. Pomegranate is known as fruit of Eden because of its taste and beneficial health properties [4]. The overall world production of pomegranate per year is 1.5 billion tons [3]. The pomegranate tree typically grows 12-16 feet height. It has many spiny branches and can be extremely long-lived known to be 200 years old. The leaves are glossy and lance-shaped, and the bark of the tree turns gray over years. The flowers are large, red, white, and have a tubular calyx that becomes the fruit. The pomegranate fruit (can be up to five inches wide with a deep red and leathery skin) is grenade-shaped and crowned by the pointed calyx. The fruit contains many seeds (arils) separated by white, membranous pericarp and each is surrounded by small amounts of tart and red juice [5].

Pomegranate peel accounts for 60% of its dry weight. Pomegranate peel has the highest antioxidant activity among pulp and seed fractions. Pomegranate peel has been used as a colorant for textiles because of their high phenolic and tannin content [6]. Pomegranate peel contains 30% of the entire fruit's anthocyanidins. Pomegranate peel can be used to increase the food oxidative stability [4]. Pomegranate peel extract was added to ice creams to increase the total phenolic level and antioxidant capacity without negatively influencing the content of *Lactobacillus casei* [7]. The treatment with pomegranate peel extract increased the stability of preserved goat fish against lipid oxidation [8]. Dietary supplementation of cattle with fresh pomegranate peel extract will promote a significant increase in feed intake and alpha-tocopherol plasma concentration, with positive tendency toward increased weight gain of bull calves and can contribute to the economic growth of cattle breeding [9]. Pomegranate peel extract can be used as natural antioxidant for the stabilization of vegetable oils thereby substituting synthetic antioxidants such as BHT and BHA [2]. Pomegranate peel extract phenolics and flavonoids act as inhibitors against food-borne pathogens. Addition of pomegranate rind powder in raw beef sausages up to 3% improves their functional characteristics [4].

Lemon (*Citrus limon* L.) is the most important species with an annual production more than 4.4 million tons worldwide. Argentina with 1.2 million tons is currently the world's largest producer of lemon [10]. The top producers of lemon are India, Mexico, Argentina, Brazil, Spain, Republic of China, United States, Turkey, Iran, and Italy [11]. India

grows lemon in about 3000 hectares largely in the states of Kerala, Uttar Pradesh, Assam, Karnataka, India's annual production is 300 to 350 tons per year [12]. Botanically, this *Citrus* fruit belongs to the family Rutaceae and the genus, *Citrus*. Lemon is a large bushy shrub or small trees which reach a height of 10-20 feet. The leaves of the lemon are dark green. The lemon has five petals white fragrant flowers. Lemons are oval *Citrus* fruits with smooth porous skin. The color range of lemon fruit is from greenish yellow to bright yellow. Lemons look very similar to limes, but lemons tend to be a little larger and are yellow when ripe, where limes are green [11].

Lemon peel has two different tissues such as flavedo and albedo. Flavedo is the peel's outer layer, whose color varies from green to yellow. It is a rich source of essential oils, which is used in flavor and fragrance industry. Albedo is the major component of lemon peel, which is a spongy and cellulosic layer and has high fiber content. Nowadays, lemon peel is used as a flavoring agent in perfumery and as stomachic and carminative and also is a source of proteins, fats, and minerals [10].

This study was designed with the objectives of recovery of polyphenolic compounds from pomegranate and lemon peels using solvent with/without mechanical stirring, acid hydrolysis, and alkali hydrolysis and analyzing total phenolic content, antioxidant activity and also to evaluate lipid peroxidation inhibition capacity in model food system.

METHODS

Sample collection

Pomegranate and lemon peels were collected from Kumbakonam during October 2015. The peels were sun dried and powdered to 1 mm particle size using lab mill at Pharmacy unit of CARISM, SASTRA University and stored for future studies.

Extraction of polyphenols

The powdered samples (10 g) were extracted in 100 ml solvent (70% ethanol) and kept at two different conditions (with and without shaking) for 5 hrs at room temperature. After every 1 hr, the contents were filtered and the filtrate was analyzed for total phenolic concentration and antioxidant activity. For acid hydrolysis, the samples (10 g) were mixed with 100 ml of 2% hydrochloric acid and for alkali hydrolysis; the samples were mixed with 2% sodium hydroxide and kept for 6 hrs at room temperature. After every 1 hr, the contents were filtered and the filtrate was analyzed for total phenolic concentration and antioxidant activity.

Total phenolic content

The total phenolic concentration was evaluated using Folin–Ciocalteu reagent method [13]. Extract (10 µl) was added to the microplate wells in duplicate and Folin's reagent (25 µl) and sodium carbonate (4.4%, 230 µl) were added. Then, the microplate was incubated for 30 min in the dark and read at 750 nm in the plate reader (make: Biotek, model: Epoch). The calibration curve was prepared with gallic acid with the linear regression formula ($Y = 0.0026x + 0.182$, $R^2 = 0.994$). The total phenol content was calculated using the formula TPC = (absorbance −c/m × dilution factor), where c is the constant obtained from gallic acid curve and m is the slope of gallic acid curve and expressed as mg gallic acid equivalents (GAE/L) extract. Total phenol yield was calculated using the formula (TPC × volume of extract/weight of sample × 100).

Antioxidant activity

Antioxidant effect of polyphenol extracts was analyzed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [14]. The DPPH reagent is prepared by dissolving 5 mg of DPPH in 100 ml of methanol. The extract (10 µl) was taken in microplate wells and 200 µl of DPPH reagent was added and incubated for 30 min in the dark. Then, the plates were read at 515 nm in a plate reader. The antioxidant activity can be calculated using the formula (absorbance of blank - absorbance of sample/absorbance of blank × 100) and expressed in percentage basis.

Lipid peroxidation inhibition

Inhibition of lipid peroxidation by the extracts was evaluated in butter sample [15]. Butter (5 g) was taken in a conical flask and 50 ml of hexane was added and shaken for 30 minutes. Then, the contents were filtered and the filtrate (lipid) was mixed with extracts (25 mg/2.5 ml) and boiled for 1 hr. No extract was added in the control while the blank was not heated. Then, 30 ml of acetic acid/chloroform (3:2 ratio) and 0.5 ml of saturated potassium iodide were added to the contents. After 2 minutes, 30 ml of distilled water was added followed by the addition of 0.5 ml of starch and titrated against 0.01 M sodium thiosulfate. The volume of sodium thiosulfate consumed was noted, and peroxide value (PV) was calculated using the formula (titration value × 0.01/weight of sample). Percentage of inhibition of lipid peroxidation by the extracts was calculated using the formula (PV control – PV extract/PV control × 100).

Purification

The samples (50 g each) were extracted with 500 ml of 70% ethanol and kept for 5 hrs at room temperature and filtered using filter paper. Slurry was prepared with dried extracts of pomegranate peel and lemon peel using silica gel. Column with 60 cm length and 2.5 cm diameter was packed with silica gel and vacuum pump was applied to speed-up the elution process. First, hexane was used to form a silica gel bed in ¾ of column length and washed with hexane (100 ml) for 2 times. Then, 2 g of the slurry was loaded above the silica bed and plugged with cotton. The extract was successively eluted with hexane, chloroform, ethyl acetate, methanol, ethanol, and water. The eluted compounds from above-mentioned solvents were collected separately in conical flasks and analyzed for total phenolic concentration.

RESULTS AND DISCUSSION

Agro-food byproducts

About 100 g of pomegranate fruit was reported to contain carbohydrate (18.7 g), protein (1.7 g), fat (1.2 g), fibre (4 g), sugar (13.7 g), calcium (10 mg), phosphorus (36 mg), iron (0.30 mg), magnesium (12 mg), potassium (236 mg), zinc (0.35 mg), vitamin C (10 mg), thiamine (0.07 mg), and riboflavin (0.05 mg) [16]. Pomegranate has been used since ancient days for various therapeutic purposes. Stomachic, inflammation, fever, bronchitis, diarrhea, dysentery, vaginitis, urinary tract infection, and malaria have been treated using various parts of pomegranate including fruit peels [17]. Pomegranate is also used to treat diabetes in Unani system of medicine which is practiced in the Middle East and India [18]. Pomegranate is found to exhibit antiviral, antioxidant, anticancer, and antiproliferative activities because of the presence of phenolic compounds [17]. Pomegranates peel, bark and leaves are used to calm the stomach disorders, diarrhea, and skin

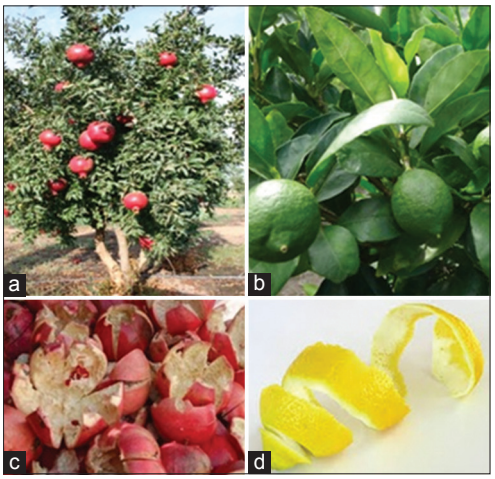

Fig. 1: Morphology of pomegranate (a) and lemon (b), plants their by products (c and d)

cancer [19]. Pomegranate is a rich source of phytochemicals such as tannins and other phenolics [17]. Pomegranate peel, an industrial waste, is a rich source of phenolic compounds such as hydrolysable ellagitannin, punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones which possess strong antioxidant activities and beneficial to our health in many ways [5].

Lemon was the primary commercial source of citric acid before the development of fermentation-based processes [20]. Lemon is used in sorbets, beverages, refreshing drinks, pickles, jams, jellies, snacks, candies, sugar boiled confectionaries and culinary and the oil extracted from its peel or skin is extensively used in soft drink concentrates, body oils, cosmetics and hair oils [11]. Lemon is used to make lemonade, soft drinks, and cocktails. Lemon juice is also used as a short-term preservative on certain foods that oxidizes quickly. The leaves of the lemon tree are used to make a tea and for preparing cooked meats and seafoods. 100 g of lemon fruit contains energy (121 kJ), carbohydrates (9.32 g), sugars (2.50 g), fats (0.3 g), dietary fiber (2.8 g), protein (1.1 g), thiamine (0.04 mg), riboflavin (0.02 mg), pantothenic acid (0.19 mg), vitamin B6 ,0.08 mg), folate (11 µg), vitamin C (53 mg), calcium (26 mg), iron (0.6 mg), phosphorus (16 mg), potassium (138 mg), and zinc (0.06 mg) [11]. It is also a source of alkaloids which are having anticancer activities and possesses antibacterial potential [21]. Lemon can be used to cure scurvy. Lemon contains flavonoids which have antioxidant, anticarcinogenic, antibiotic, and detoxifying properties which help in healing peptic and oral ulcers [11]. *Citrus* flavonoids have a large spectrum of biological activity such as antibacterial, antifungal, antidiabetic, anticancer, and antiviral activities [22]. Flavonoids can function as direct antioxidants [23] and they can act as a defense system [24]. Lemon contains alkaloids, flavonoids, tannins, phenols, and saponins. Flavonoids are present in higher percentage compared to others. They also contain liminoids and amines such as octopamine, synephrine, and tyramine [25]. Lemon peel contains flavonoids, eriocitrin, epigenin, luteolin, chrysoeriol, quercetin, isorhamnetin, limocitrin, limocitrol, isolimocitrol and hesperidin.

Total phenolic content

Polyphenols are a large class of chemical compounds found in plants. They are characterized by the presence of more than one phenol unit or building block per molecule [26]. Polyphenols are the secondary metabolite of plants which play a key role in defense system. Table 1 shows the yield of polyphenols from pomegranate and lemon peels at different conditions. From the data, the solvent extraction without agitation revealed maximum phenolic concentration (369.75 mg GAE/L) at 4th hrs, whereas in solvent extraction with agitation, the maximum phenolic concentration (224.24 mg GAE/L) was observed at 3rd hour in the case of pomegranate peels. Among all the treatments, acid hydrolysis has shown maximum concentration of 732.75 mg GAE/L at 6th h for pomegranate peels.

In the case of lemon peel, the solvent extraction without agitation exhibited maximum phenolic concentration at 1th hr (83.00 mg GAE/L) as per the data whereas in solvent extraction with agitation, the maximum phenolic concentration was observed at 6th hrs (1257.50 mg GAE/L). Acid and alkali hydrolysis showed maximum concentration of 740.50 mg GAE/L and 94.00 mg GAE/L at 1st and 5th hrs, respectively. Among these four methods, the maximum concentration of polyphenols (1257.50 mg GAE/L) was observed in lemon peel at 6th hr during ethanol extraction with agitation.

Antioxidant activity

Antioxidants are substances that can prevent or delay some types of cell damage. Higher the antioxidant activity, greater the chance of preventing cell damage [27]. Antioxidant activity of pomegranate peel at different conditions are shown in Fig. 2 which shows that the pomegranate peel

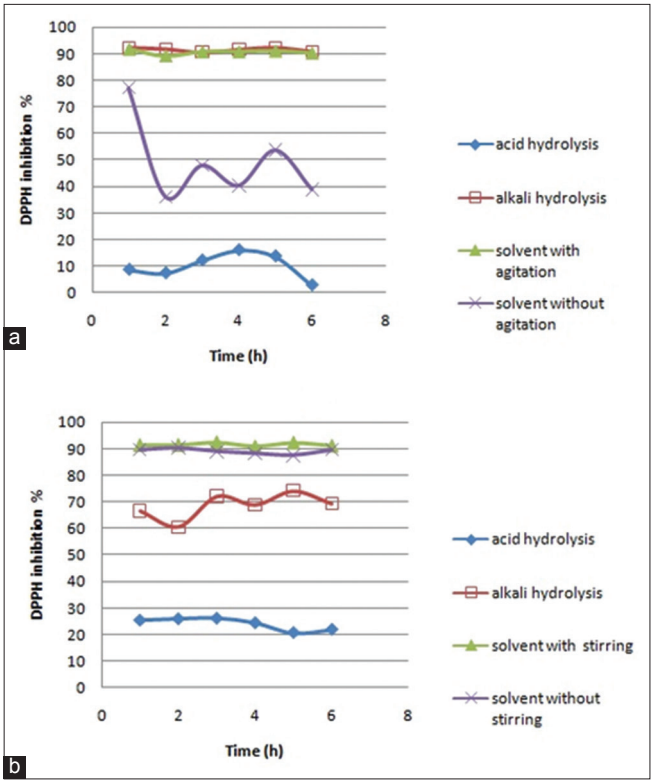

Fig. 2: Antioxidant activity of solvent extracts of pomegranate (a) and lemon (b) peels at different time intervals

Table 1: Recovery of phenolic compounds from pomegranate and lemon peels during different conditions at different time intervals

Extraction time (h)	Total phenolic concentration of pomegranate peel (mg GAE/L)			
	Solvent without agitation	Solvent with agitation	Acid hydrolysis	Alkali hydrolysis
1	86.25±1.06	72.50±22.63	319.50±33.94	0
2	231.00±21.92	125.75±46.32	429.50±70.71	0
3	249.75±0.35	224.25±199.05	414.75±2.47	0
4	369.75±17.32	136.25±64.70	303.75±69.65	0
5	330.75±34.29	85.50±38.89	665.25±37.83	0
6	258.25±70.36	152.75±6.72	732.75±45.44	0
Total phenolic concentration of lemon peel (mg GAE/L)				
1	83.00±28.28	1241.00±609.53	740.50±97.58	51.50±19.09
2	0	941.00±120.21	650.00±175.36	63.75±1.06
3	0	882.00±74.95	556.00±246.07	92.50±33.94
4	0	941.00±45.25	290.25±82.38	83.50±15.56
5	0	948.50±57.28	384.00±82.73	94.00±3.54
6	0	1257.50±6.36	315.75±3.89	76.50±4.24

GAE: Gallic acid equivalents

has the maximum antioxidant activity of 77.24% (1st hr) in ethanolic extracted prepared without agitation which was lesser than the maximum antioxidant activity (91.85% 1st hr) observed during solvent extraction with agitation maximum. Acid and alkali hydrolysis showed maximum antioxidant activity of 16.01% and 92.33% at 4th and 5th hr, respectively. Among these four conditions, maximum antioxidant activity (92.33%) was observed during alkali hydrolysis at 5th hr.

From Fig. 2, it is evident that the lemon peel showed antioxidant activity of 90.51% (2nd hr) for the ethanolic extract which was prepared whereas in same solvent extract prepared with agitation also revealed maximum antioxidant activity at 3rd hr (92.44%). Acid and alkali hydrolysis have maximum antioxidant activity of 74.06% and 26.16% at 5th and 3rd hrs, respectively. Among these four conditions, maximum antioxidant activity (92.44%) was observed during solvent extraction with agitation at 3rd hr. Antioxidant can be used to reduce oxidation in the food system to prevent food spoilage [28].

Lipid peroxidation inhibition

A lipid is a substance which is insoluble in water but soluble in alcohol. Lipids contain hydrocarbons and make up the building block of living cells. Lipid peroxidation is process in which the free radicals attack lipid containing carbon-carbon double bond which results in cell damage [29]. Fig. 3a shows the effect of pomegranate peel extract on the PV of butter sample. The PV of pomegranate peels treated butter (0.0098) was lower when compared to control sample (0.039). It is evident that the pomegranate peel shows high percentage of inhibition of lipid peroxidation (74.87%).

Fig. 3b shows the effect of lemon peel extract on the PV of butter sample. The PV of lemon peels treated butter (0.008) was lower when compared to control sample (0.039). From the data, it is evident that the lemon peel shows higher percentage of inhibition of lipid peroxidation (79.48%). Lipid peroxidation is the process in which the free radicals steel the electrons from the lipids in the cell membranes. PV is commonly used to determine the rancidity of the fat or oil when subject to oxidation [30].

Purification of the extracts

Column chromatography is a technique used for purification of individual chemical component from mixtures of compounds. In this study, column chromatography was done to purify the polyphenols present in the pomegranate peel extract. In the case of pomegranate, four fractions were collected (chloroform, ethyl acetate, methanol, and ethanol), among which the maximum phenolic concentration was noted in ethyl acetate (810 mg GAE/L) (Fig. 4). In case of lemon peel extract, only one fraction (methanol) was collected with total phenolic concentration of 155.75 mg GAE/L.

CONCLUSION

Industrially, peels of lemon (*C. limon* L.) and pomegranate (*P. granatum* L.) are considered as agricultural waste byproducts. Even though these byproducts have minor uses, still they are considered as waste and also cause environmental pollution if not handled properly. Hence, due to the presence of high amounts of polyphenolic compounds, they could be explored as natural food preservatives. Hence, this

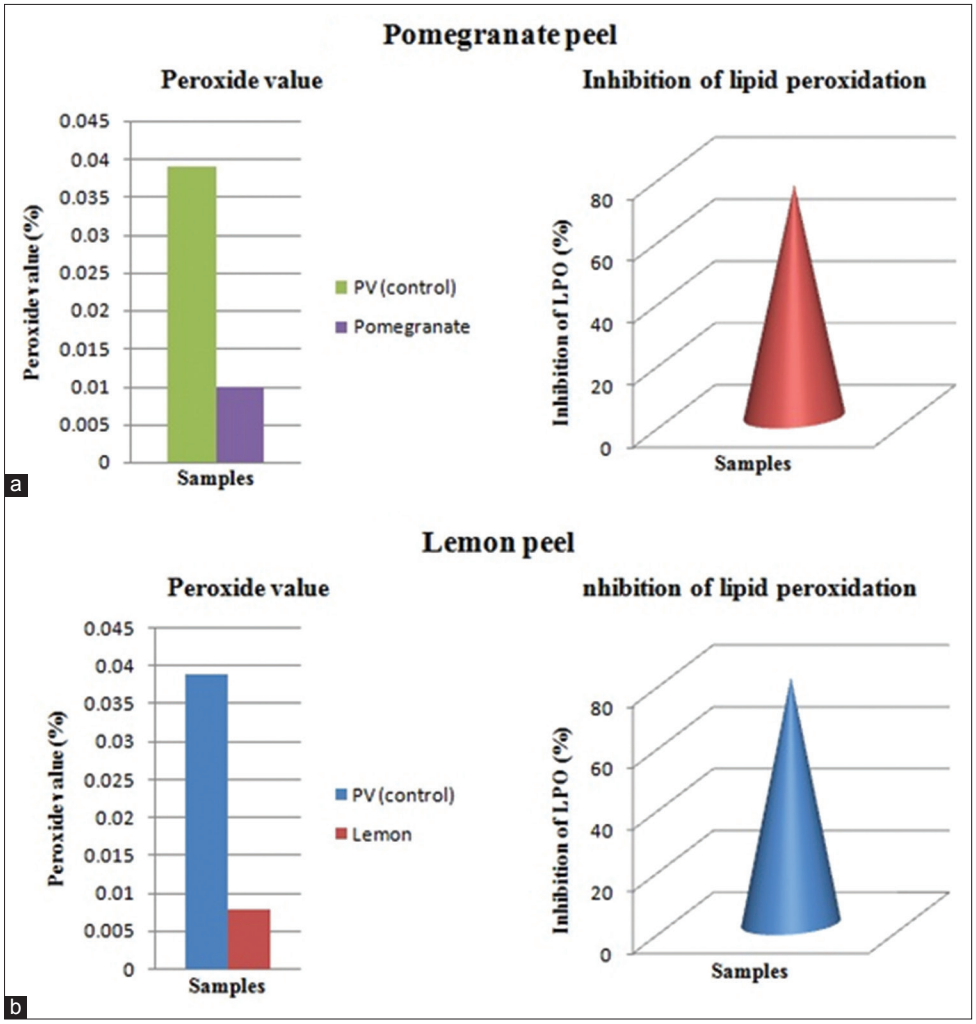

Fig. 3: Inhibition of lipoid peroxidation by the solvent extract of pomegranate (a) and lemon (b) peels

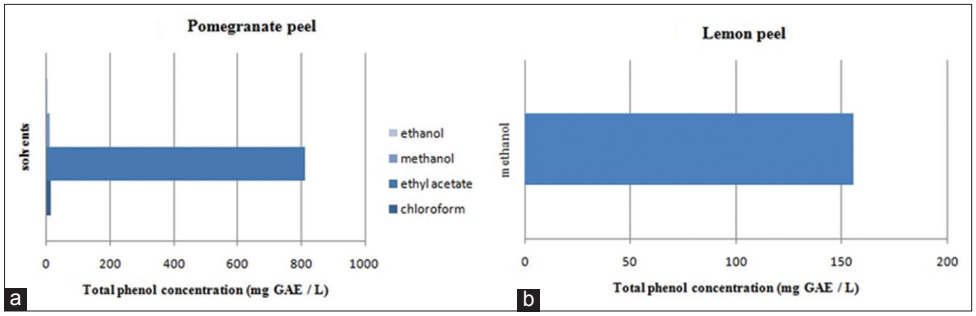

Fig. 4: Total phenolic content of column fractions of pomegranate (a) and lemon (b) peel extracts

work was emphasized to investigate the polyphenolic concentration of lemon and pomegranate peels during solvent extraction, acid and alkali hydrolysis and evaluation of their antioxidant potential. Higher yield of phenolic compounds was noted in lemon during ethanol extraction with agitation (1257.50 mg GAE/L) and in acid hydrolysis for pomegranate peels (732.75 mg GAE/L). The results of antioxidant assay revealed maximum activity for ethanolic extract of lemon which was prepared with agitation (92.44%), whereas the alkali hydrolysate of pomegranate peel revealed maximum activity (92.28%). These phenolic extracts were used in the butter food sample to estimate the inhibition of lipid peroxidation. Phenolic-rich lemon peel extract showed higher inhibition of lipid peroxidation (79.48%) when compared to pomegranate extract (74.87%). Column chromatography results showed that ethyl acetate was the ideal solvent for the elution of phenolic compounds of pomegranate peel and methanol in the case of lemon peel. Both pomegranate and lemon peels were considered as rich source of phenolic compounds with remarkable antioxidant activity. Thus, the selected samples could be considered as a potential source of natural antioxidants and could be used in food industries to prevent the oxidative spoilage of foods after evaluating the toxicity of the extracts.

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Research Article

PHARMACOGNOSTIC EVALUATION OF *ZINGIBER OFFICINALE* ROSCOE RHIZOME

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ABSTRACT

Objectives: According to the Siddha classical texts ginger (*Zingiber officinale* Roscoe) is one among the 108 kayakarpa plants (promoting health as well as prophylactic medicine). Therapeutically, it is used for respiratory and digestive problems such as cough, wheezing, sinusitis, headache, fever, indigestion, loss of appetite, gastritis, gastric reflexions, gastric ulcer, flatulence, diarrhea, and anemia. Even though there are several research studies have been conducted on the essential oils of ginger, the antioxidant and anti-inflammatory activities of ethanolic extract are not yet revealed.

Methods: Hence, in this work, we have analyzed the powder microscopic features, physiochemical properties, phytochemical profile, total phenol content, antioxidant, and anti-inflammatory properties of ethanol extract of dry ginger (rhizome) powder along with gas chromatography mass spectrometry (GC-MS) analysis. Powder microscopic analysis provided certain characteristic features of ginger rhizome powder, which could be used as botanical standards to authenticate this drug.

Results: Physicochemical properties revealed 4.5% of total ash, 0.504% of acid insoluble ash, loss on drying (7.35%), alcohol soluble extractive 3.199%, and water soluble extractive (14.81%). All the pharmacognostic characters observed from this study have matched with the Ayurvedic Pharmacopoeia, and thus, we have authenticated the plant drug. Phytochemical profile revealed that presence of sterols, terpenes, flavonoids, tannins, saponins, and coumarine. Ethanolic extract was found to contain appreciable total phenolic content (179 mg gallic acid equivalents/100 g), antioxidant (80%), and anti-inflammatory activities (34.35%) in terms of 2,2-diphenyl-1-picryl hydrazyl free radical property and red blood cell membrane stabilization capacity, respectively. 61 compounds were detected in GC-MS analysis, among which gingerol is the predominant phytochemical (40%).

Conclusions: Due to the noticed phytochemicals and bioactivities, ginger is found to be active in many Siddha herbal formulations.

Keywords: *Zingiber officinale*, Ginger, Pharmacognosy, Phytochemicals, Antioxidant, Gas chromatography-mass spectrometry analysis.

INTRODUCTION

Zingiber officinale Roscoe belongs to the Zingiberaceae family. In Tamil, it is called as chukku, naagaram, vidamudiya amirtham, and arkkam. It is called as ardraka, Katubhadra, and sringavera in sanskrit. In French language, it is called gingembre, ingwer in German, zanjabil in Arabic. Its name in Hindi is sonth, Bengali-ada, Gujarati-adu, Maharastri-sunttel-sonti, Malayalam-chukku, and Telungu sonti [1]. Ginger is cultivated in many parts of India in warm, moist regions. Indian ginger contains an aromatic volatile oil with light yellow color and characteristic odor. The essential oil and resin that occurs just beneath the skin, to which ginger owes its pungent flavor. The pungent principles of ginger are not volatile in steam to any appreciable extent, which is called as gingerol [1].

In Siddha system of medicine ginger (*Z. officinale* Roscoe) is used for the diseases of gastrointestinal tract such as indigestion, gastric reflexion, gastric ulcer, flatulence, diarrhea, vomiting, and respiratory problems such as cough, cold, wheezing, head ache, head heaviness tonsillitis and sinusitis. It acts as a stomachic, carminative and stimulant and also used for ear pain, rectal problems, abdominal tuberculosis, anemia, body pain, viral fever, and tooth pain. External application of its paste for joint swelling, head ache and throat pain [2]. In siddha system, the outer layer of ginger is considered as poisonous, so we should scrape the rhizome and used for extracting the juice, in which only the supernatant should be used [2,3].

It is the main ingredient in many Siddha formulations such as showbakia chundi legium [2], chukku choornam, thayir chundi choornam, and chukku thylam [4]. Thirikadugu choornam is a main and most important medicine prepared from dry ginger with black pepper and long pepper. All these formulations are used as internal medicine for digestive, carminative, expectorant, febrifuge (a medicine used to reduce fever), antiperiodic, cough, and bronchitis [5]. In coma and drowsiness, a small quantity of powder of these formulations are mixed in water and dropped in the nostrils or as a snuff [2].

Kayakarpam is ambrosial medicine taken by the Siddhars for the prevention of death and the decay of body, thereby protecting themselves from decrepitude, death, hunger, thirst, fatigue, sleep, etc. They rejuvenate the system and enable one to live for hundreds of years on earth with the youthful body [6]. There are 108 herbs available as karpam and ginger is one among that. Consumption of ginger powder with sugarcane juice every day morning will give kayakarpam effect [2]. Another method of administration is giving the ginger powder with equal quantity of *Vitex negundo* immature leaf [7].

In Ayurveda ginger is s named as "Mahaushadhi," which means, use of this great herb improves growth of body, used for the treatment of digestive disorders, appetizer, aphrodisiac, and arthritis [8]. The ginger is used as major ingredient in Trikatu, a famous Ayurvedic remedy for the treatment of digestive disorders. In Ashtanga Hridaya, the ginger has been used in rasna saptak quath (a decoction based on seven medicinal herbs) and used as a traditional remedy for arthritis.

Local application of ginger in ointment form for in pains in hypolipidemic and antiemetic effect [8]. Paul *et al*., 2012 confirmed that it has hypolipidemic activity in animal experiment [9]. Srivastava and Mustafa (1992) proved that it is useful for rheumatism and musculoskeletal disorders through clinical trials [10]. White (2007) evaluated the effectiveness of ginger in patients with osteoarthritis, rheumatoid arthritis, and muscular discomfort [11]. It acts as analgesic and anti-inflammatic relievers. Other research paper explained that ginger powder is effective for neurological disorder like migraine head ache [12]. Katiyar *et al*., (1996) explained that it possesses antioxidant,

anti-inflammatory and anti-skin-tumor promoting effects through *in vitro* study [13]. Another *in vivo* study showed it has preventive effect against atherosclerosis through its free radical scavenging, prostaglandin inhibitory, and fibrinolysis enhancing properties [14].

METHODS

Preparation of the drug

Raw drug was procured from local market, Thanjavur, Tamil Nadu, India, and identified in the NABL accredited lab of CARISM, SASTRA University and authenticated using macroscopic and microscopic studies. Ginger rhizome powdered (particle size 1 mm) using a lab mill and used for further analysis.

Microscopic studies

The powder microscopic characters of ginger rhizome powder also studied according to the method of the WHO (1998) [15]. The presence of calcium carbonate crystals was observed by taking a pinch powdered material and treated with acetic acid (60 g/L), and the preparation was increased and observed under microscope. The presence of fats and fatty oils was analyzed by taking one pinch of powdered material with 1-2 drops of sudan red solution, heated lightly and the preparation was irrigated with ethanol (750 g/L) and the slides were mounted and noted under microscope. For Starch test, a pinch of powdered material was treated with iodine (0.02 M) solution and the slides were mounted and observed under microscope.

Chemical standardization

The pH of the aqueous solution of ginger powder (1%, W/V) was measured using the pH meter at 24.4°C. The determination of the total ash content of ginger rhizome powder was done by the method of Joshi and Aeri (2009) [16]. Powder (1.0896 g) is added to a preweighed silica crucible and heated in the muffle furnace at 400°C for about 3 hrs. Then the crucible was carefully placed in the desiccator and allowed to cool to room temperature and the weight is finally measured. The percentage weight of the ash is calculated using the formula (weight of the ash/weight of the drug × 100). The percentage of acid insoluble ash is calculated using the formula, weight of the residue weight of the powder × 100, where the weight of the residue is the net weight of ash.

The loss on drying (LOD) was calculated by taking 1.0605 g of powder in a dish (which was preweighed and kept in the hot plate at a temperature of 105°C and the LOD was evaluated by using the formula (weight of the dish before LOD - weight of the dish after LOD/weight of the sample × 100). The alcohol and water soluble extractives (WSEs) of the powder were analyzed according to the methodology written by Joshi and Aeri (2009) [16]. Dry powder (1.0034 g) was taken in two beakers individually and 50 ml of alcohol in the first beaker and 50 ml of water was added in the second one and shaken well manually. The beakers were kept away for 24 hrs and thereafter 10 ml of the solution was taken and placed in hot air oven at 105°C. Finally, the percentage weight of the extract is calculated using the formula (weight of residue/ weight of the drug × 100).

Extract preparation

For preparing extract, 10 g of dry powdered material was taken with 100 ml of ethanol in a conical flask. The mixer was kept for 24 hrs at room temperature (37°C). Then the contents were filtered through a filter paper placed on the funnel and the volume of the extract was noted and the extracts thus collected were used for phytochemical screening.

Phytochemical screening

Phytochemical profile of herbal drugs extract was analyzed as per the methodology of Harborne [17]. The presence of phenolic compounds was identified by taking 1 ml of extract with 5 ml alcohol and a pit of ferric chloride. The presence of alkaloids was identified using Dragendorff's test, in which, 0.5 ml of extract was taken with 0.2 ml of acetic acid and 1 ml of Dragendorff's reagent and shaken well. The existence of flavonoids was detected by adding 2 ml of extract with 1 ml of hydrochloric acid and a pinch of magnesium turnings and heated for few minutes. The terpenoids were observed by taking 0.5 ml of extract with tin pellet and 0.2 ml of thionyl chloride and heated gently. The extract (0.5 ml) was added with 0.1 ml of lead acetate and observed for tannins. To identify the presence of saponins, 0.5 ml of extract was mixed with 5 ml of distilled water and shaken vigorously. For confirming the presence of steroids, the extract (0.5 ml) and 0.5 ml of acetic anhydride were taken and few drops of concentrated sulfuric acid were added. To know the presence of quinones, 0.5 ml of extract was added with 0.1 ml of sulfuric acid. For coumarins test, 0.5 ml of extract was mixed with 0.2 ml of sodium hydroxide. The extract (0.5 ml) was mixed with Fehling's (A and B) to reveal the presence of sugars.

Total phenol content

The total phenolic concentration of ethanolic extract of ginger rhizome powder was estimated according to the modified method of Singleton *et al*. (1999) [18]. Extract (10 µl) was taken in a 96 well microplate and 25 µl of folin reagent and 230 µl of 4.4% of Na_2CO_3 were added and incubated for 30 minutes in dark place. Then the absorbance was measured at 750 nm in the ELISA plate reader (Make: Biotek, Model: Epoch). A calibration curve was prepared using standard gallic acid (100-1000 mg/L, R^2 = 0.9978) and used to express the results as gallic acid equivalents (GAE).

Antioxidant activity

The antioxidant activity of ethanolic extract was analyzed using 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging assay [19]. Extract (10 µl) was taken in the 96 well microplates and 200 µl of DPPH solution (2.5 mg/100 ml) and incubated for 30 min in dark place. Then the absorbance was measured at 515 nm in the ELISA plate reader (Make: Biotek, Model: Epoch). The radical scavenging activity of tested sample was calculated using the formula (antioxidant activity = Abs control - Abs test/Abs control × 100) and expressed on percentage basis.

Gas chromatography-mass spectrometry (GC-MS) analysis

For extract preparation, 10 g of dry powdered drug was taken with 100 ml of ethanol in conical flasks. The mixer was placed for 24 hrs at room temperature (37°C). Then the contents were filtered through a filter paper placed on the funnel and the volume of the extract was noted. The extracts were kept in the water bath 3-hrs for drying. After drying the methanol extract was analyzed using gas chromatographic system coupled with mass spectrometry (Perkin Elmer, Model: Clarus-500). Silica capillary column (30 m × 0.25 mm, 0.25 µm film thicknesses, elite-5 MS nonpolar fused) was used. Oven temperature was programed with an increase of 6°C/minutes to 150°C; injector temperature was 280°C; carrier gas was helium with the flow rate of 1 ml/minutes. Sample (1.4 µl) was injected with split ratio of 1:10. Ionization energy 70 eV was used in the electron ionization mode; ion source temperature was set at 160-200°C, mass was scanned in the range of 40-450 amu. The resulted mass spectrum was compared with inbuilt NIST library database and fragments of various compounds present in the extracts were identified.

RESULTS AND DISCUSSION

Microscopic studies

The results of powder microscopic studies of ginger powder were showed in Fig. 1. The powder microscopic characterization studies of the *Z. officinale* revealed the presence of simple and compound, round, oval, and polygonal starch grains. Rarely unicellular covering trichomes with smooth surface were seen. Xylem vessels with spiral thickening and septate fibers were observed. Elongated irregular epidermal cells with wavy cell walls were also noticed. Polygonal parenchyma cells are filled with brown colored resins and few filled with simple and compound round, oval, elongated and polygonal starch grains with striated margins (Fig. 1).

Chemical standard values are shown in Table 1. LOD indicates the amount of unstable matter (i.e., water aeration off from the material). The LOD of ginger powder material was 7.356%, which falls within

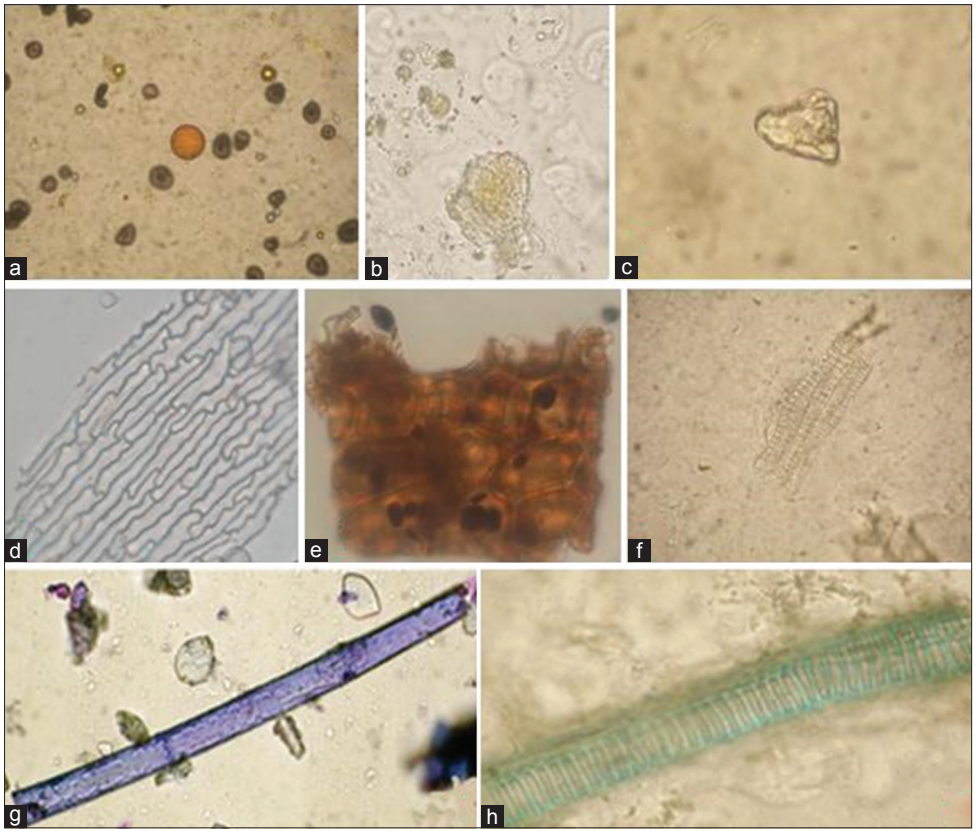

Fig. 1: Powder microscopic features of *Zingiber officinale* rhizome ([a] starch grains and oleoresin, [b] parenchyma cell, [c] calcium oxalate crystal, [d] epidermal cells, [e] brown colored cells with starch grains, [f] xylem vessel, [g] septate fiber and [h] xylem vessel with scalariform thickening)

Table 1: Chemical standardization of ginger rhizome powder

S. No.	Parameters	Results	Range	Reference of test methods [20]
1.	Appearance	Light yellow colored fine powder	-	IP Vol-I, 1996, p7
2.	pH (1% w/v solution)	4.80	4-14	IP Vol-I, 2014, p169
3.	Loss on drying at 105°C (%, w/w)	7.356	1-20	IP Vol-I, 2014, p162
4.	Total ash (%, w/w)	4.504	1-25	IP Vol-I, 2014, p98
5.	Acid insoluble ash (%, w/w)	0.504	0.1-10	IP Vol-I, 2014, p98
6.	WSE (%, w/w)	14.81	4-85	IP Vol-I, 2014, p277
7.	ASE (%, w/w)	3.199	4-85	IP Vol-I, 2014, p277

ASE: Alcohol soluble extractive, WSE: Water soluble extractive

the normal range. Low level of moisture content in the material will be useful to avoid the microbial spoilage of herbal drugs. Total ash and acid-insoluble ash of ginger powder were found to be 4.504% and 0.504%, respectively, which are in agreement with the levels of normal limit [21]. Water soluble and alcohol soluble extractives (ASEs) of ginger powder were found to be 14.81% and 3.199%, respectively, and these results fall within the range denoted by Ayurvedic Pharmacopoeia of India [21]. WSE content is higher than ASE in ginger, which shows the presence of high amount of high polar compounds in the sample.

Phytochemical screening results are shown in Table 2. Phytochemical screening revealed the presence of sterols, terpenes, flavonoids, tannins, saponins, and coumarins in the ethanol extract of ginger rhizome powder. The presence of these phytochemical constituents might be responsible for the therapeutic properties exhibited by this plant. Sterols have anticholesterol and anticancer activity [22], anti-inflammatory, antineoplastic, antipyretic, and immune-modulating activities [23]. Important therapeutic uses of terpenoids are antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory, antioxidants, antiparasitic, immunomodulatory, and as skin permeation enhancer [24]. Flavonoid compounds possess antioxidant activity, free radical scavenging capacity, coronary heart disease prevention, hepatoprotective, anti-inflammatory, and anticancer activities, while some flavonoids exhibit potential antiviral activities also [25,26]. Tannins are used as an antiviral, antibacterial, antiparasitic, antidiarrheal, hemostatic agents gastritis, esophagitis, enteritis, irritable bowel disorders, heal burns, and protect kidneys [27]. Saponins have antimicrobial [28], antifungal, antioxidant, anti-inflammatory, hypercholesterolemia, hyperglycemia, and weight loss properties [29]. Coumarins have so many medicinal values such as anticoagulant, antineurodegenerative, anticancer, antioxidant, antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, analgesic, antidiabetic, and antidepressive properties [30].

Total phenol content of ginger whole rhizome is found to be higher (444.35 mg GAE/100 g) when compared to ginger without skin (267.30 mg GAE/100 g) and ginger skin (345.76 mg GAE/100 g) (Fig. 2). Results on antioxidant activity also revealed that ginger skin is having high free radical scavenging activity (inhibitory concentration-50 [IC-50] value 386.9 mg/ml) when compared to rhizome tuber without skin (IC-50 value 912.7 mg/ml) and whole rhizome (IC-50 value 701.1 mg/ml) (Fig. 3).

Table 2: Phytochemical screening of ethanolic extract of ginger

S. No.	Compounds	Phytochemicals
1.	Sterols	+
2.	Terpenes	+
3.	Sugars	-
4.	Alkaloids	-
5.	Phenols	-
6.	Flavonoids	+
7.	Tannins	+
8.	Saponins	+
9.	Quinones	-
10.	Coumarins	+

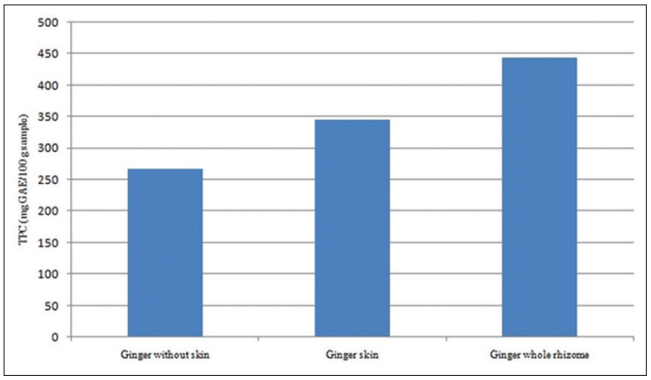

Fig. 2: Total phenolic content of ginger whole rhizome with and without skin

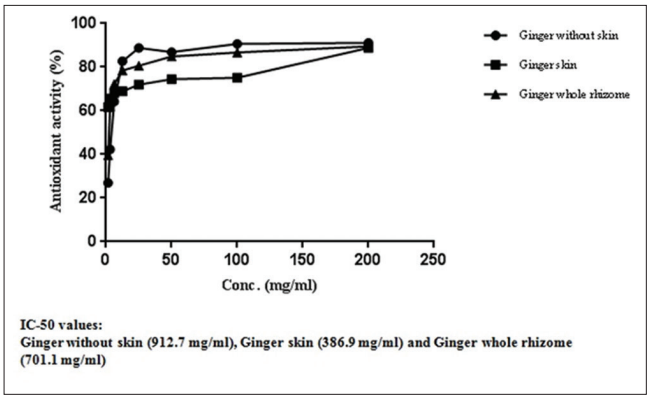

Fig. 3: Antioxidant activity of ginger rhizome with and without skin

Actually, in Siddha drug preparations, skin is removed and only the tuber portion was used, but this study results revealed that skin is rich source of total phenols than tuber portion. Hence, instead of use of rhizome tuber alone, if we could use whole rhizome along with skin, more amount of polyphenols will be available which could exhibit better medicinal value. However, inclusion of ginger skin in herbal drug formulation should be evaluated for its toxicity in animal models. Total phenol and antioxidant activity of ginger was already reported by Maizura *et al*., 2011, in their research article [31]. The value is nearly same.

GC-MS analysis

The results of the GC-MS analysis of ethanol extract of *Z. officinalis* rhizome were presented in Fig. 4 and Table 3. A total of 61 compounds were identified among which nonvolatile oil and recorded 40% peak area is identified as gingerol (retention time 37.93, 48.11, 40.19, and 38.64 with peak area of 18.8163, 12.1938, 6.4146, and 2.5871, respectively). Major phytochemicals are gingerol, zingerone, phenol-2-methoxy-3-(2-propenyl), and capsaicin.

Gingerol is a nonvolatile phenylpropanoid-derived compound and its chemical name is 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl) decan-3-one. The pungency odor and flavor of ginger is due to gingerol. It is formed in the plant from phenylalanine, malonate, and hexanoate [32-34]. It is a digestion stimulator, increase the secretion of peptic juices, such as gastric juice, bile, pancreatic and intestinal juices, raise the fat metabolism, prevents gastric mucosal damage, antioxidant, exhibits antitumor, anticancer activities, induces apoptosis in gastric cancer [33]. Another research article proves that gingerol has antioxidant, anti-inflammatory, antilipid, antidiabetic, analgesic, antipyretic, and antitumor activity [35]. Gingerol is an effective therapeutic agent providing protection against ultraviolet-B-induced skin disorders [36].

Another major compound is phenol 2-methoxy-3-(2-propenyl) peak area is 10.9641% and the retention time is 14.32. Its other names are 3-allylguaiacol and O-eugenol. Already, this compound was identified in a Siddha herbometallic preparation namely Kandhaga rasayanam [37], piper betle [38], fresh peels of three *Citrus* species namely *Citrus sinensis*, *Citrus Limetta,* and *Citrus limon* [39]. Its major uses are food preservation, cosmetics, and medicine as they exhibit a strong antioxidant, antibacterial, and antifungal activity against food-borne pathogens [39]. Sugumaran *et al.* (2011) has proved that it has antimicrobial activity [40].

Other major bioactive compound is zingerone, which is also called as 2-butanone 4-(4-hydroxy-3-methoxyphenyl) and vanillyl acetone. Zingerone is a nontoxic compound with varied pharmacological activities. It is the slight pungent component of ginger. Zingerone is absent in fresh ginger but cooking or heating transforms gingerol to zingerone. Zingerone very much related to vanillin from vanilla and eugenol from clove. It has potent anti-inflammatory, antidiabetic, antilipolytic, antidiarrheic, antispasmodic properties besides it gives growth and immune stimulation. It acts as appetizer, anxiolytic, antithrombotic, radiation protective, and antimicrobial. Furthermore, it inhibits the reactive nitrogen species which are important in causing Alzheimer's disease and many other disorders [41]. Benzene (2,6,6 - Trimethylcyclohex-1-enylmethanesulfonyl) is already identified in flower of Artemisia austroyunnanensis by Chen-Xing *et al*. (2014) [42] and leaves of *Eupatorium catarium* by Wang *et al*. (2014) [43].

Capsaicin is a unique alkaloid present primarily in the fruit of the *Capsicum* genus and it provides its spicy flavor [44]. Capsaicin is the active element in pepper, which accounts for its prominent pharmaceutical and antioxidant properties [45]. In therapeutically, it acts as a vasodilator, increasing gastric mucous blood flow and reduced adipose tissue in rodents by enhancing energy and lipid metabolism, increasing catecholamine secretion from the adrenal medulla in response to activation of the sympathetic nervous system. Externally for neuropathic pain, postherpetic neuralgia, musculoskeletal pain, diabetic neuropathy, osteoarthritis, and rheumatoid arthritis [44].

3-cyclohexene-1-methanol-4-trimethyl-acetate which is otherwise called as α-terpineol acetate and terpinyl acetate. α-terpinyl acetate is identified in the aerial parts of *Artemisia rupestris* plant [46]. This phytochemical is used as insecticidal, antimicrobial, antispasmodic, and immune-stimulant [47]. 6-octen-1-yn-3-ol, 3,7-dimethyl called as dehydrolinalool or linalool. This compound presents in *Coriandrum sativum*, *Nepeta flavida*, *Nepeta italica*, *Origanum majorana*, *Origanum onites*, *Salvia sclarea*, *Thymus longicaulis, Thymus sibthorpii,* and *Thymus zygioides*. It has antimalarial and antimicrobial activities against human and plant pathogenic microorganisms and used frequently in perfume product produce [48]. 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-4-en-1-yl)pent-3-en-2-one, other names are β-ionon-5,6-epoxide, β-ionone epoxide. 1,3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*,S*)] other names zingiberene; α-zingiberene. It is derived from fresh leaf of *Citrus medica limonum* [49], it obtains from the *Eugenia* species of *Eugenia austin-smithii, Eugenia cartagensis, Eugenia haberi, Eugenia zuchowskiae, Eugenia monteverdensi* [50], fruit of *Lycopersicon*

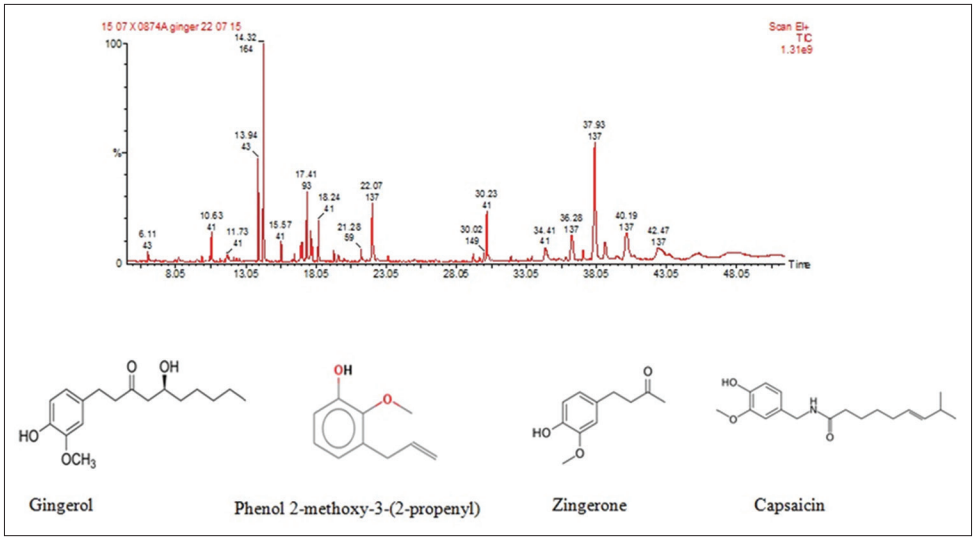

Fig. 4: Gas chromatography-mass spectrometry analysis of phytochemicals in ethanolic extract of ginger

Table 3: Phytocomponents detected in the ethanol extract of ginger rhizome powder

S. No.	Peak name	Retention time	Percent of peak area	Type of compound
1.	Name: Decanal ($C_{10}H_{20}O$) MW: 156	10.63	1.1974	Hydrocarbon
2.	3-Cyclohexene-1-methanol, à,à,4-trimethyl-, acetate $C_{12}H_{20}O_2$ MW: 196	13.94	4.1117	Terpenyl acetate
3.	Phenol, 2-methoxy-3-(2-propenyl)-$C_{10}H_{12}O_2$ MW: 164	14.32	10.9641	Flavor compound
4.	1,3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-,[S-(R*, S*)]- C15H24 MW: 204	17.41	2.9620	Sesquiterpene
5.	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*, S*)]-$C_{15}H_{24}$ MW: 204	18.24	2.1679	Sesquiterpene hydrocarbon
6.	5-Azulenemethanol, 1,2,3,4,5,6,7,8-octahydro-à,à,3,8-tetramethyl-$C_{15}H_{26}O$ MW: 222	21.28	1.1220	Sesquiterpenoid
7.	2-butanone, 4-(4-hydroxy-3-methoxyphenyl)-$C_{11}H_{14}O_3$ MW: 194	22.07	5.3914	Terpene
8.	6-octen-1-yn-3-ol, 3,7-dimethyl- $C_{10}H_{16}O$ MW: 152	30.22	3.9706	Acyclic monoterpenoid
9.	E, Z-2,13-Octadecadien-1-ol$C_{18}H_{34}O$ MW: 266	34.41	2.8163	Fatty alcohol
10.	(2,6,6-trimethylcyclohex-1-enylmethanesulfonyl) benzene $C_{16}H_{22}O_2S$ MW: 278	36.27	4.4054	In organic compound
11.	Acetic acid, 1-[2-(2,2,6-trimethyl-bicyclo[4.1.0] hept-1-yl)-ethyl]-vinyl ester($C_{16}H_{26}O_2$) MW: 250	37.10	1.0795	Terpenes
12.	Gingerol ($C_{17}H_{26}O_4$) MW: 294	37.93	18.8163	Pungent principles
13.	Gingerol ($C_{17}H_{26}O_4$) MW: 294	38.64	2.5871	Pungent principles
14.	Gingerol $C_{17}H_{26}O_4$ MW: 294	39.51	0.6000	Pungent principles
15.	Gingerol ($C_{17}H_{26}O_4$) MW: 294	40.19	6.4146	Pungent principles
16.	Capsaicin $C_{18}H_{27}NO_3$ MW: 305	42.47	4.1439	Alkaloid
17.	4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-4-en-1-yl) pent-3-en-2-one ($C_{14}H_{20}O_2$) MW: 220	45.35	3.8297	
18.	Gingerol $C_{17}H_{26}O_4$ MW: 294	48.11	12.1938	Pungent principles

esculentum. Alpha-zingiberene reduces nausea due to it has the direct action on the vomiting [51].

Cyclohexene, 3-(1, 5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*,S*)], is a hydrocarbon commonly called as β-sesquiphellandrene. It is also identified in the following plants *Curcuma longa* by Tyagi *et al.* 2015 [52], *Alpinia galanga* by Hamad *et al*., 2016 [53], leaves and stem of *Eryngium caucasicum* [54]. It has antioxidant and antimicrobial activity [55].

Decanal is a hydrocarbon commonly called as caprinaldehyde. Decanal is an important compound of family Rutaceae and is used in fragrances and flavoring [56]. It derived from plant sources of *Cinnamomum camphora, Citrus aurantium, C. sativum* and *Juniperus Sabina* [57], *Atalantia racemosa, Atalantia wightii, Atalantia monophylla* [56].

5-azulene methanol, 1,2,3,4,5,6,7,8 - octahydro - à,à,3,8-tetramethyl commonly called as guaiac acetate and bulnesol. It also found in *Commiphora ornifolia* and *Commiphora parvifolia.* It has antimicrobial activity [58].

CONCLUSIONS

In this study, we have investigated the pharmacognostic properties of a well-known herbal drug, ginger and evaluated its antioxidant property through *in vitro* model. Microscopic and chemical properties

of ginger rhizome powder will be useful in identifying this plant drug and these standards could be employed in drug authentication process. Phytochemical screening revealed the presence of various bioactive compounds in the ethanolic extract of ginger. GC-MS analysis also exhibited the major phytochemicals such as gingerol and zingiberene in the ethanolic extract. Quantification of total phenolic content in different fractions of ginger indicated the presence of higher levels of phenolic compounds with remarkable antioxidant activity in ginger skin than rhizome without skin. Hence, use of rhizome with skin with rich phenolic content could be considered in drug formulation after conducting toxicological evaluation.

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Research Article

COMPARATIVE EVALUATION OF ANTIOXIDANT ACTIVITY OF AQUEOUS EXTRACTS OF THREE DIFFERENT *SALACIA* SPECIES COLLECTED FROM SOUTH INDIA

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ABSTRACT

Objective: The genus *Salacia* belongs to the family Hippocrataceae and so far 18 *Salacia* species have been identified in India. Various medicinal uses of different *Salacia* species were mentioned in various Siddha literatures. Distribution of *Salacia* species in southern regions of India and Sri Lanka were reported in the Flora of the Carnatic Tamil Nadu. This genus is a perennial straggling plant found in foot hills of the mountain of Eastern and Western Ghats of Tamil Nadu and Kerala up to 1000 m. Roots of *Salacia* species are astringent and used as an antidiabetic drug in the indigenous system of medicine.

Methods: In this study, we have comparatively analyzed the total phenolic content and antioxidant activity of three different species of *Salacia chinensis*, *Salacia oblonga,* and *Salacia reticulata*.

Results: Based on total phenolic content (1970 mg GAE/L), phosphomolybdate reducing power (1458 ascorbic acid equivalent activity), ferric reducing power (0.939), radical scavenging activities against 2,2-diphenyl-1-picrylhydrazyl (81%), superoxide (48%), hydrogen peroxide (95%) and hydroxyl radicals (90%), *S. reticulata* was found to be more effective in exhibiting antioxidant activity when compared to other two species.

Conclusion: Hence, *S. reticulata* could be explored as natural antioxidant to prevent/treat variety of oxidative stress-related diseases.

Keywords: *Salacia* species, *Salacia chinensis*, *Salacia oblonga*, *Salacia reticulata,* Phenols, Antioxidants.

INTRODUCTION

The genus *Salacia* belongs to the family Hippocrataceae. So far 18 *Salacia* species have been identified in India [1]. Out of 18 species, we could only trace 5 species such as *Salacia chinensis, Salacia reticulata, Salacia oblonga, Salacia roxburghii,* and *Salacia grandiflora* in South India including Tamil Nadu [2]. The medicinal use of *S. oblonga* and *S. reticulata* was mentioned in various Siddha literatures [3-5] while the therapeutic value of *S. chinensis* was reported in the Flora of the Carnatic Tamil Nadu [6].

S. oblonga Wall. is widely distributed in south region of India and Sri Lanka. It is a perennial straggling plant found in foot hills of the mountain of Eastern and Western Ghats of Tamil Nadu and Kerala up to 1000 m. It is a climbing shrub, leaves are elliptic in shape, flowers are small, axillary, short pedunculate, head or branched cymes with 5-6 cm in dia. The root of *S. oblonga* is used for the treatment of rheumatoid arthritis and allergic conditions. The fruits are used as a dietary supplement and as a tribal antidiabetic medicine [7]. Root is extensively used for treating diabetes mellitus by Siddha Practitioners and also it has been used as a remedy for gonorrhea, rheumatism, itch, asthma, thirst and ear diseases in Tamil Nadu and Kerala. *S. oblonga* is proved to be antiseptic, besides nephroprotective, cardiac fibrosis inhibitor, hypolipidemic and also useful in hepatic steatosis and as an antimicrobial, anti-inflammatory, antioxidant, and antidiabetic agent [8]. Matsuura *et al*. [9] revealed the sucrose inhibitory effect of herbal tea that containing *S. oblonga* was claimed to be beneficial as a dietary supplement. Similarly, Collene *et al*. [10] determined the nutraceutical value of *S. oblonga* extract and suggested that it could be a good supplement to decrease glycemia. The glucose lowering capacity of *S. oblonga* was evaluated in streptozotocin-induced diabetic rats [11]. *In vitro* antioxidant activity of methanolic extract of *S. oblonga* was investigated by Basu *et al*. [12].

S. reticulata is a climbing shrub with blackish, prominently lenticellate, and terete. Leaves are coriaceous, ovate, reticulate 6-9 × 4-5 cm in size. Small flowers are in each axillary short cymes. Calyx is lobed, short, and obtuse. Petals are broad at the base, imbricate, greenish yellow, oblong, 0.5 mm long, apex acute and recurved. The roots are acrid, bitter, thermogenic, useful in urinary tract infection, astringent, anodyne, and anti-inflammatory. They are also useful in vitiated conditions of vatha, diabetes, hemorrhoids, rheumatism, gonorrhea, and skin diseases. There are reports that mugs made of the wood of this species is used to drink water by diabetic patients [1]. This plant is scientifically investigated for hypoglycemic activity, anti-inflammatory activity, free radical scavenging activity, antiobesity, and antibacterial activity [13]. Nikale and Mulani [14] proved the antidiabetic potential of *S. reticulata* and *S. macrosperma* in STZ-induced rat model.

S. chinensis is widely distributed, but scattered in India, Sri Lanka, Burma, Thailand, Indo-China, China and Malaysia. In India, it is found in evergreen forests of Western Ghats and in Andaman Islands at an altitude up to 750 m. It is a shrub, leaves are subopposite, oblong, nerves obscure, mid nerve prominent below, glabrous, glossy above, base subacute, margin serrate-crenate. Inflorescence is axillary umbels, flowers are 7 mm in diameter and fruit is berry, globose, and solitary seeds. Root of this plant is astringent and used as an antidiabetic drug in the indigenous system of medicine. It is also used as an abortifacient and a decoction is useful in amenorrhea, dysmenorrhea and in venereal disease [1]. Research studies revealed the bio-active properties of this plant including antihyperlipidemic, antihyperglycemic, radical scavenging, hypotensive, hepatoprotective, anticaries, anticancer and alpha-glucosidase activities [15]. The antidiabetic potential of *S. chinensis* was proved in STZ-induced rat model [14]. Phytopharmacological aspects of *S. chinensis* were investigated by Deokate and Khadabadi [16].

Even though few reports are available on the medicinal properties of *Salacia* species, especially their antidiabetic property, studies on antioxidant potential of *Salacia* species are found to be meager. In this

study, we have comparatively investigated antioxidant activity of three different *Salacia* species (*S. chinensis*, *S. oblonga,* and *S. reticulata*) which are available in South India with a view to select the potential source of natural antioxidants.

METHODS

Preparation of aqueous extract

Different *Salacia* species (*S. chinensis*, *S. oblonga,* and *S. reticulata*) were collected from South India and authenticated using the Flora of the Carnatic Tamil Nadu by the Botanist Professor P. Brindha, Associate Dean and Co-ordinator, Centre for Advanced Research in Indian System of Medicine, SASTRA University. The root samples of all the three species were shade-dried and powdered in a lab mill. Powdered sample (10 g) was taken with 100 ml of distilled water and kept on magnetic stirrer (500 rpm) for 60 minutes. Then, the extract was separated using filtration, and the volume of the extract was measured. The contents were frozen and then lyophilized and the dried extract powder was redissolved with distilled water in the ratio of 1 mg/ml and then used for further analysis.

Analysis of total phenolic content

The total phenolic content was analyzed using Folin–Ciocalteu reagent method with some modifications [17]. The sample (50 µl opportunely diluted) is added to 250 µl of Folin–Ciocalteau reagent in a test tube and vortexed. Then, 4.7 ml of 2.2% sodium carbonate solution are added and the mixture is vortexed again. A blank is prepared with 50 µl of the sample solvent instead of the sample. The tubes are incubated at 40°C for 30 minutes in the dark. The absorbance is read at 750 nm against the blank using spectrophotometer (Perkin-Elmer, Model). A calibration curve was prepared with standard ferulic acid (200-1600 mg/L, $R^2 = 0.9978$) and used to express the results as ferulic acid equivalents (FAE). The total phenolic content of the sample was then calculated and expressed on dry weight and fresh weight basis.

Ferric reducing power

The reducing power of extract was determined according to the method of Oyaizu [18]. Samples (2.5 ml) in phosphate buffer (2.5 ml, 0.2 M, pH 6.6) were added to potassium ferricyanide (2.5 ml, 1.0%) and the mixture was incubated at 50 C for 20 minutes. Trichloroacetic acid (2.5 ml, 10%) was added, and the mixture was centrifuged at 650 × g for 10 minutes. The supernatant (5.0 ml) was mixed with distilled water (5.0 ml) and ferric chloride (1.0 ml, 0.1%), and then the absorbance was read spectrophotometrically at 700 nm. Based on the absorbency value, the ferric reducing power of extract was expressed.

Phosphomolybdate assay

The antioxidant activity of extracts was evaluated according to the method of Prieto *et al*. [19]. An aliquot of 100 µl of extract was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) in a screw-capped vial. The vials were closed and incubated in a water bath at 95°C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The results expressed as ascorbic acid equivalent antioxidant activity.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was analyzed for each by following Sanchez-Moreno *et al*. [20] method. The extract (100 µl) was added to 3.9 ml of DPPH solution (0.025 g/L) and the reactants were incubated at 25°C for 30 minutes. Different concentrations of ferulic acid were used as a positive control and ethanol was used instead of extract in blank. The decrease in absorbance was measured at 515 nm with a spectrophotometer. The radical scavenging activity of tested samples was calculated and expressed on percentage basis.

Superoxide radical scavenging activity

The capacity of rice hull extracts to scavenge the superoxide anion radical was measured according to the method described by Zhishen *et al*. [21]. The reaction mixture was prepared using 3×10^{-6} M riboflavin, 1×10^{-2} M methionine, 1×10^{-4} M nitroblue tetrozolium chloride and 0.1 mM EDTA in phosphate buffered saline (pH 7.4). For the analysis, 3.0 ml of the reaction mixture was taken with 100 µl of extract in closed tubes and illuminated for 40 minutes under fluorescent lamp (18 W). The absorbance was then read at 560 nm against the un-illuminated reaction mixture. Results were expressed as superoxide radical scavenging activity on percentage basis.

Hydrogen peroxide scavenging activity

The effect of *Salacia* extracts on hydrogen peroxide was analyzed according to the method proposed by Ruch *et al*. [22]. The extract (100 µl) was mixed with 2.5 ml of 0.1 M phosphate buffer (pH 7.4) containing 45 mM hydrogen peroxide. The reaction mixture was mixed well and the absorbency was measured at 230 nm after 5 minutes. The extract with phosphate buffer is used as a blank and the level of hydrogen peroxide degradation in the solution was calculated and expressed on percentage basis.

Hydroxyl radical scavenging activity

The hydroxyl radical quenching activity of rice hull extracts was evaluated according to the method of Hagerman *et al*. [23]. The reaction mixture consists of 10 mM phosphate buffer (pH 7.4), 2.8 mM deoxyribose, 2.8 mM hydrogen peroxide, 0.025 mM ferric chloride, 0.1 mM EDTA and 0.1 mM ascorbic acid in a total volume of 3 ml. With the reaction mixture, 100 microliter of extract was added and incubated at 37°C for 15 minutes. Then the reaction was terminated by the addition of 1 ml of 2.5% ice-cold trichloroacetic acid and 1% thiobarbituric acid (TBA). The reactants were mixed well and heated at 90°C for 15 minutes in a water bath and cooled to room temperature. The chromogen was extracted with 1-butanol and absorbency was measured at 530 nm. Based on absorbency value, the hydroxyl radical scavenging activity of extracts was calculated and expressed on percentage basis.

RESULTS AND DISCUSSION

Total phenolic content

Phenolic compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer are widespread groups of substances in flowering plants. They are secondary metabolites which are derived from pentose phosphate, shikimate, and phenylpropanoid pathways in plants. Plant genetics, cultivar, soil composition, growing conditions, maturity state and post-harvest conditions could alter the quantity and quality of the polyphenols present in plant samples. In this study, the total phenolic compounds were quantified by Folin–Ciocaltue reagent method. This assay is based on the reduction of Folin's reagent by the phenolic compounds. Under alkaline pH, phenols dissociate into phenolate anion and proton, hence phenolate anion can reduce the Mo (VI), which is a major component of Folin's reagent, by single electron transfer. Due to this reduction, a blue colored complex $(PMoW_{11}O_{40})^{4-}$ is formed with absorption max at 750 nm.

The total phenolic content of three different *Salacia* species was shown in Fig. 1. Among the presently analyzed samples, *S. reticulata* extract exhibited higher levels of total phenolic concentration (2462.86 mg FAE/L) when compared to *S. chinensis* (977 mg FAE/L) and *S. oblonga* (555 mg FAE/L). Based on back calculation, it was observed that the root materials of *S. reticulata* registered as the rich source of phenolic compounds, which was followed by *S. chinensis* and *S. oblonga* based on their total phenolic contents of 1970.29, 782.29, and 388.50 mg FAE/100 g sample, respectively.

Antioxidant activity

When the molybdenum (VI) is reduced to Mo (V) by an antioxidant, it forms a green colored complex at acidic pH in the presence of phosphorous with the absorption maxima at 695 nm. This assay

evaluates the reducing or electron donating power of the antioxidant to molybdenum and the intensity of PMo (V) complex is proportional to antioxidant power of the extract. Among the three different samples, *S. reticulata* exhibited maximum reducing power (1458.25 ascorbic acid equivalent antioxidant activity) when compared to *S. chinensis* (228 ascorbic acid equivalent antioxidant activity) and *S. oblonga* (41.50 ascorbic acid equivalent antioxidant activity) (Fig. 2a).

In ferric reducing assay, Fe (III) is reduced to Fe (II) by the antioxidant compound through electron transfer. The reduced Fe (II) forms the Pearl's blue complex, which can be measured at 700 nm. The results of ferric reducing assay also revealed that the *S. reticulata* possess higher level of reducing power (0.939 absorption units) similar to the reference compound ferulic acid (0.852 absorption units) (Fig. 2b).

Antioxidants can reduce DPPH through hydrogen transfer into its nonradical form (DPPH-H) and hence the absorption disappears at 515 nm. The decrease in absorbency at 515 nm may be due to the reaction between phytochemicals and DPPH, which indicates the antioxidant power. Among the investigated samples, *S. reticulata* showed higher level of DPPH radical scavenging power (81.01%) when compared to *S. oblonga* (62.69%) and *S. chinensis* (43.44%) (Fig. 3a).

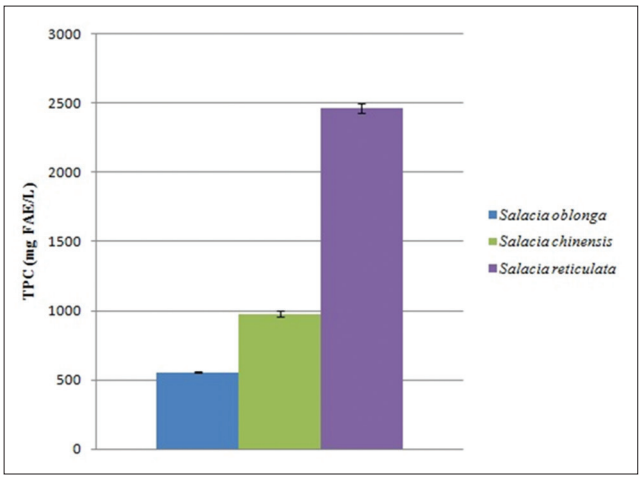

Fig. 1: Total phenolic concentration of water extract of different *Salacia* species

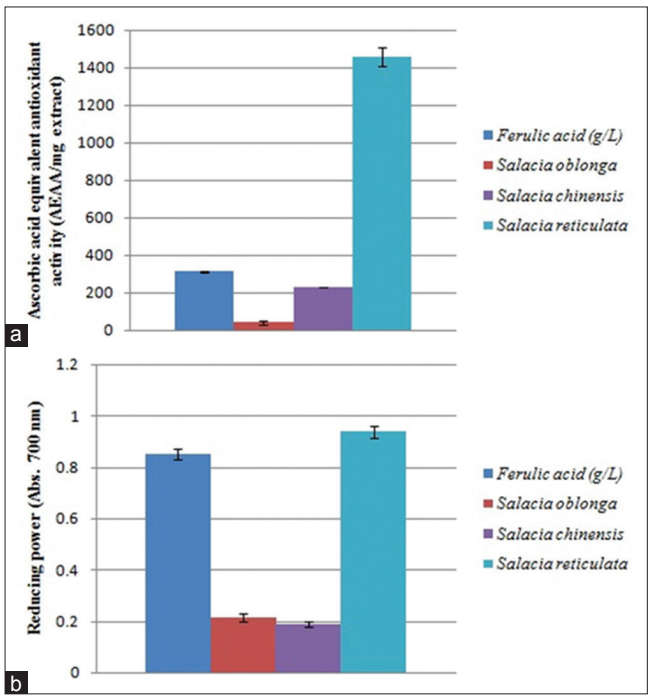

Fig. 2: Reducing power of aqueous extract of different *Salacia* species, (a) phosphomolybdate, (b) ferric reducing power

The superoxide radical scavenging activity of samples was investigated by generating superoxide through photo-induced reduction of riboflavin, which can generate superoxide radical in the presence of methionine. The generated superoxide radical reduce the NBT into purple color formazan, which was measured at 560 nm. In the presence of antioxidant, the generated superoxide radicals were scavenged, and hence, formation of purple color formazan is minimum or nil. Superoxide radical scavenging power of 85%, 48%, and 20% was noticed in *S. reticulata, S. chinensis,* and *S. oblonga*, respectively (Fig. 3b).

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. Thus, removing hydrogen peroxide is very important for protection of cellular system. The hydrogen peroxide can decompose into water by accepting two electrons and protons. The level of hydrogen peroxide in buffer solution can be detected spectrometrically at 230 nm. If antioxidants (electron donors) are added to the reaction mixer, they can accelerate the conversion of hydrogen peroxide into water. Highest level of degradation of hydrogen peroxide was caused by *S. reticulata* root extract (95%), which is followed by *S. chinensis* (76%) and *S. oblonga* (25%) (Fig. 3c).

Hydroxyl radicals are produced by the Fenton reaction between Fe(II)-EDTA and hydrogen peroxide. The hydroxyl radicals (OH.) degrade deoxyribose and produce malondialdehyde (MDA), which can be measured by TBARS reaction. The TBA can react with MDA in acidic medium to form pink color chromogen, which could be extracted with 1-butanol and read at 530 nm. OH radicals may attack various biomolecules including proteins, lipids, and DNA and cause oxidative damage to the cellular components and hence it is considered to be biologically dangerous free radical. Both *S. reticulata* and *S. chinensis* were revealed higher levels of hydroxyl radical scavenging activity of 90% and 81%, respectively, that the reference compound ferulic acid (65%) (Fig. 3d) and thus expected to protect the biological molecules from the attack of highly reactive hydroxyl radicals under *in vivo* conditions.

CONCLUSION

Although the antidiabetic activity of different *Salacia* species has been popular and being used in Indian system of medicine, their antioxidant potentials have not yet revealed through deeper studies. In this connection, we have comparatively analyzed the antioxidant property of aqueous extracts of root materials of three different *Salacia* species (*S. chinensis*, *S. oblonga*, and *S. reticulata*). Based on the data obtained from this study, *S. reticulata* was noticed to be the rich source of polyphenols and also recorded remarkable antioxidant activity in terms of reducing power as well as free radical scavenging activities. There was a positive correlation has been observed between total phenolic content and antioxidant activity of aqueous extracts of *S. reticulata*. Thus, consumption of *S. reticulata* as a source of natural antioxidants in the form of herbal tea or extract could be recommended after evaluating the toxicity, which will prevent the oxidative-stress-induced human diseases.

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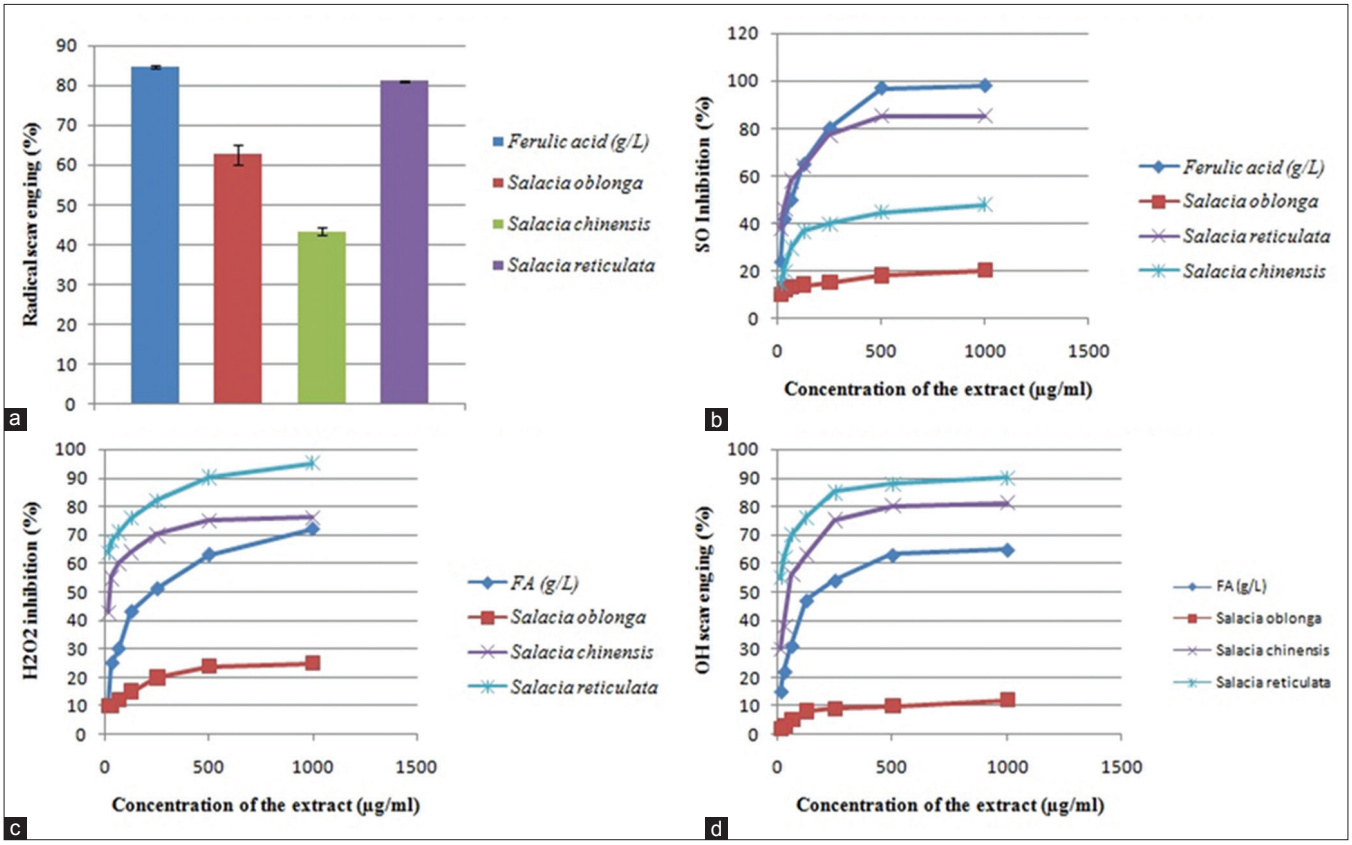

Fig. 3: Radical scavenging capacity of different *Salacia* species, (a) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, (b) superoxide radical scavenging activity, (c) hydrogen peroxide scavenging activity, (d) hydroxyl radical scavenging activity

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Research Article

INHIBITORY POTENTIAL OF PHYTOCOMPOUNDS FROM *BARLERIA MONTANA* AGAINST MITOGEN ACTIVATED PROTEIN KINASE

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ABSTRACT

Objective: This study aims to evaluate the inhibitory potential of phytocompounds obtained from *Barleria montana* against mitogen activated protein kinase (MAPK) using an *in silico* approach.

Methods: The ethanolic leaf extract of *B. montana* was subjected to gas chromatography-mass spectrometry (GC-MS) analysis using Perkin Elmer Clarus 500 and the constituents obtained were identified after comparison with those available in the inbuilt library (NIST) attached to the GC-MS instrument. *In silico* analysis of the phytocompounds was done by AutoDock software. Indomethacin was used as the standard reference compound.

Results: Docking results revealed that the amino acid residue methionine was involved in interaction with phytol in the active site of MAPK 1 with a bond length of 1.811 Å and inhibitory concentration 50% (IC_{50} value of 1.21 µm). The amino acid residues methionine and threonine interacted with the second ligand phenol 2, 4 bis (1,1-dimethylethyl) with a bond length of 1.884 Å and 1.934 Å, respectively, and the IC_{50} was found to be 4.72 µm. The reference drug indomethacin showed two interactions with the amino acid residues methionine and lysine at a bond length of 2.165 Å and 2.007 Å with an IC_{50} value of 9.21 µm.

Conclusion: The results obtained revealed that there was effective hydrogen bonding established between the ligands and target which fell within the acceptable and effective range of >1.5 Å and <3.2 Å. The IC_{50} values of the ligands chosen were lesser than the reference drug which proved its efficacy in exhibiting a strong anti-inflammatory activity. Based on the *in silico* results of this study, further in depth *in vitro* and *in vivo* analysis can be carried out to establish the anti-inflammatory potential of *B. montana*.

Keywords: Mitogen activated protein kinase, *Barleria montana*, Phytol, Phenol 2, 4 bis (1,1-dimethylethyl), Indomethacin.

INTRODUCTION

In recent years signal transduction mechanisms responsible for the initiation of inflammatory responses have been identified and are being reviewed as attractive targets for drug development. One of the best studied signaling routes is the mitogen activated protein kinase (MAPK) signal transduction pathway which plays a key role in many aspects of immune mediated inflammatory responses. MAPK signaling pathways have been implicated in the pathogenesis of a variety of human diseases [1]. MAPKs are members of intracellular serine/threonine specific kinases that integrate several environmental and extracellular stimuli into a variety of fundamental cellular processes [2].

Inflammatory responses, in general, are characterized by a common gamut of genes and endogenous mediators such as growth factors, inflammatory cytokines and toxic molecules such as nitric oxide or free radicals [3]. Macrophages that are activated in response to inflammatory stimuli subsequently activate intracellular signaling pathways like MAPK pathways which in turn holds the signal needed to activate the production of inflammatory mediators [4].

On activation of the MAP kinases, transcription factors present in the cytoplasm or nucleus such as nuclear factor (NF)-κB or activator protein-1 are phosphorylated and activated, leading to expression of target genes resulting in a biological response [5]. MAPKs, through these transcription factors, play a key role both in the expression and activation of pro-inflammatory cytokines such as interferon-γ, tumor NF-α, interleukin-1β (IL-1β), and IL-8, both at the transcriptional and the translational levels [6].

The first MAPK to be discovered was extracellular signal regulated kinases (ERK1) or MAPK3 in mammals followed by ERK2 or MAPK1. ERK1 and ERK2 are 83% identical with most of the differences falling outside the kinase core. Since the ERK signaling pathway is involved in both physiological and pathological cell proliferation, it is natural that ERK1/2 inhibitors would represent a desirable class of antineoplastic agents.

In this study, attempts have been made to evaluate the docking effect of some phytocompounds against MAPK1.

METHODS

Plant material

Plant source selected for this study was *Barleria montana* Wight and Nees and its leaves were collected from Kolli Hills, Eastern Ghats, Tamil Nadu, India. Geographically it is situated between 11°10,00 to 11°30,00 N and 78°15,00 to 78°30,00 E. The altitude of this hill ranges from 180 m above mean sea level (MSL) at the foot hill to 1415 m above MSL on top. The plant was identified with the help of Prof. Dr. P. Jayaraman, Director, Institute of Herbal Botany, Plant Anatomy Research Centre, Chennai, Tamil Nadu, India and authenticated with the specimen deposited at Rapinat Herbarium (Voucher No. SJCBOT 1202), the Department of Botany, St. Joseph's College (Autonomous), Tiruchirappalli, Tamil Nadu, India.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS was performed using Perkin Elmer Clarus 500 equipped with a column type capillary column elite-5 (5% phenyl, 95% dimethylpolysiloxane), with a column length of 30 m/0.25 mm and a film

thickness of 250 µm, respectively. The residue was diluted (1 µl/ml) and 1 µl was taken as the injection volume. They were injected in the split mode with 10:1 ratio. The oven was programed at 50°C @ 8°C/minutes to 220°C (2 minutes) @ 8°C/minutes to 290°C (10 minutes) and the injector was maintained at 290°C. Helium was used as a carrier gas with a constant flow at 1 ml/minutes. The ionization voltage was 70 eV. The constituents were identified after comparison with those available in the computer library (NIST) attached to the GC-MS instrument and the results obtained are reported [7].

Selections of ligands and target molecule

Phytocompounds with proven anti-inflammatory activity, identified through GC-MS analysis of ethanolic extract of *B. montana* were selected as ligands. They were retrieved from the PubChem database based on literature studies, drawn in ChemSketch and optimized. The structure of MAPK1 was retrieved from the Protein Data Bank (PDB) database and molecular docking studies were carried out by targeting its active amino acid residues. Indomethacin was used as the reference compound.

RESULTS AND DISCUSSION

GC-MS analysis of ethanolic extract of *B. montana* revealed the presence of 26 compounds. A thorough literature study was carried out on these 26 compounds and 2 compounds phytol and phenol 2, 4 bis (1,1-dimethylethyl) were selected for *in silico* studies based on their proven anti-inflammatory ability. The 2 chemical constituents along with their molecular formula, molecular weight, retention time and percentage area are tabulated in Table 1.

Protein preparation

AutoDock is a suite of automated docking tool. It is designed to predict the binding of small molecules, such as substrates or drug candidates, to a receptor of known three-dimensional (3D) structure [8]. The protein MAPK1 (Fig. 1) was assigned with Kollmann charges that aided in addition of hydrogens and side chains were optimized for hydrogen bonding. The energy minimized protein was then saved in PDB format. Using MGLTools - 1.4.6 the nonpolar hydrogens were merged, AutoDock atom type AD4 and gasteiger charges were assigned, and finally saved in protein.pdbqt format [9].

Table 1: GC-MS phytocompounds of *B. montana*

S. No.	Peak name	Retention time	Peak area	Percent of peak area
1.	Name: Phenol, 2,4-bis (1,1- dimethylethyl) Formula: $C_{14}H_{22}O$ MW: 206	17.35	2160237	0.5535
2.	Name: Phytol Formula: $C_{20}H_{40}O$ MW: 296	26.39	2345264	0.6009

B. montana: *Barleria montana,* GC-MS: Gas chromatography-mass spectrometry

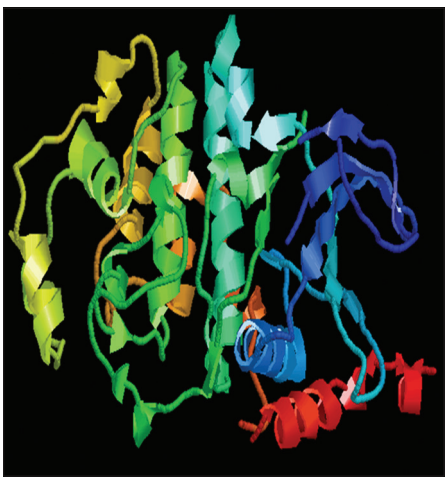

Fig. 1: Rasmol view of mitogen activated protein kinase 1

Ligand preparation

Structure of ligands was drawn using Chemsketch, optimized with 3D geometry and the two-dimensional structure of phytol, phenol 2, 4 bis (1,1-dimethylethyl) and indomethacin were converted into 3D structure (Figs. 2-4) using the Open Babel format molecule converter [10] and

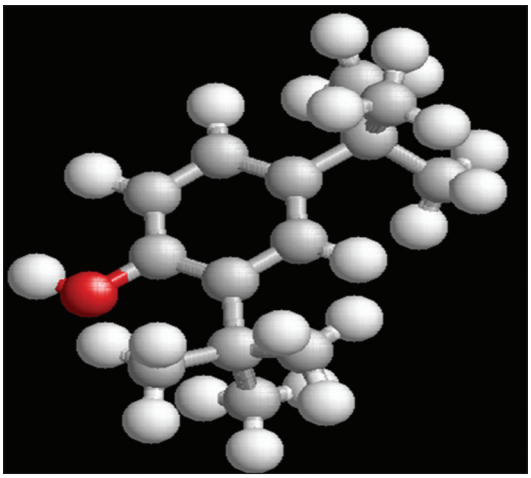

Fig. 2: Phenol 2, 4 bis (1, 1-dimethylethyl)

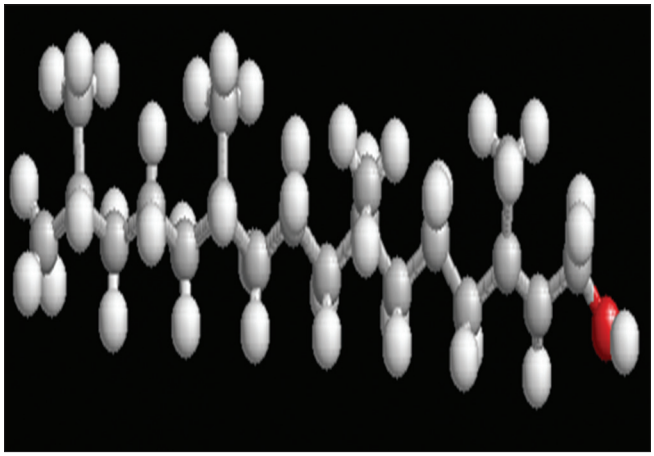

Fig. 3: Phytol

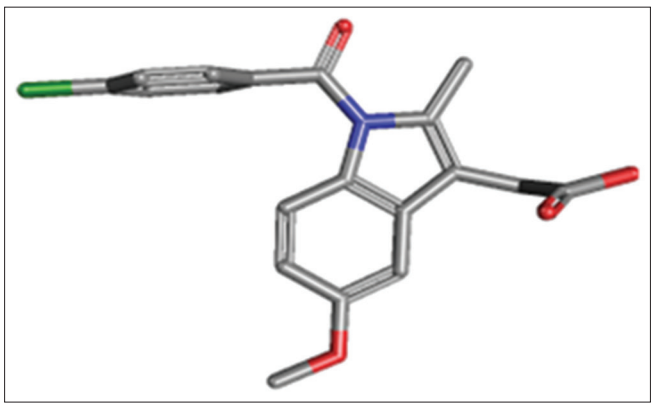

Fig. 4: Indomethacin

saved in PDB format for AutoDock compatibility. MGLTools-1.4.6 (The Scripps Research Institute) was used to convert ligand.pdb files to ligand.pdbqt files.

Active site prediction

The active site of the protein is its binding site or usually a pocket at the surface of the protein that contains residues responsible for substrate specificity which often act as proton donors or acceptors. Identification and characterization of binding site is the key step in structure-based drug design. The binding site was identified by computational and literature reports. The active site region of the protein was identified by Q - site [11]. These servers analytically furnished the area and the volume at the probable active site of each pocket to envisage the binding site.

Docking protocol

Grid parameter files (protein.gpf) and docking parameter files (ligand.dpf) were written using MGLTools-1.4.6. Receptor grids were generated using 90×60×60 grid points in xyz with grid spacing of 0.375 Å. Grid box was centered and cocrystallized ligand map types were generated using autogrid4. Docking of macromolecule was performed using an empirical free energy function and lamarckian genetic algorithm, with an initial population of 250 randomly placed individuals, a maximum number of 106 energy evaluations, a mutation rate of 0.02, and a crossover rate of 0.80. 100 independent docking runs were performed for each ligand. Results differing by 2.0 Å in positional root mean square deviation were clustered together and represented by the result with the most favorable free energy of binding.

Absorption, distribution, metabolism, excretion (ADME) toxicity prediction

Plant compound inhibitor can also be optimized by computational ADME prediction as it is crucial in inhibitor designing. Inadequate ADME properties are the cause of many drug development failures [12]. Hence, in this investigation, care was taken to study if the selected compounds exhibited appreciable ADME properties to take it further for inhibitor designing. The results so obtained (Table 2) possessed all the characteristics of the plant molecule such as molecular weight, computed dipole moment of the molecule, Ionization potential (IP), Electron

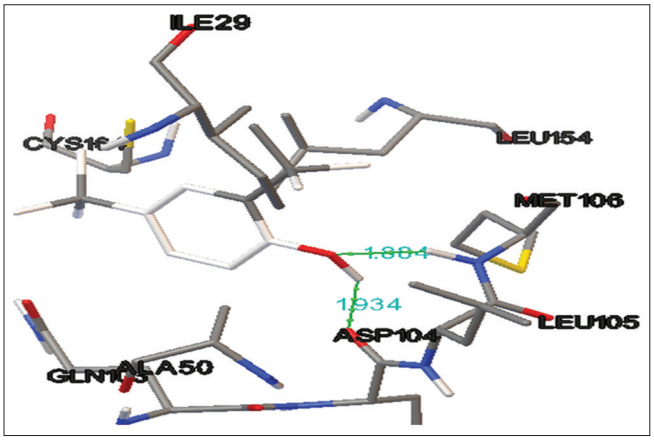

Fig. 5: Docking of phenol 2, 4 bis (1, 1-dimethylethyl) with mitogen activated protein kinase 1
Pictorial representation of docking of phenol 2, 4 bis (1, 1-dimethylethyl) with mitogen activated protein kinase 1 (Note: MET106 N-H...O; bond length 1.884; ASP104 O-H...O; bond length 1.934; auto dock score: - 6.2 K cal/mol; IC_{50} value - 4.72)

Cluster rank	**=1**
Estimated free energy of binding	**=−6.33 kcal/mol**
Final docked energy	**=−6.2 kcal/mol**
(1) IC_{50} value	**=4.72**
(2) H-bond interaction	**=MET106 N-H...O**
(3) Bond distance	**=1.884 Å**
Final docked energy	**=−6.2 kcal/mol**
(1) IC_{50} value	**=4.72**
(2) H-bond interaction	**=ASP104 O-H...O**
(3) Bond distance	**=1.934**

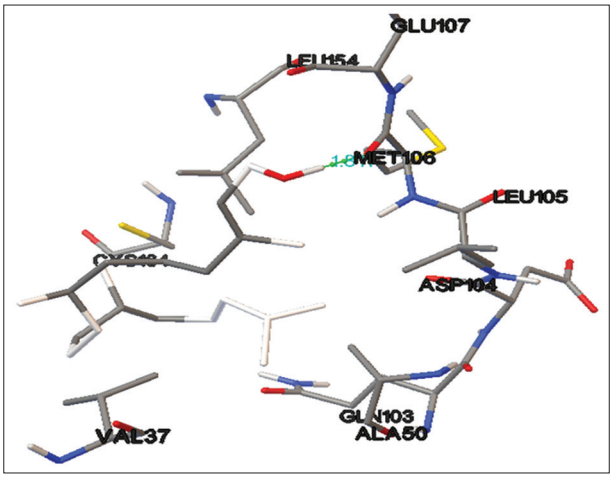

Fig. 6: Docking of phytol with mitogen activated protein kinase 1
Pictorial representation of docking of phytol with mitogen activated protein kinase1 (Note: MET106 N-H...O; bond length 1.811; auto dock score: −7.2 K cal/mol; IC_{50} value - 1.21)

Cluster rank	**=1**
Estimated free energy of binding	**=−6.00 kcal/mol**
Final docked energy	**=−7.2 kcal/mol**
(1) IC_{50} value	**=1.21**
(2) H-bond interaction	**=MET106 N-H...O**
(3) Bond distance	**=1.811 Å**

Table 2: ADME toxicity prediction for the selected plant molecules

S. No.	Compound name	Mol. Weight	Log P	tPSA	Rotatable bonds	Rigid bonds	HB donors	HB acceptors
1	Phytol	296.53	4.19	20.23	13	1	1	1
2	Phenol 2, 4 bis (1, 1 - dimethylethyl)	206.32	4.32	34.46	10	4	2	4
3	Indomethacin	357.79	4.00	68.53	4	19	1	5

ADME: Absorption, distribution, metabolism, excretion, HB: Hemoglobin

Table 3: Molecular docking studies of plants compounds with MAPK1

S. No.	Compound name	Docking score	IC_{50} value	H-bond interaction	Distance
1	Phytol	−7.2	1.21	MET106 N-H...O	1.811
2	Phenol 2, 4 bis (1, 1 - dimethylethyl)	−6.2	4.72	MET106 N-H...O	1.884
				ASP104 O-H...O	1.934
3	Indomethacin	−5.78	9.21	MET106 N-H...O	2.165
				LYS112 N-H...O	2.007

MAPK: Mitogen activated protein kinase

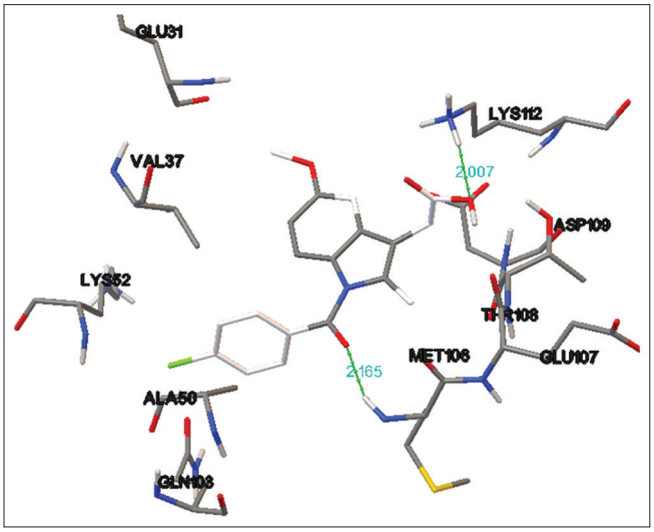

Fig. 7: Docking of indomethacin with mitogen activated protein kinase 1
Pictorial representation of docking of indomethacin with mitogen activated protein kinase 1 (Note: MET106 N-H…O; bond length 2.165; LYS112 N-H…O; bond length 2.007; auto dock score: −5.78 cal/mol; IC_{50} value - 9.21)

Cluster rank	**=1**
Estimated free energy of binding	**=−6.67 kcal/mol**
Final docked energy	**=−5.78 kcal/mol**
(1) IC_{50} value	**=9.21**
(2) H-bond interaction	**=MET106 N-H…O**
(3) Bond distance	**=2.165 Å**
Final docked energy	**=−5.78 kcal/mol**
(1) IC_{50} value	**=9.21**
(2) H-bond interaction	**=LYS112 N-H…O**
(3) Bond distance	**=2.007**

affinity (EA), π (carbon and attached hydrogen) component of the solvent accessible surface area) (PISA), Weakly polar component of the solvent accessible surface area (WPSA), Polar surface area (PSA), volume, #rotor, donor hemoglobin (HB), accept HB which were very essential to determine if the selected ligand possessed the desired biological activity.

Docking studies

Study on domain region of a protein forms the platform of understanding the positioning of amino acid residues in its active sites. Interestingly, amino acid residues in the domain region claim to play a central role in its functions and establish a structure-function relationship of a protein in its tertiary conformation. In this study, the amino acid residues methionine (MET106), aspartate (ASP104), lysine (LYS112), isoleucine, and leucine were involved in the active site of MAPK1.

Docking analysis of the selected ligands and the target (Table 3) showed that the amino acid residue MET106 was involved in interaction with phytol in the active site of MAPK1 with a bond length of 1.811 Å and the inhibitory concentration 50% (IC_{50}) value of this compound was 1.21 µm (Fig. 5).

The amino acid residues MET106 and Thr 108, interacted with the second ligand phenol 2, 4 bis (1,1-dimethylethyl) with a bond length of 1.884 Å and 1.934 Å, respectively, and the IC_{50} was found to be 4.72 µm (Fig. 6).

The bonding of the chosen ligands with the active sites of MAPK1 was compared with the reference drug indomethacin which showed two interactions with the amino acid residues MET106 and LYS112 at a bond length of 2.165 Å and 2.007 Å with an IC_{50} value of 9.21 µm (Fig. 7).

The results obtained showed that there was effective hydrogen bonding established between the ligands and target which fell within the acceptable and effective range of >1.5 Å and <3.2 Å. The IC_{50} values of the ligands chosen were lesser than the reference drug which proved its efficacy in exhibiting a strong anti-inflammatory activity.

CONCLUSION

This study showed that 2 phytocompounds from the ethanolic leaf extract of *B. montana* exhibited appreciable inhibitory potential against MAPK1. The docking score, binding energy, and IC_{50} values all indicated that the selected phytocompounds had a much better docking potential against MAPK1 than the reference compound indomethacin.

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Research Article

STANDARDIZATION STUDIES ON THE FRUITS AND WOOD OF *TERMINALIA CATAPPA* L.

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ABSTRACT

Objective: The tremendous increase in the use of medicinal plants warrants the quality control studies of herbal preparations to produce safe and efficacious herbal products. Hence, in this study, efforts were made to develop botanical and chemical standards for *Terminalia catappa* L. fruits and wood.

Methods: Organoleptic, macroscopic, and powder microscopic standards were developed. Moisture content, ash values, and extractive values were determined.

Results: Preliminary phytochemical analysis revealed the presence of certain important phytochemical constituents. Fluorescence analysis revealed the presence of chromophores. Using standard procedures, primary and secondary metabolites, vitamins and elements were estimated.

Conclusions: The selected plant sources were found to be devoid of heavy metal contamination. High performance thin layer chromatography chromatogram revealed the presence of gallic acid. The data of the results obtained could find use in deciding the genuineness and quality of the plant drugs.

Keywords: Botanical, Chemical, Standardization, *Terminalia catappa* L.

INTRODUCTION

Plants are considered as the key reservoirs of natural entities having tremendous therapeutic value. With the progress in chemical techniques, crude drugs came to be replaced by pure chemical drugs and the developed countries witnessed a decline in the popularity of medicinal plant therapy. However, during the recent past, the pendulum has swung again and there is a resurgence of interest in study and use of medicinal plants. At present medicinal plants as a whole occupy a stable position in modern medicine. The revival of interest in plant derived drugs is mainly due to current widespread belief that "Green Medicine" is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects. Phytotherapy acts as a bridge between traditional medicine and modern medicine. The need to control and assure the quality of the herbal medicine through systematic scientific studies including chemical standardization, biological assays and validated clinical trials is gaining tremendous importance, as a large majority of population of developed as well as developing countries relies on these medicines.

Terminalia catappa L., a large spreading tree belongs to the family Combretaceae, is distributed throughout the tropics in coastal environments. The dried leaves are used as an alternative to antibiotics to control fish pathogens [1]. Various extracts of different parts of *T. catappa* have been reported to exhibit antimicrobial [2-5], anti-inflammatory [6-8], antioxidant and antitumor [9], anti-HIV [10] and hepatoprotective [11] and antidiabetic properties [12] besides being aphrodisiac [13]. The moderate consumption of the seed kernel is useful in treating sexual dysfunction among men, primarily for premature ejaculation [13]. The ethanol extract of the leaves of *T. catappa* inhibits osmotically-induced hemolysis of human erythrocytes in a dose-dependent manner [11]. Punicalagin and punicalin isolated from the leaves are used to treat dermatitis and hepatitis as both have strong antioxidative activity [6]. The potential medicinal use of this tree has not been fully studied and present work is undertaken to contribute toward the quality control studies on the fruit and wood of *T. catappa*.

METHODS

Collection and authentication of plant materials

Selected plant parts such as fruits and wood of *T. catappa* Linn. For the proposed study were collected from in and around Mannargudi, Tamil Nadu, India. The identity of the plant specimens was confirmed using Flora of Presidency of Madras [14]. The botanical identity was authenticated by comparing with the herbarium specimen deposited at RAPINAT Herbarium, St. Joseph's College, Tiruchirappalli, Tamil Nadu, India (Voucher specimen number P. N. 001/2012).

Organoleptic and macroscopic features

The organoleptic characters of the samples were evaluated based on the textual methods [15]. Macroscopic features were determined by simple observation.

Powder microscopic studies

A pinch of powdered material was placed on a microscopic slide. A small amount of chloral hydrate and few drops of water added [16]. The slide was warmed over a water bath for few minutes, then washed thoroughly and mounted carefully in 30% glycerol. The salient microscopic features of the drug were observed under Carl Zeiss microscope using various staining agents. Photomicrographic images were taken using ProgRes Digital Camera.

Determination of physicochemical constants

The procedures recommended in Indian Pharmacopoeia were followed for determining loss on drying at 110°C, total ash, acid insoluble ash, water soluble ash, and alcohol and ethanol extractive values [17].

Preliminary phytochemical and fluorescence analysis

It involves testing of plant powder and different extracts of selected parts of *T. catappa* for the presence of different classes of compounds and to determine their chemical profile. The methods used for detection of various phytochemicals were done as per standard texts [18-20]. Fluorescence analyses of the plant powder and extracts were carried out according to standard procedure [21].

High performance thin layer chromatography (HPTLC)
HPTLC was performed with a view to develop chemical standards. Flavonoids were analyzed by HPTLC according to the method of Sethi [22]. In this work analysis of gallic acid was performed with the help of HPTLC instrument. The HPTLC system (Camag, Muttenz, Switzerland) consists of (1) TLC scanner connected with software under MS Windows NT, (2) Linomat V Sample applicator, and (3) photo documentation system Camag, Reprostar III.

About 100 mg of hydroalcoholic extract and aqueous extract was refluxed with 2 N hydrochloric acid. Filtered and extracted with diethyl ether. The extracts were evaporated to dryness. The dried residue was dissolved in suitable volume of solvent and is used for TLC analysis. Dissolved 25 mg of gallic acid in 25 ml of methanol and used as standard. Toluene:ethyl acetate:formic acid (5:5:1) was used as mobile phase. Chamber saturation was done for 18 hrs. TLC plate precoated with silica gel 60 F_{254} was used as stationary phase, obtained from Merck. The thickness of the plate was 0.2 mm. The samples and standard were prepared. The TLC plate was activated by heating at 120°C for about 30 minutes before use. Methanol extract of standard and hydroalcoholic and aqueous extracts of sample were applied with Linomat V applicator. The mobile phase used was toluene:ethyl acetate:formic acid (5:5:1). No prewashing of the plate was done. Chamber saturation time was 18 hrs. The TLC plate was kept for development to a migration distance of 8 cm. The plate was dried in hot air oven at 108°C for 10 minutes and scanned at 254 nm. The R_f and peak area of the spots were interpreted using software. The plate was photo documented under 254 nm and 366 nm light using Camag Reprostar 3, equipped with 12 bit CCD camera.

Estimation of inorganic constituents
Inorganic constituents are estimated using atomic absorption spectrometric method (Perkin Elmer model 400, 2008). This procedure is based on flame absorption and depends on the fact that metal atoms will be absorbed strongly at discrete characteristic wavelength, which coincide with emission spectra line of a particular metal.

Estimation of primary and secondary metabolites
Total carbohydrate content was estimated as per the method described by Plummer [23]. Total proteins were estimated using the method of Lowry *et al*. [24]. Total lipids were estimated using the method described by Cox and Pearson [25]. Secondary metabolites such as alkaloids [26], flavonoids [27], tannins [28], lignin [29], glycosides [30], total terpenoids [26], saponins [31], and phenols [29] were estimated using standard protocols.

Estimation of vitamins
Estimation of vitamin A [32], vitamin C [33], and vitamin E [34] was carried out using the prescribed procedures.

RESULTS AND DISCUSSION

Several concerns regarding the safety and quality of the herbal medicines have become mandatory because of the tremendous increase in the usage of medicinal plants globally. The safety and efficacy profiles of numerous plants worked out through animal studies and clinical trials are very expensive and time consuming. Reverse pharmacology can be used to identify bioactive constituents from herbal formulations. Hence, in this work, attempts are made to determine the chemical standards for an interesting herbal drug *T. catappa* fruits and wood.

To ensure the reproducible quality of the herbal products, proper identification, and authentication of the plant drug is very much essential [35]. Plant identification and authentication is the most essential part of any pharmacognostical study. Hence, in the present dissertation also after proper identification and authentication, the plant drug was subjected to preliminary phytochemical and analytical studies. Organoleptic study forms an important part of powder analysis and is a technique that is helpful in the qualitative evaluation of the test drug. Its utility in plant identification has been reported in many studies [36-38]. In this work, organoleptic standards were determined for the selected plant drug and presented in Table 1.

Macroscopic features of *T. catappa* observed were summarized in Table 2. Sessile drupe fruits and strong pliable wood are very much characteristic to *T. catappa*.

Powder microscopic features of the selected plant parts were observed and studied using Carl Zeiss microscope and photographs were taken with the help of ProgRes Digital camera and microscopic standards determined were presented in Figs. 1 and 2.

Tests for identity, purity, and strength
Physicochemical data of the drugs are important parameters to detect identity, purity, and strength of the selected parts of *T. catappa*.

Foreign matter
The term "Foreign matter" is used to designate any matter, which does not form part of the drug as defined in the monograph. Drug should be free from molds, insects, animal fecal matter, and other contaminants such as earthy material, stone or any other extraneous material. In this study, fruit and wood samples contained 0.01% and 0.43% foreign matter, respectively (Table 3). This indicates the purity of the parts selected for the studies.

Loss on drying
Loss on drying was determined for the bark, fruit, and wood powder of *T. catappa* as per the method described in Indian pharmacopeia. Deterioration time of the plant material depends on the amount of water present in it. If the water content is high, the plant material can be easily deteriorated due to contamination by fungal colonies [20]. The loss on drying determined at 105°C for the selected plant drug was found to be 17.5% for fruits and 9.9% for wood (Table 3). The drug must be dried properly before it enters any formulation/preparation.

Ash values
Ash values are a significant pharmacognostic tool which aids to decide quality and purity of crude drugs. The ash content is generally considered as a residue remaining after incineration, which simply

Table 1: Organoleptic features of the plant powder

S. No.	Character	Observance	
		Fruits	**Wood**
1	Color	Pink	Yellowish white
2	Odor	Pleasant odor	No characteristic odor
3	Taste	Vinous taste	Bland

Table 2: Macroscopic features of *Terminalia catappa*

S. No.	Parts	Features
1	Fruits	Sessile, laterally compressed, and ovoid to ovate, smooth-skinned drupe
2	Wood	Strong and pliable

Table 3: Physicochemical constants of different parts of *Terminalia catappa*

S. No.	WHO parameters	*Terminalia catappa* (% yield W/W)	
		Fruits	**Wood**
1	Foreign matter	0.01±0.00	0.43±0.01
2	Loss on drying	17.5±0.01	9.9±0.01
3	Total ash	11.53±0.02	1.39±0.01
4	Acid insoluble ash	0.49±0.01	0.10±0.01
5	Water soluble ash	10.16±0.01	0.75±0.01
6	Sulfated ash	12.62±0.01	1.82±0.01

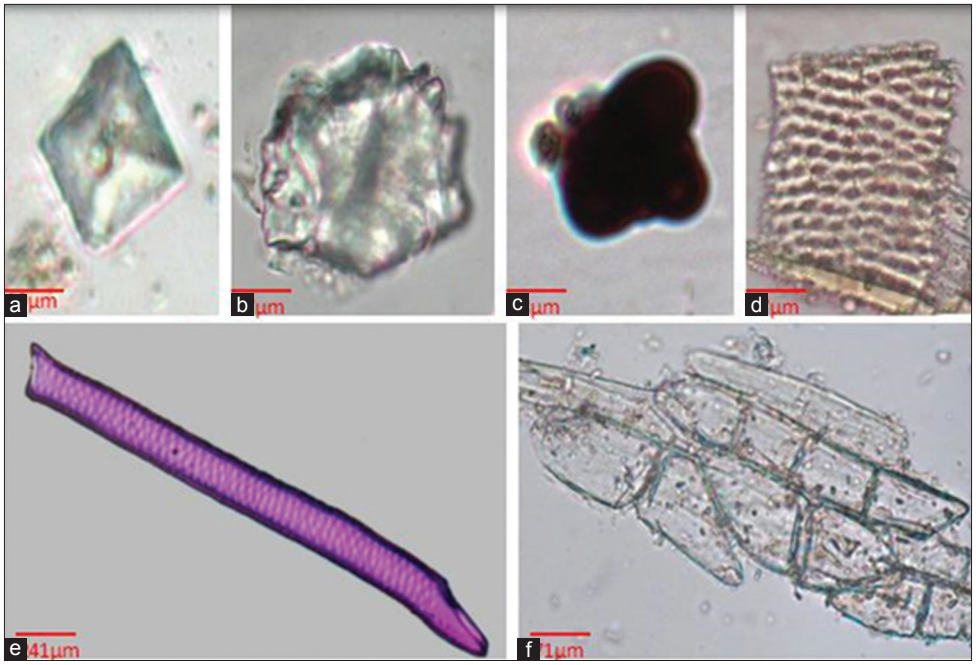


Fig. 1: Powder microscopic features of *Terminalia catappa* L. fruits. (a) Prismatic calcium oxalate crystal (×400), (b) druses calcium oxalate calcium crystal (×1000), (c) compound starch grains (×100), (d and e) xylem vessels with simple pitted thickening, (f) parenchyma cells

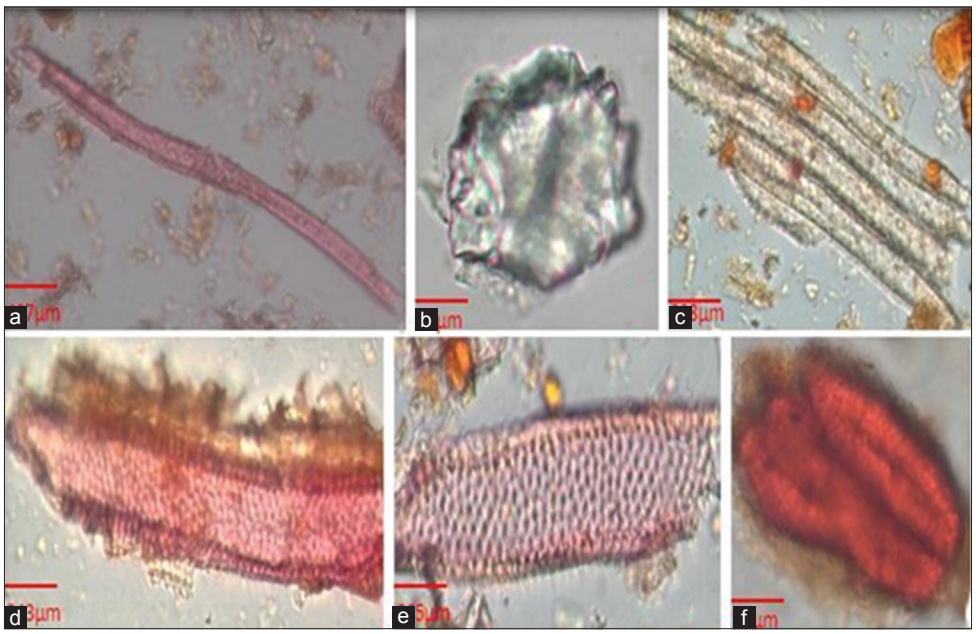


Fig. 2: Powder microscopic features of *Terminalia catappa* L. wood. (a) Aseptate fiber (×400), (b) druses calcium oxalate calcium crystal (×1000), (c) tracheary fibers (×400), (d and e) xylem vessels with pitted thickening (×400), (f) sclereids (×400)

represents inorganic salts, naturally occurring in crude drug or adhered to it or deliberately added to it in the form of adulteration. Total ash, acid-insoluble ash and water soluble ash percentage were determined for the selected drugs as per the WHO protocols. The total ash method is employed to measure the total amount of material remaining after ignition. This includes both "physiological ash" which is derived from the plant tissue itself and "nonphysiological ash," which is the residue of the extraneous matter adhering to the plant surface. Acid-insoluble ash is a part of total ash and measures the amount of silica present, especially, as sand and siliceous earth. Water soluble ash is the water soluble portion of the total ash [20]. The data of the ash values (Table 3) obtained in this work revealed higher values of total ash content and lesser acid-insoluble ash in all the three samples selected for this study, which indicated the purity and the presence of higher inorganic contents in these test drugs.

Extractive values

Extractive values are primarily useful for the determination of exhausted or adulterated drugs and is an important tool to check quality and variation in chemical constituents of the drug. In the selected plant drugs, it is noted that water extractive values are higher than all other extractive values suggesting the presence of high polar compounds (Table 4). Among the three parts selected, water extractive value of fruit is found to be maximum (50.24%) than wood, suggesting that fruits are rich in chemical content.

Preliminary phytochemical screening

The therapeutic effect or the medicinal value of any particular plant drug depends on the nature of secondary metabolites present in it. Determination of phytochemical profiles of plants thus helps in qualitatively detecting the class of compounds present in them [39]. Data obtained from the results of preliminary phytochemical screening of drug powders (Table 5) revealed the presence of certain important phytochemical constituents such as tannins, phenols, flavonoids, and alkaloids in all the parts selected and water extract proved to be rich in chemical contents.

Table 4: Percentage yield of various extracts of different parts of *Terminalia catappa*

S. No.	Solvent	*T. catappa* (% yield W/W)	
		Fruits	Wood
1	Hexane	0.21590±0.03	0.0320±0.01
2	Chloroform	0.7599±0.03	0.2959±0.01
3	Ethyl acetate	1.6639±0.02	0.2639±0.01
4	Ethanol	29.7484±0.54	0.7199±0.02
5	Water	50.2379±0.95	1.81±0.01

Table 5: Preliminary phytochemical analysis of various extracts of *Terminalia catappa* L.

Parameters	*Terminalia catappa* L.									
	Fruits					Wood				
	H	CH	EA	E	W	H	CH	EA	E	W
Protein	-	-	-	+	+	-	-	-	-	+
Coumarins	-	+	+	-	+	-	-	-	+	+
Fats and oil	-	-	-	-	-	-	-	-	-	-
Sterols	-	+	+	+	-	-	+	-	-	-
Terpenoids	+	+	+	+	+	+	+	+	+	+
Sugar	-	-	+	+	+	+	+	+	+	+
Flavonoids	-	+	+	+	+	-	-	-	+	+
Alkaloids	-	+	+	+	-	-	-	+	+	-
Glycosides	-	-	+	+	+	-	-	-	-	-
Saponins	-	-	-	-	-	-	-	-	-	-
Tannins	-	-	+	+	+	-	-	-	+	+
Phenols	-	-	-	+	+	-	+	+	+	+
Quinones	-	-	-	-	-	-	-	-	-	-
Lignin	-	-	-	+	+	-	+	-	+	-
Gum test	-	-	-	-	-	-	-	-	-	-

+: Present, -: Absent, H: Hexane extract, CL: Chloroform extract, EA: Ethyl acetate extract, E: Ethanol extract, W: Water extract

Fluorescence analysis

Fluorescence analysis of the drug powder treated with different reagents or solvents helped to detect various chromophores present in the test drugs [40]. Fluorescence behavior of the selected plant powders and their successive extracts were observed under day and ultraviolet (UV) light at 254 and 366 nm after treatment with aqueous and alcoholic acids and bases. The extracts and powder as such fluorescence various shades of yellow, green, and brown in day as well as UV light. Different solvent extracts exhibited various shades of yellow and green fluorescence in day light and UV light, respectively (Tables 6 and 7). Yellow chromophores observed in this study indicate the presence of flavones and green chromophore indicates the presence of sterols in the selected plant drugs.

HPTLC

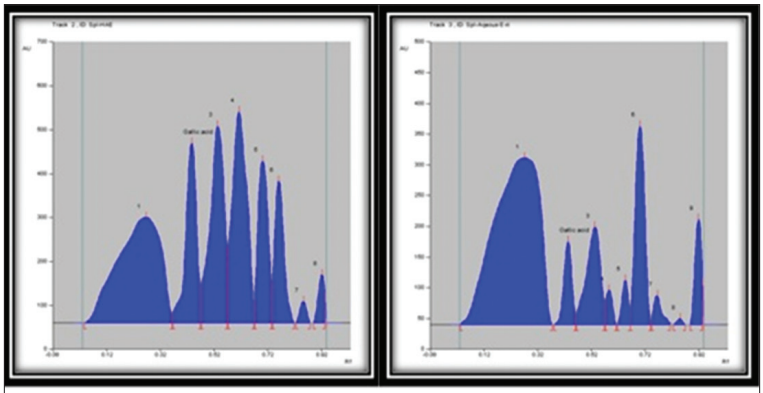
HPTLC is a valuable quality assessment tool for the chemical evaluation of botanical materials. HPTLC fingerprints of the hydroalcoholic and aqueous extracts of the selected plant drugs were determined using single mobile phases for detecting gallic acid equivalents. In all the three plant drugs tested, a band corresponding to gallic acid is visible in test solution tracks. Gallic acid is commonly used in the pharmaceutical industry. It is used as a standard for determining the phenol content of various analytes by the Folin-Ciocalteu assay; results are reported as gallic acid equivalents. The pharmaceutical and nutraceutical industries are nowadays confronted with adulteration and cheating [39]. Determination of salient standards for herbal drug is inevitable in this field to check adulteration and substitution. HPTLC finger printing observed in this study could serve as a chemical standard to check the quality and genuineness of the selected plant drug.

HPTLC fingerprint of *T. catappa* fruit at 366 nm revealed one spot at Rf 0.55 (black) at standard solution Track (S1). Test solution Track 1 (T1) corresponding to 10 µl of hydroalcoholic extract of *T. catappa* fruit, revealed 8 spots at Rf 0.37, 0.47, 0.74 (blue); 0.57 (black), 0.67, 0.82, 0.93 (red) and 0.88 (green). Test solution Track 2 (T2), corresponding to aqueous extract revealed 9 spots at Rf 0.38, 0.46, 0.61, 0.74, 0.87 (all blue), 0.57 (black), 0.67, 0.81, 0.93 (all green). A band (Rf 0.55) corresponding to gallic acid is visible in both standard solution (Track 1) and test solution tracks (Track 2 and 3) (Fig. 3).

Table 6: Fluorescence features of different parts of *Terminalia catappa* L. powder

Treatment of drug powder with various reagents	Fruits			Wood		
	Day light	UV light (nm)		Day light	UV light (nm)	
		254 nm	366 nm		254 nm	366 nm
Powder as such	P	BN	BK	LY	BN	B
1 N NaOH (in H_2O)	G	BN	G	LY	BN	B
1 N NaOH (in ethanol)	G	BK	B	G	BK	B
50% HCl	P	BK	BN	DB	BN	BN
50% H_2SO_4	P	BK	P	G	BN	BN
50% HNO_3	YB	BK	Y	BN	BK	Y
Ammonia	YB	BK	BK	BN	BK	B
Iodine	R	BN	BK	Y	BN	Y
$FeCl_3$ solution	BK	BK	BN	Y	BN	BN
Acetic acid	BK	BK	BN	BN	BK	G
Petroleum ether	P	BN	P	Y	BN	BN
Chloroform	PL	BK	R	BN	BK	B
Potassium hydroxide	BK	BN	BK	BN	BN	B
H_2O	P	BK	B	Y	BN	BN
Ethyl acetate	R	BN	PL	BN	BN	G
n Hexane	P	BK	B	BN	BK	B
Picric acid	P	BN	BK	Y	BN	G
Methanol	P	BN	B	BN	BN	G

B: Blue, BK: Black, BN: Brown, DB: Dark brown, G: Green, LY: Light yellow, P: Pink, PL: Purple, R: Red, Y: Yellow, YB: Yellowish brown



Rf	Height	Area %
0.37	22.4	29.43
0.47	87.8	12.31
0.57	175.1	17.52
0.67	49.7	19.43
0.74	95.9	10.40
0.82	0.1	8.24
0.88	0.2	0.90
0.93	70.7	1.78

a

Rf	Height	Area %
0.38	0.3	59.13
0.46	18.3	5.08
0.57	39.2	10.49
0.61	0.4	1.70
0.67	26.9	2.10
0.74	7.7	14.33
0.81	2.8	1.78
0.87	0.3	0.25
0.93	75.3	5.16

b

Fig. 3: High performance thin layer chromatography chromatogram of hydroalcoholic, (a) and aqueous extract, (b) of *Terminalia catappa* fruit

Table 7: Fluorescence features of extracts of *Terminalia catappa*

Extract	Fruits			Wood		
	Day light	UV light (nm)		Day light	UV light (nm)	
		254	366		254	366
H	Yellow	Colorless	Red	Colorless	Colorless	Blue
CL	Dark green	Green	Red	Colorless	Colorless	Blue
EA	Yellow	Dark Brown	Blue	Colorless	Colorless	Blue
E	Dark green	Brown	Red	Colorless	Colorless	Blue
W	Brown	Dark brown	Dark brown	Brown	Light brown	Light brown

H: Hexane extract, CL: Chloroform extract, EA: Ethyl acetate extract, E: Ethanol extract, W: Water extract

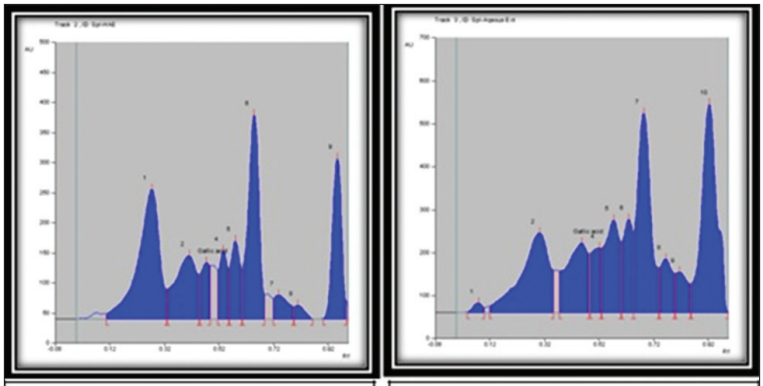
Table 8: Elemental analysis of aqueous and ethanol extracts of different parts of *Terminalia catappa* L.

Elements	*Terminalia catappa* L.			
	Fruits		Wood	
	Aqueous	Ethanolic	Aqueous	Ethanolic
Organic carbon %	0.86	0.82	0.81	0.72
Total nitrogen %	1.09	0.98	1.29	1.22
Total phosphorus %	0.22	0.16	0.48	0.42
Total potassium %	3.32	3.21	5.62	5.62
Total sodium %	0.32	0.30	0.24	0.18
Total calcium %	6.36	8.32	5.13	5.24
Total magnesium %	5.66	6.32	3.02	3.02
Total sulfur %	0.43	0.41	0.67	0.63
Total zinc ppm	1.11	1.06	2.19	2.35
Total copper ppm	0.06	0.12	0.04	0.08
Total iron ppm	152.36	142.03	136.25	123.65
Total manganese ppm	7.54	7.62	8.13	8.65
Total boron ppm	0.01	0.03	0.02	0.06
Total molybdenum ppm	0.02	0.02	0.01	0.03

HPTLC fingerprint of *T. catappa* wood at 366 nm revealed one spot at Rf 0.55 (black) at standard solution Track (S1), Test solution Track 1 (T1) corresponding to 10 µl of hydroalcoholic extract of *T. catappa* wood, revealed 9 spots at Rf 0.33, 0.56, 0.61, 0.69, 0.87 (all blue), 0.45, 0.49, 0.80, 0.99 (all green). Test solution Track 2 (T2), corresponding to aqueous extract revealed 10 spots at Rf 0.10, 0.36, 0.53, 0.60, 0.65, 0.86, 1.00 (all blue), 0.49 (green), 0.74 (light blue), and 0.80 (black). A band (Rf 0.55) corresponding to gallic acid is visible in both standard solution (Track 1) and test solution tracks (Track 2 and 3) (Fig. 4).

Elemental analysis

It is a known fact that plant growth is regulated by various biochemical processes involving inorganic elements present in the soil. Trace quantities of these elements are essential for the enzyme activity involved in the biological processes. These elements are derived from plants for human health care. Their presence is vital for the maintenance of health and to prevent diseases [41]. Quantitative estimation of inorganic constituents in the selected plant drugs revealed the presence of high and moderate levels of iron, manganese, calcium, magnesium, and zinc (Table 8).



a

Rf	Height	Area %
0.33	49.1	27.53
0.45	73.4	13.44
0.49	88.6	4.94
0.56	83.6	5.72
0.61	83.4	7.21
0.69	39.9	21.99
0.80	19.7	3.43
0.87	0.1	1.36
0.99	30.9	14.37

b

Rf	Height	Area %
0.10	11.9	0.65
0.36	98.7	16.38
0.49	135.8	10.76
0.53	149.2	4.56
0.60	163.7	10.06
0.65	187.1	6.53
0.74	103.9	19.54
0.80	90.2	4.82
0.86	65.9	3.65
1.00	1.0	23.05

Fig. 4: High performance thin layer chromatography chromatogram of hydroalcoholic, (a) and aqueous extract, (b) of *Terminalia catappa* wood

Table 9: Heavy metal analysis of powder of *Terminalia catappa*

S. No.	Elements	*Terminalia catappa* (ppm)	
		Fruits	Wood
1	Lead	0.02	0.09
2	Mercury	Nil	0.02
3	Cadmium	0.03	0.06
4	Arsenic	Nil	Nil
5	Selenium	0.01	0.11
6	Chromium	0.02	0.05
7	Cobalt	0.01	0.03

Table 10: Estimation of vitamins present in the aqueous extract of *Terminalia catappa*

S. No.	Vitamins (µg/Kg)	*Terminalia catappa* L.	
		Fruits	Wood
1	Vitamin A	693	152
2	Vitamin C	279	26
3	Vitamin E	397	48

Heavy metal analysis

The data of the results obtained for heavy metal analysis revealed that all the three herbal drugs tested were free from arsenic. Lead, mercury, and cadmium were found to be below the permissible limits (Table 9).

Analysis of vitamins

Vitamins are important immunomodulators and play a vital role in alleviating the burden of diseases (Table 10).

Table 11: Estimation of primary and secondary metabolites of *Terminalia catappa* L.

Constituents	*Terminalia catappa* L. (mg/g)			
	Fruits		Wood	
	Aqueous	Alcoholic	Aqueous	Alcoholic
Total carbohydrates	1.22	1.32	0.98	1.05
Total proteins	0.43	0.41	0.31	0.32
Total fats	0.02	0.03	0.02	0.03
Total alkaloids	1.26	1.08	0.98	0.96
Total flavonoids	3.87	3.26	2.29	2.19
Tannin	0.22	0.54	0.75	0.63
Lignin	0.13	0.42	0.72	0.65
Glycosides	0.12	0.03	0.06	0.08
Terpenoids	0.05	0.05	0.02	0.03
Saponins	0.03	0.04	0.03	0.03
Phenols	0.08	0.13	0.08	0.19

Quantitative analysis of primary and secondary metabolites

Medicinal plants possess a variety of pharmacological activities based on the type and amount of secondary metabolites present in them. Primary and secondary metabolites present in the selected plant drugs were estimated using standard protocols. Among the secondary metabolites, estimated, flavonoid content was more in fruits than wood (Table 11).

CONCLUSION

To conclude present work depicted presence of interesting phytochemicals, nutraceutical elements, and molecules which could

very well be used as marker molecules for the identification of useful parts of fruits and wood of *T. catappa* and could act as chemical standards for the selected herbal drug.

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Review Article

HEPATOPROTECTIVE SIDDHA PLANTS AND THEIR MAJOR CHEMICAL CONSTITUENTS: A REVIEW

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ABSTRACT

The liver is a vital organ that is vulnerable to various diseases such as hepatitis A, B, C, and E, alcoholism, fatty liver, cirrhosis, cancer and injury due to exposure to several chemical compounds. Liver disease is still a serious health problem worldwide. According to World health organization viral hepatitis kills about 1 million people every year and has estimated that more than 80% of the world's population use routinely traditional medicine to meet their primary care health. Since time immemorial plants formed the major source of medicine and plants mentioned in Siddha system of medicine are known for their wide spectrum of therapeutic potentials. The Siddha system of medicine became significantly more popular all over the globe because of its curative property with minimal or no serious side effects. Hepatoprotective and antioxidant potentials of single herbs and polyherbal Siddha formula are available that can protect liver from various toxic substances and can also inhibit deleterious oxidative changes. Among many plants that are used to treat liver disorders, in this review, herbs are classified based on their significant hepatoprotective and antioxidant actions. In this review, ten herbs are discussed along with their phytoconstituents having hepatoprotective action and antioxidant properties include *Phyllanthus amarus, Eclipta prostrata, Curcuma longa, Tinospora cordifolia, Glycyrrhiza glabra, Terminalia chebula, Boerhavia diffusa, Cassia fistula, Phyllanthus emblica,* and *Cuminum cyminum.*

Keywords: Hepatoprotective, Phytomolecules, Siddha medicine, Medicinal plants.

INTRODUCTION

The use of plants for medicinal purposes is a practice that has been used since time immemorial, and plants from the main source of medicine. Today medicinal properties of a plant are attributed to the substances of different chemical nature (secondary metabolites), present in plants which vary according to plant species, but are bestowed with therapeutic benefits.

The World Health Organization (WHO) has estimated that more than 80% of the world population uses routinely traditional medicine to meet their primary health care and much of traditional treatments involve use of plant extracts or their active ingredients [1,2].

A key origin comes from South India where the use of medicinal plants constitute an important part in ancient traditional practice known as Siddha system of medicine. In Tamil Nadu, more than 10000 preparations are commonly used for various disorders. This medicine is also practiced among people of Sri Lanka, Malaysia, and in other Tamil speaking lands.

The Siddha system of medicine became significantly more popular all over the globe because of its curative property, less toxicity, and has no side effects [3,4]. In recent years, it gained greater importance. Many single plants and polyherbal formulations are used in Siddha system for treating many diseases, including liver diseases.

Liver is a vital organ that is vulnerable to various diseases such as hepatitis A, B, C and E, alcoholism, fatty liver, cirrhosis, and cancer due to exposure of several chemical compounds [5]. Liver diseases are still a serious health problem worldwide. According to the WHO, viral hepatitis kills about 1 million people every year. In addition, an estimated 500 million people experience chronic illness from their infection with hepatitis; it is a major cause of liver cancer and liver cirrhosis [6].

Free radicals have been implicated in many disease processes including liver disease, cardiovascular, and cancer [7]. Even though various drugs such as antiviral drugs, corticosteroids, immunosuppressant, and other synthetic drugs are available in modern medicine to treat hepatitis; the prognosis is unfavorable and also causes serious adverse effects [8].

Medicines from Siddha system are much beneficial than the modern powerful synthetic drugs. Hepatoprotective and antioxidant therapy in Siddha system of medicine include usage of single herb to polyherbal formulas that can protect the liver from various toxic substances and also can inhibit deleterious oxidative changes and is considered as a very important tool for the treatment of liver disorders.

Among many plants that can be used to treat liver disorders, present review establishes a classification based on hepatoprotection and antioxidant actions.

LIST OF HERBS USED AS A HEPATOPROTECTIVE AGENTS

1. *Phyllanthus amarus* [9A]
2. *Eclipta prostrata* [9B]
3. *Curcuma longa* [9C]
4. *Tinospora cordifolia* [9D]
5. *Glycyrrhiza glabra* [9E]
6. *Terminalia chebula* [9F]
7. *Boerhavia diffusa* [9G]
8. *Cassia fistula* [9H]
9. *Phyllanthus emblica* [9I]
10. *Cuminum cyminum* [9J]

Phyllanthus amarus

Botanical name: *Phyllanthus amarus*
Family: Euphorbiaceae
Tamil name: *Keezhanelli*
Part used: Whole plant
Phytoconstituents: Phyllanthin [10].

Uses given in Siddha text: *P. amarus* leaves and root are ground and mixed in buttermilk. This cures jaundice.

Recent research: Hepatoprotective effects of aqueous extract from *P. amarus* is evaluated on ethanol-induced hepatotoxic rats. Hepatic injury was studied in *in vitro* model where *P. amarus* increased

the 3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide reduction assay and decreased the release of aspartate transaminase (AST) and alanine transaminase (ALT) in rat primary cultured hepatocytes treated with ethanol. The results revealed that treatment of rats with *P. amarus* extract orally brought cell recovery in ethanol-induced liver injury by bringing the levels of AST, ALT, high-sensitivity human thyroglobulin, and tumor necrosis factor-α to normal. Histopathological study confirmed the beneficial effect of *P. amarus* with its potential antioxidant activity [11].

Eclipta prostrata
Botanical name: *Eclipta prostrata*
Family: Asteraceae
Tamil name: *Karisalai*
Part used: Whole plant
Phytoconstituents: Wedelolactone [10].

Uses given in Siddha text: Give 2 g of *Eclipta prostrate* powder is given twice a day for 5 days with honey to cure jaundice.

Recent research: Therapeutic efficacy of *E. prostrata* was evaluated using male Wistar rats and it was found that the curative effect of *E. prostrata* was comparable to that of the standard drug Silymarin. The optimum effective dosage was found to be 200 mg of ethanolic extract per body weight of the experimental animals used. In this study, the plant *E. prostrata* L. was found to produce significant liver protection. The drug was standardized pharmacognostically and the optimum dosage was found to be 200 mg/kg body weight of the animal. Technically and economically feasible protocol was developed for optimization of the secondary metabolite of *E. prostrata* [12].

Curcuma longa
Botanical name: *Curcuma longa*
Family: Zingiberaceae
Tamil name: *Manjal*
Part used: Rhizome
Phytoconstituents: Curcumin [10].

Uses given in Siddha text: 30- 60 ml *C. longa* decoction twice daily to cure jaundice. This has to be taken regularly till the symptoms subside.

Recent research: Curcumin possesses hepatoprotective and choleretic properties. Curcumin has been demonstrated *in vivo* to prevent lipid peroxidation from diverse agents such as carbon tetrachloride, and aflatoxin from *Aspergillus parasiticus* [13]. In animal models, curcumin proved to be a potent choleretic, increasing bile output by almost 100% in one study [14].

Tinospora cordifolia
Botanical name: *Tinospora cordifolia*
Family: Menispermaceae
Tamil name: *Seenthil*
Part used: Stem wood
Phytoconstituents: Tinosporin [10].

Uses given in Siddha text: Extract of *T. cordifolia* is called *Seenthil sarkarai*. This *sarkarai* is dried and 2 g taken regularly twice a day for 5 days with milk.

Recent research: *T. cordifolia* plant material exerts protective action against CCl4 induced hepatocellular alterations through synthesis of proteins, or due to deactivation of CCl4 accelerated detoxification. The potential of *T. cordifolia* to minimize the effects of free radicals including the peroxy radicals in association with the inhibition of lipid peroxidation, the Siddha drug can be considered as hepatoprotective agent because the combined synergistic effect of its constituents and micronutrients rather than any single factor through free radicals activity [15].

Glycyrrhiza glabra
Botanical name: *Glycyrrhiza glabra*
Family: Fabaceae
Tamil name: *Adhimadhuram*
Part used: Root
Phytoconstituents: Glycyrrhizin, glycyrrhetinic acid [10].

Uses given in Siddha text: Roots of *G. glabra* and *Azima tetracantha* taken in equal ratio and ground with lemon juice for 3 days. This mixture is consumed twice a day for 3 days.

Recent research: Biochemical studies indicated that glycyrrhizinates inhibit 11-hydroxysteroid dehydrogenase, the enzyme responsible for inactivating cortisol. The continuous, high-level exposure to glycyrrhizin compounds was reported to produce hyper mineralocorticoid such as effects in both animals and humans. These effects were found to be reversible on withdrawal of licorice or glycyrrhizin. Other *in vivo* and clinical studies have reported beneficial effects of both licorice and glycyrrhizin consumption which revealed good responses including antiulcer, antiviral, and hepatoprotective responses [16].

Terminalia chebula
Botanical name: *Terminalia chebula*
Family: Combretaceae
Tamil name: *Kadukkai*
Part used: Fruit rind
Phytoconstituents: Chebulic acid, chebulinic acid, chebulagic acid, gallic acid, corilagin, and ellagic acid [10].

Uses given in Siddha text: 2 g of powder in water twice a day for 3 days cures jaundice.

Recent research: Ethanolic extract of *T. chebula* fruit showed strong hepatoprotective activity [17]. It also showed similar property against antituberculosis drug rifampicin, isoniazid, and pyrazinamide (combination) induced toxicity. This is because of its prominent antioxidative and membrane stabilizing activities [18]. Protective effects of aqueous extract of *T. chebula* fruit on the tert-butyl hydroperoxide-induced oxidative injury was observed in cultured rat primary hepatocytes and rat liver models [19,20].

Boerrhavia diffusa
Botanical name: *Boerrhavia diffusa*
Family: Nyctaginaceae
Tamil name: *Mookirattai*
Part used: Root
Phytoconstituents: Punarnavine [10].

Uses given in Siddha text: Decoction of this root consumed regularly cures jaundice. About 30-60 ml of this decoction is taken twice a day for 5 days.

Recent research: The hepatoprotective activity of roots of different diameters collected in three seasons, rainy, summer and winter, and was evaluated in thioacetamide intoxicated rats. The results showed that an aqueous extract (2 ml/kg) of roots of diameter 1-3 cm, collected in the month of May (summer), exhibited marked protection of a majority of serum parameters, i.e., glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, acid phosphatase, and alkaline phosphatase (ALP), but not glutamate dehydrogenase and bilirubin, thereby suggesting the proper size and time of collection for *B. diffusa* roots for obtaining desirable results. Further, the studies also proved that the aqueous form of drug (2 ml/kg) administration has more hepatoprotective activity than the powder form; this is probably due to the better absorption of the liquid form through the intestinal tract. The hepatoprotective activity of *B. diffusa* roots showed marked protection of serum parameters in thioacetamide toxicity in rats. Furthermore, the aqueous extract of thin roots collected in the summer has more activity suggesting the proper time and type of root collection for the

most desirable result. The investigation also validates scientifically the use of *B. diffusa* roots in hepatic ailments by the several tribes in India [21]. An alcoholic extract of whole plant of *B. diffusa* given orally exhibited hepatoprotective activity against experimentally induced carbon tetrachloride hepatotoxicity in rats and mice. The extract also produced an increase in normal bile flow in rats suggesting a strong choleretic activity. The extract showed no signs of toxicity up to an oral dose of 2 g/kg in mice [22].

Cassia fistula

Botanical name: *Cassia fistula*
Family: Caesalpiniaceae
Tamil name: *Sarakkondrai*
Part used: Tender leaf, flower
Phytoconstituents: Aurantimide acetate, B-sitosterol, free rhein, glucoside and sennosides A and B [10].

Uses given in Siddha text: Grind flowers or flowers along with tender leaves. This mixture is taken twice a day for 3 days to cure jaundice.

Recent research: The hepatoprotective activity of the n-heptane extract of *C. fistula* leaves was evaluated scientifically. The extract at a dose of 400 mg/kg body weight exhibited significant protective effect by lowering serum levels of transaminase (serine glutamic- oxaloacetate transaminase [aspartate aminotransferase] and serine glutamic pyruvic transaminase [alanine aminotransferase]), bilirubin and ALP. The protective effect is comparable to that of a standard hepatoprotective agent [23].

Phyllanthus emblica

Botanical name: *Phyllanthus emblica*
Family: Euphorbiaceae
Tamil name: Nelli
Part used: Fruit rind
Phytoconstituents: Sesquiterpene lactone [10].

Uses given in Siddha text: The fruit is ground well and 5 g of the mixture is given with 250 ml of butter milk to cure jaundice.

Recent research: *P. emblica* extract was investigated on ethanol induced rat hepatic injury. Protective roles of this against induced liver injury were evaluated scientifically. *P. emblica* decreased the severity of hepatic fibrosis induced by thioacetamide and carbon tetra chloride. *P. emblica* effectively reversed profibrogenic events possibly due to its antioxidant activity. Hepatic activity of *P. emblica* against antituberculosis drugs induced hepatic injury is also reported. *P. emblica* exhibits hepatoprotective activity due to its membrane stabilizing antioxidative and CYP 2E1 inhibitory property [24].

Cuminum cyminum

Botanical name: *Cuminum cyminum*
Family: Apiaceae
Tamil name: *Seeragam*
Part used: Fruit
Phytoconstituents: Cuminol and cymene [10].

Uses given in Siddha text: Dry *C. cyminum* in *P. amarus* juice and powder it. 4 g of this powder consumed along with 2 g of dried ginger cures jaundice.

Recent research: Aqueous ethanolic extract of dried seeds of *C. cyminum* revealed hepatoprotective activity in nimesulide intoxicated albino rats. *C. cyminum* extract reduced the level of liver markers ALP, serum oxaloacetic transaminase, serum glutamic-pyruvic transaminase, and tuberculosis. *C. cyminum* extract at a dose level of 200 mg/kg, significantly ($p<0.001$) reduced the level of all elevated parameters as compared to other doses. *C. cyminum* extract in 300 mg/kg showed better reduction in the level of all the parameters as compared to the dose of 100 mg/kg but less than dose of 200 mg/kg [25].

SIDDHA HERBAL MEDICINES USEFUL IN THE TREATMENT OF LIVER DISEASES

1. Siru vilvathy ilagam
2. Elathy Chooranam
3. Seeragathy Chooranam
4. Sanjeevi maathirai
5. Naagarathy nei [26]
6. Sarabaraja maathirai [27]
7. Thengaai Legiyam
8. Legiya kuzhambu
9. Vilvathy Legiyam [28]
10. Siringi Perathi Chooranam [29]
11. Pitha Kamaalai Chooranam [30]

CONCLUSION

Hepatic disorders stand as one of the foremost health problem throughout the world. Therapies developed along modern medicine are often limited in efficacy and also carry risk of adverse effects. Therefore, treating liver diseases with plant derived compounds are essential. This study reveals plant extracts with hepatoprotective properties against toxic chemicals that cause injury to liver. These plants may offer new alternatives to limited therapeutic options that exist in the present treatment for liver diseases. This review dealt with the plant extracts and their isolated phytoconstituents on the liver protection. Effective formulations need to be developed from the treasure of Siddha medical system with proper pharmacological evaluations and clinical trials which can contribute in treating many degenerative diseases and disorders include liver disorders.

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Review Article

PHYTOCONSTITUENTS FROM SIDDHA PLANT DRUGS IN THE MANAGEMENT OF ALZHEIMER'S DISEASE: "A STRANGE DISEASE OF THE CEREBRAL CORTEX"

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ABSTRACT

In Siddha system of medicine, many single and polyherbal formulations have been used to treat cognitive disorders such as Alzheimer's disease (AD) and other neurodegenerative diseases and memory related disorders. In this review, a detailed approach is attempted on Siddha herbal drugs that could help us in identifying potential new drugs for memory disorders. There are numerous plants available in Siddha system of medicine and chemical constituents present in the plants and are now in clinical use, especially in AD therapy. Siddha medicines have the potential to be developed into optimum pharmaceuticals for AD because of their broad spectrum characteristics. In this review, plants and their constituents that possess pharmacological actions which could be used in the treatment of cognitive disorders, including enhancement of cholinergic activity in the central nervous system, anticholinesterase enzyme and antioxidant are discussed. In this review, seven Kayakalpa (Rejuvenating herbs) herbs namely *Centella asiatica, Strychnos potatorum, Emblica officinalis, Solanum trilobatum, Amaranthus tricolor, Zingiber officinale, Achyranthes bidentata,* and anticholinesterase drugs such as *Bacopa monnieri, Citrus medica, Urtica dioica* were presented and discussed. Although scientific evidence to prove the efficacy of herbal preparations in treating AD is increasing, the current available data in support of their use is inadequate. The chemical compositions of medicinal plants and their efficiency in reducing symptoms of AD or in altering the disease mechanism need to be further studied.

Keywords: Siddha system of medicine, Alzheimer's disease, Anticholinesterase, Antioxidant herbs, Cognitive disorder.

INTRODUCTION

Alzheimer's disease (AD) is a disease affecting neurons in brain causing cognitive impairment. It is one of the most widespread forms of known dementia, causing progressive decline of cognitive functions and behavior. According to a recent survey, it has become the sixth leading cause of death and the fifth leading cause of death in the elderly over 65 years [1-4]. Recent research suggests processes that are involved in the formation of senile plaques and neurofibrillary tangles [5].

Etiopathogenesisis

The deregulation of metabolism of amyloid precursor protein in the central nervous system leading to the formation of neuritic or senile plaques, corresponding to abnormal amyloid extra neuronal deposits in center, surrounded by dystrophic neuritis and inflammatory components [6].

Current treatments offer moderate symptomatic relief but there is no treatment available to delay or prevent the progression of the disease.

This abnormal amyloid accumulation is also responsible for a sequence of pathological processes, not well determined, and is expressed in the formation of neurofibrillary tangles, corresponding to intraneuronal deposits of cytoskeletal protein with greater reactivity and glia inflammatory reaction and finally leads to cell death [7,8].

Oxidative stress is another pathophysiological mechanism that intervenes in the neuronal degeneration of AD which has led to therapeutic trials employing vitamin E, selegiline, estrogen, and drugs from traditional practices [9].

Siddha system of medicine has a long history of prevention and treatment in the management of cognitive impairment. Although AD is a modern pathological entity and has no direct analogue in the ancient and classical literature of Siddha medicine, memory disorders, and cognitive deficits are referenced as dementia in various Siddha literatures. Important herbal sources and formulations from the treasure of Siddha system of medicine in treating AD are discussed in sequence and belong to three major categories as,

1. Kayakalpa drugs.
2. Plants specific for AD especially in improving cognitive function.
3. Plants as acetyl cholinesterase inhibitors.

KAYAKALPA HERBALS (REJUVENATING HERBS)

Kayakalpa plants mentioned in Siddha text revitalizes the body protecting every cell and helps to attain longevity. It has also been used for centuries in Siddha system of medicine to prevent ageing and protects the body from diseases.

Following list of plants are known as kayakalpa drugs:

1. *Centella asiatica* (*Vallarai*) [10A,11]:

Phytochemicals: Asiaticoside A and B, brahmic acid, brahmoside.
Uses: Leaves are used as memory enhancers and as brain tonic.

2. *Withania somnifera* (*Ammukira kizhangu*) [10B,12A]:

Phytochemicals: Withaferin -A, withanolide E, F, I, K, L, M and S.
Uses: Roots are used as nervine tonic and help the person to attain longevity.

3. *Solanum trilobatum* (*Thoodhuvalai*) [10C,12B]:

Phytochemicals: Solasodine.
Uses: Leaves and flowers give clarity to the brain, and makes the aged look young.

4. *Amaranthus tricolor* (*Sirukeerai*) [10D,12C]:

Phytochemicals: Amaranthine, isoamaranthine, stigmasterol, sitosterol.
Uses: When cooked with pepper and salt and consumed enhances sharpness of brain.

5. *Zingiber officinale* (*Inji*) [10E,12D]:

Phytochemicals: Gingerol, Shagaol, gingiberine, gingerine.
Uses: Sliced ginger soaked in honey taken for 45 days gives longevity, vigor and vitality.

There are many plants and plant extracts containing several compounds that can have positive effects on cells in the brain and body. They are antioxidants which protect cell membranes and regulate neurotransmitter function properties.

PLANTS USEFUL IN AD

Following plants are found to improve the cognitive function in AD as per Siddha literature:

1. *Celastruspaniculatus* (*Vaaluzhuvai*) [10F,13A]:
   Phytochemicals: Celapanine.
   Uses: Seeds are used as nervine tonic

2. *Ipomoea mauritiana* (*Nilapoosani*) [10G,13B]:
   Phytochemicals: β- sitosterol, palmitic, stearic, linoleic, linolenic acid.
   Uses: Root improves knowledge and skill.

3. *Andrographispaniculata* (*Nilavembu*) [10H,13C]:
   Phytochemicals: Lactones- andrographolide, andrographin, andrographane.
   Uses: Whole plant gives clarity to the mind.

4. *Gossypium arboreum* (*Sembaruththi*) [10I,13D]:
   Phytochemicals: Gossypetin - 8-0- rhamnoside, quercetin - 3-0- glucoside.
   Uses: Two teaspoons of seed powder taken daily with milk strengthens the brain and nerves.

5. *Hibiscus rosasinensis* (*Sembaraththai*) [10J,13E]:
   Phytochemicals: Sesquiterpenoids, hibiscones A-D, hibisquinones A-D.
   Uses: Flowers are coolant and calms the brain.

6. *Butea monosperma* (*Palaasu*) [10K,13F]:
   Phytochemicals: Palasonin, methylallophanic acid.
   Uses: Seeds improves knowledge.

7. *Averrhoa carambola* (*Thamaraththam*) [10L,13G]:
   Phytochemicals: Rutin, cyanidin glucoside.
   Uses: Fruit cures mania, unwanted fear and insanity.

ACETYL CHOLINESTERASE INHIBITOR DRUGS OF PLANT ORIGIN

The scientific literature indicates that consuming certain plants as food or medicine benefits people with AD and could be as useful as acetyl cholinesterase inhibitor drugs such as donepezil [14]. According to the Siddha literature following plants are used in Siddha system of medicine for centuries and has properties similar to those of cholinesterase inhibitors.

1. *Bacopa monnieri* (*Neer Bhrami*) [15]:
   Phytochemicals: Bacoside A, bacosiide B, betulinic acid, stigma sterol [16].
   Action: It inhibits cholinergic degeneration and displayed cognition enhancing effect in rat model [15].

2. *Glycyrrhiza glabra* (*Adhimathuram*) [17]:
   Phytochemicals: Glycyrrhizin, glycyrrhizic acid, glycyrrhetinic acid, and glucuronic acid [18].
   Action: It has shown inhibitory property against cholinesterase enzyme [17].

3. *Convolvulus pluricaulis* (*Vishnu kirandi*) [19]:
   Phytochemicals: Convolvine, convolidine, confoline, convasine, kaempferol, and steroids [20].
   Action: This plant produced a dose-dependent increase in acetylcholinesterase activity [19].

4. *Terminalia chebula* (*Kadukkai*) [21]:
   Phytochemicals: Arjunglucoside I, arjungenin, chebulosides I, II, chebulinic acid, gallic acid, ethyl gallate, punicalagin [22].
   Action: It exerts acetylcholinesterase inhibitory activity and it may be good in the treatment of AD [21].

5. *Urtica dioica* (Perunkanchori) [23]:
   Phytochemicals: Acetylcholine, histamine, 5- hydroxyl tryptamine, protein, fat, fiber, etc. [24].
   Action: It enhances the cholinergic system in the brain and may be useful in treating AD [23].

Naturally expressed oils are far superior to refined oil. Cold pressed virgin coconut oil is proved effective in Alzheimer's. The neurons (nerve cells) in the brains of people with AD are unable to use glucose and to produce energy properly. Coconut oil may act as an alternative energy source, and can prevent the neuron from starving.

Latest therapies for AD are "acetyl cholinesterase inhibitors" and they work by reducing the levels of tau protein. Coconut oil when digested provides fuel (necessary energy) to keep the brain nourished. It improves brain health and is an antioxidant. This is why it is important to ensure that any potential treatment must be ascertained for its safety before it can be approved for widespread use in treating AD [25].

Like any other vegetable fats, cold pressed virgin olive oil might be useful to maintain healthy brain cells, and can help in preventing the progression of Alzheimer's and even reversing some of its symptoms. Omega-3 fatty acids rich flax seeds (*Aalivithai*), beans, sea fish like sardine, tuna and mackerel helps in preventing ageing. Memory boosting food like spinach, *C. asiatica* used as green vegetables such as radish, broccoli, berries, and dark skinned fruits can also be consumed to prevent AD [26,27].

CONCLUSION

Several herbal remedies and dietary supplements are available in Siddha system of medicine which was suggested as effective in the treatment of AD and related disorders. In Siddha system, many plants are said to possess phytoconstituents that can enhance memory and could favor the positive approaches in treating dementia. To treat Alzheimer's various single and polyherbal formulations are subjected to preclinical and clinical trials.

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Review Article

THE MULTI-FACETED ROLE OF *URAI MATHIRAI*: THE IMMUNE PILL OF SIDDHA

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ABSTRACT

In this post-antibiotic era, the general public and researchers are focusing their research to find solutions for treating their ailments and research purposes (respectively) through traditional systems of medicine. With the emergence of newer infections and allergens day by day, treatment options available today are often short lived and there is an urgent need for finding out new drugs to maintain human health. Scientists report that world health-care system will face a serious antibiotic resistance in the forthcoming centuries, in spite of the development of newer strains of microbes and newer antibiotics to cope up it is always advised to improve overall Immune power of the individuals especially from their childhood itself. In that way, *"Urai mathirai,"* a polyherbal Siddha formulation promises to be a very good immune booster, prevents contagious diseases besides being effective against *Maantham* (indigestion), *Kanai* (primary complex), chronic cough and fever. This tablet is recommended to children attending the OPD of all Government Siddha Hospitals and PHCs thereby ensuring the boosting up of the immune status of the individuals. This review paper deals with all the aspects of "*Urai mathirai,"* its ingredients and their supremacy as both prophylactic and therapeutic agent. To conclude, "*Urai mathirai"* has the greater ability to cure diseases ranging from pediatrics to geriatrics.

Keywords: Urai Marundhu, Skin care, Immune booster.

INTRODUCTION

In Siddha system, *Urai mathirai* - The Immune pill has a major role in pediatric and childhood immune-modulatory process. Many geographical and cultural evidence reveal that the pediatric classical drug *Urai mathirai* is used in Southern part of India (especially in Kerala and South Tamil Nadu) and even used in Srilanka. *Urai* means rubbing and *Mathirai* denotes its physical form (the tablet) [1,2]. The name itself denoted the mode of administration. It is to be given by rubbing and mixing with mother's milk and directly applying it to the babies' tongue [3,4]. The drug helps to improve the self-defense mechanism and boosts the whole immune system [5].

The immune system is an organization of different cells with specified role in defending the Infection. Clinically, children are more prone to the infections/allergens due to lack of immune stabilization and competency [6,7]. As per the Siddha literature, the diseases of pediatrics and children were classified based on its occurrence due to either external (*Pura karanangal*) internal (*Aga karanangal*) reasons. The common childhood problems are *Suram* (fever), *Erumal* (cough), *Kanam* (respiratory infections), *Kirumi* (worm infestations), *Seetha kazhichal* (dysentry), *Lasuna thabitham* (tonsilitis), *Kaamaalai* (jaundice), etc. [8,9].

INTEGRATED MANAGEMENT OF CHILDHOOD ILLNESS

As per WHO global health observation data, globally 5.9 million children died under the age of five in 2015 which is approximately 16,000 every day (Fig. 1). Out of that 83% of deaths are mainly due to the infections and nutritional conditions [10].

The concept of integrated management is also of mounting more awareness among scientists and Physicians around the world. WHO and UNICEF are also developing a solution to reduce the mortality by increasing child health improvisation strategy and by creating more awareness for the integrated health-care approach [7,11].

It is the novel time to search for a new solution to boost the cells of immune system. Keeping this in view, authors attempted the initial step with the literature review on aged, old and classically used immune modulating drug "*Urai mathirai."*

The combination of this drug varies by different authors [12,13]. Finally, we decided and choosed the combination used in the OPD department, Government Siddha Medical College, Palayamkottai, Tirunelveli (Table 1).

Scientific reports of each ingredient mentioning their actions especially immuno-modulatory activity, anti-allergic activity are reviewed. Literatures revealed that combination of polyherbal drug is effective in immune enhancing mechanism. Focus is made on pharmacological investigations of the polyherbal formulation.

Zingiber officinale

Z. officinale Rosc. (Zingiberaceae) belong to the family Zingiberaceae which is commonly known as *chukku* in Tamil. Ginger contains more volatile oils. It has been reported that it acts as a potent immune-modulating agent [16,17] in cell-mediated response studies [18] and Antiallergic drug [19]. The cellular and humoral immune responses in cyclophosphamide - immunosuppressed mice is observed. Experimental animals recovered the humoral immune responses in a dose-dependent manner when administrated with the test drug. Apart from its vital action, it is also reported to possess many pharmacological activities such as anti-inflammatory activity [20,21], antipyretic activity, carminative, antiviral activity, analgesic [21], antitussive, and expectorant activity.

Glycyrrhiza glabra

G. glabra is a tall perennial herb belonging to the family Fabaceae. It is widely spread in Meditarian regions of Asia and India [22]. It has been widely used for treating various ailments. Immune-modulatory activity is evaluated by its effect on leukocyte count [23] and phagocytosis (Carbon clearance method). Increased cell-mediated immune response and heameagglutination antibody count have revealed its immune-modulatory activity in efficacy of this plant drug with sheep blood cells [23]. Antiallergic such as anti-scratching behavior and IgE production-inhibitory activity were also studied. The plant drug also inhibited degranulation of RBL-2H3 cells induced by IgE with the antigen

(DNP-HSA) in animal models. It also posses antiviral and immune stimulant activity [24]. Beside this is scientifically proved antioxidant and antibacterial agent [25], anti-inflammatory activity [26], antiulcer activity [27], and anticancer [27,28] were also reported for this plant.

Anacyclus pyrethrum

Sharma *et al*. evaluated the immunomodulatory activity of petroleum ether extract of *A. pyrethrum* [29]. It is an important herb widely used for treating respiratory tract ailments. Petroleum extracts of *A. pyrethrum* were evaluated for immunomodulation activity by observing the total and differential leukocyte count, cyclophosphamide-induced immunosuppression and survival rate against *Candida albicans* infection, delayed type hypersensitivity reaction, percentage neutrophil adhesion, and phagocytic activity [29]. The data of the results suggested significant immune-modulatory activity [30]. It also possesses potent anti-bacterial activity [31], anticonvulsant [32], anabolic activity [33], antioxiadant activity [34], cognitive [35], and antidiabetic [33] activity.

Acorus calamus

A. calamus is commonly known as sweet flag. It is an important drug for the management of pediatric diseases. It has a pleasant smell, which repels the insects. In several villages, it is being used as insect repellent by holding it with a small thread in baby's hand. Rajagopal *et al*. (2011) evaluated the immuno-modulatory activity of *Vachadhatryadi avaleha* in albino rats. They evaluated the cell-mediated immunity and humoral immunity response by antibody formation against sheep red blood cells [36]. The results showed significant and enhanced secretion of cytokines like interleukin-4 (IL-4) and tissue growth factor-b both of which stimulate B-lymphocytes to proliferate [37]. It also posses anti-inflammatory and analgesic [38], Antibacterial

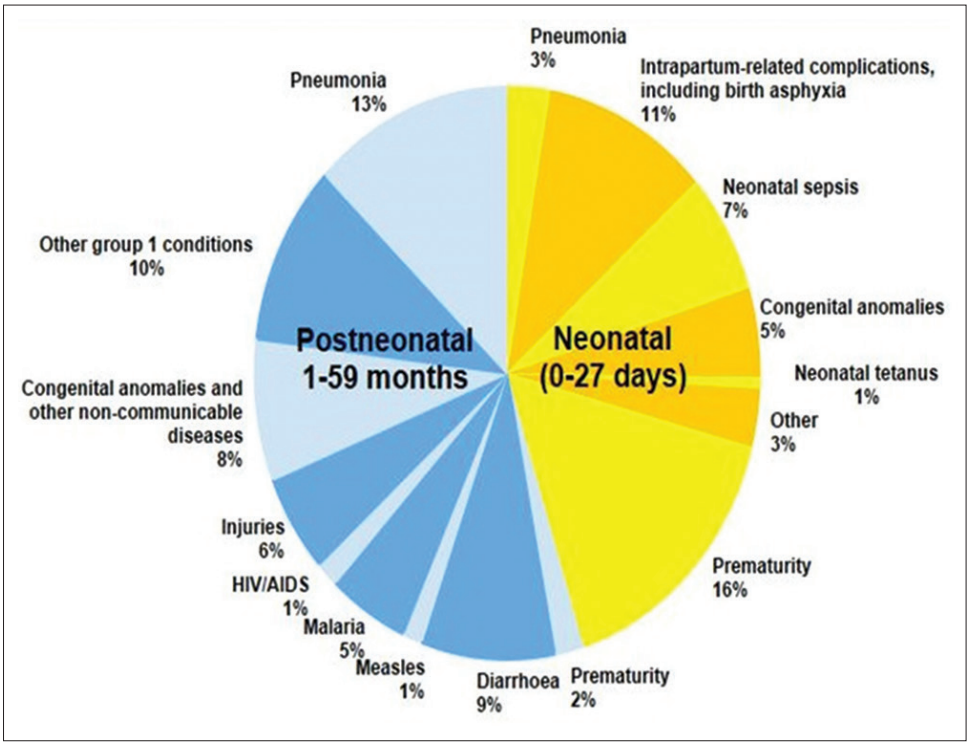

Fig. 1: Causes of death among children under 5 years of age during 2015

Table 1: Ingredients of the drug *Urai mathirai* [1,2,4,14,15]

Tamil name/common name	Botanical name/family	Parts used	Phyto-constituents	Uses in Siddha
Chukku/dry ginger	*Zingiber officinale*/Zingiberaceae	Rhizome	Gingerol, zingiberin	Stimulant, stomachic, carminative
Athimadhuram/liquorice	*Glycyrrhiza glabra*/Fabaceae)	Roots	Glycyrrizin, glycyrric acid	Emollient, demulcent, mild expectorant
Akkrakaram/pellitory	*Anacyclus pyrethrum*/Asteraceae	Stem	Pellitorin, anacyclin, sesamin	Stimulant, expectorant
Vasambu/sweet flag	*Acorus calamus*/Acoraceae	Rhizome	Asarone, aristolene	Anti-periodic, disinfectant, germicide
Jathikkai/nutmeg	*Myristica fragrans*/Myristicaceae	Seed coat	Myristicin, myristic acid, nectandrin-B	Tonic, carminative, stimulant
Maasikkai/oakgall	*Quercus infectoria*/Fagaceae	Galls	Gallic acid, ellagic acid, gallo tannins	Astringent, styptic, tonic
Kadukkai/chebulicmyrobalan	*Terminalia Chebula*/Combretaceae	Fruit	Chebulinic acid, chebulin, terchebin	Laxative, hepatoprotective
Perunkayam/asafoetida	*Ferula asafoetida*/Apiaceae	Resin	Asaresino tannols -A and B, umbelliferone	Antispasmodic, expectorant, diuretic
Thippili/long pepper	*Piper longum*/Piperaceae	Fruit	Piperidine amide, Piperine	Stimulant, carminative
Poondu/garlic	*Allium sativum*/Amaryllidaceae	Bulb	Alliin, ajoene, flavanoids	Digestive, carminative

activity [39], anthelmintic [40], antispasmodic activity [41]. Its hepatoprotective [42], antiulcer activity [43], and anticonvulsant [44] activities were also evaluated.

Myristica fragrans

Checker *et al*. evaluated the immuno-modulatory and radioprotective effects of lignans derived from fresh nutmeg mace (*M. fragrans*) in mammalian splenocytes [45]. They identified the special ligand in nut mug, which is therapeutically useful for immuno-modulation and other activities. The leukocytes count was brought to normal and the data obtained in significant. Antibacterial, antimicrobial [46], antifungal [47], antidiabetic [48], hepatoprotective [49], anti-inflammatory [50], and antioxidant [51] efficacies of the plant drug were also evaluated scientifically.

Quercus infectoria

Q. infectoria is a variety of Oak, traditionally used in Ayurveda and Siddha systems of medicine. It is a gall and styptic in nature [52]. It has antibacterial action and removes oral pathogens [53], antimicrobial activity [54], antioxidant [55], cognitive [56], anticancer activity [57], neuroprotective [57], wound healing activity [58], anti-inflammatory [59], antidiabetic activity [60], anthelmintic [61], and antiulcer [58] activity were studied for this plant source.

Terminalia chebula

T. chebula Retz. (Combretaceae) commonly named as myrobalan. It is an important herbal drug in Siddha Pharmacopoeia. Literature denotes the importance of Kadukkai with a quote as Mother Nurturing a Child. This drug is likely to aid for a healthy life and destroys all the wastes from body [1,2]. Aher and Wahi evaluated the immune-modulatory activity of alcoholic extract of *T. chebula* and noted the expressions of cytokines, viz., IL-2, IL-10, and TNF-α [62,63]. *T. chebula* is reported to be antimicrobial [64], hepatoprotective, antispasmodic and antidiarrheal [65] besides being antihyperglycemic [66,67], antioxidant [68], and antiamoebic [69].

Ferula asafetida

Mahima *et al*. (2012) evaluated the immune-modulatory activity of *F. asafetida* and overviewed the concepts of its indigenous use [70]. This herb comes under the WHO recognized plant drugs. Asafoetida is very useful in the digestion process. Many researchers proved its efficacy in gastric ailments [71] such as ulceration, gastric irritation, and acid peptic diseases. It is also being used as an antispasmodic and digestive [72]. Apart from that, it is a potent anti-oxidant and Anticancer agent especially for the breast cancer and also possess antifungal activity (Fatehi *et al*.).

Piper longum

Sunila and Kuttan evaluated the immuno-modulatory and antitumor activity of *P. longum* Linn. and its compound piperine. The results showed an increase in leukocytes level and plague formation number increased significantly [73]. Furthermore, cardioprotective [74] besides *P. longum* possess platelet enhancing effect [75], antiasthmatic effect [76], cognitive [77], antioxidant [78], antitumor [79], carminative and hypocholesterolemic [80], antiallergic [81], anti-inflammatory activity [82], and anthelmintic [82] efficacies.

Allium sativum

In India, consumption of garlic in diet is often noticed. Everyone believed that it helps digestion and prevents infection. Tende *et al*. (2014) evaluated the immuno-modulatory effect of the *A. sativum* in Wistar rats. The study findings showed that no significant change is observed in the leukocyte levels and also helps in destroying foreign pathogens. Besides it has anti-inflammatory [83], antimicrobial [84], antiallergic [85], antioxidant [86,87], and antiulcer activities [88].

CONCLUSION

"*Urai mathirai"* has been in use from time immemorial. It has found its place in almost all household remedies of Tamil Nadu and Kerala. Review of Siddha literature for the drug. "*Urai mathira"* indicates that the ingredients of this drug have potent and proven immuno-modulatory, anti-inflammatory, antiallergic, and digestive properties. Its role in the overall prophylactic and therapeutic action with regard to child health care is noteworthy. It is the right time to carry out extensive pre-clinical and clinical studies on this drug for the betterment of human healthcare, especially for child healthcare.

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Review Article

PHYTOMOLECULES OF TRADITIONAL DRUGS IN THE MANAGEMENT OF RHEUMATOID ARTHRITIS: A SYSTEMATIC REVIEW

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ABSTRACT

The term "arthritis" means inflammation of the joint that produces pain, stiffness, redness, and swelling. The type of the most common arthritis is rheumatoid arthritis (RA), which produces an inflammation of the lining of the joints. It is the most common cause of inflammatory arthritis and it is estimated that globally this condition affects between 0.5% and 1.0% of world population. If RA is not controlled by medicines, it can end up damaging the bones, ligaments and tendons. Currently, no specific treatment is available to cure RA. Modern medical system provides various effective drug treatments which includes nonsteroidal anti-inflammatory drugs, corticosteroids, disease-modifying antirheumatic drugs (DMARDs), biologic response modifiers (biological DMARD). However, all these drug treatments have been associated with adverse or side effects. Because of these and other limitations, patients with RA are likely to seek Siddha system of medicine to manage pain and inflammation. They control RA better than modern drugs with little or no side effects. In the treatment of RA, Siddha medicine has been identified as a potential benefit therapy. There are several herbal remedies that are considered beneficial in the treatment of RA as they help to reduce pain and inflammation. In this review, ten antiarthritic herbs *Hemidesmus indicus, Boerhaavia diffusa, Caesalpinia sappan, Butea monosperma, Costus speciosus, Euphorbia antiquorum, Withania somnifera, Semecarpus anacardium, Vitex negundo,* and *Smilax chinensis* are discussed. This article provides a systematic review depicting the effects of Siddha treatment for managing RA, and major substances present in these plants.

Keywords: Siddha, Inflammation, Rheumatoid arthritis, Antiarthritic herbs.

INTRODUCTION

The term "arthritis" means inflammation of the joint that produces pain, stiffness, redness, and swelling. The arthritis can affect any joint in the body such as hips, knees, spine, and other weight-bearing joints. The type of the most common arthritis is rheumatoid arthritis (RA), which produces an inflammation of the lining of the joints. But can also affect other extraarticular organ systems such as heart, kidney, lungs, eyes, skin, blood vessels, and other connective tissue present throughout the body. It is the most common cause of inflammatory arthritis and it is estimated that globally this condition affects between 0.5% and 1.0% of the overall world population. However, there are still many people affected with RA who are undiagnosed.

The incidence is higher in women than in men. Although RA may affect any person at any age, it is most commonly seen in the age group of 30-50 years. Eventhough there is a significant development in the field of immunopathology, the definite cause of RA remains uncertain [1].

If RA is not controlled by medicines, it can end up damaging the bones, ligaments, and tendons that surrounds the joint which then lead to progressive joint deformity and loss of functions of the affected joint. Currently, no specific treatment is available to cure RA. However, disease management and treatment modalities for RA are available and allow good control of the disease. The ideal treatment will aim to remove the pain, control the inflammation process, and prevent joint deformities in maintaining functional independence. Some scientific data in the recent years revealed that reactive oxygen species and other free radicals may trigger the pathogenesis of RA as well as other degenerative diseases [2-4].

Modern medical system provides various effective drug treatments which includes nonsteroidal anti-inflammatory drugs, corticosteroids, disease-modifying antirheumatic drugs (DMARDs), biologic response modifiers (biological DMARD). However, all these drug treatments have been associated with adverse or unwanted side effects which include nausea, vomiting, skin rash, alopecia, headache, mouth sores, muscle aches, hepatic and renal toxicity leading to life threatening conditions. A recent medical article showed that corticosteroids may actually increase the pain in the long run.

Because of these and other limitations, patients with RA are likely to seek for alternative therapy such as Siddha system of medicine to manage pain and inflammation. They sometimes relieve comparatively better than modern drugs with little or no side effects. According to scientific reports, 60-90% of dissatisfied arthritis patients are likely to seek the option of traditional therapy [5,6].

Many therapeutic measures from Siddha system of medicine are available today that can provide remarkable remedy to relieve symptoms and can improve prognosis, beside gives a very positive impact on the quality of life of those affected with RA. The use of medicinal herbs for prevention and treatment are major strategy existing in the ancient traditional medical practices. Through centuries, "Siddhars" have accumulated knowledge and experience in handling the medicinal plants and this traditional knowledge was passing orally from generation to generation. Later, with the development of writing this knowledge was compiled through palm leaf manuscript and is the only resource that could provide curative or preventive care for human society.

The growing popularity of Siddha medicine appears to be particularly common among people with chronic diseases. In the treatment of RA, Siddha medicines have been identified as a good therapy. There are several herbal remedies that could be useful for those affected, as they help to reduce pain and inflammation. This article provides a systematic review of Siddha treatments existing for RA.

HERBS HAVING ANTIARTHRITIC ACTIVITY

- *Hemidesmus indicus*
- *Boerhaavia diffusa*

- *Caesalpinia sappan*
- *Butea monosperma*
- *Costus speciosus*
- *Euphorbia antiquorum*
- *Withania somnifera*
- *Semecarpus anacardium*
- *Vitex negundo*
- *Smilax china.*

Hemidesmus indicus
- Common name: Indian Sarsaparilla
- Tamil name: Nannari
- Family: Asclepiadaceae
- Distribution: Throughout India
- A perennial, slender, lactiferous, wiry shrub with woody root-stock. The tuberous root is dark brown. It has strong central vasculature and a pleasant smell and taste.
- Parts used: Roots, leaves, and stem.

It has alterative, tonic, demulcent, diuretic, and diaphoretic action. It cures chronic Vadha disease. The stems are very useful in inflammations [7].

The leaves contain tannins, flavonoids, hyperoside, rutin, B-sitosterol, hemidesminine, hemidesmin 1 and 2.

It has protective activity against arthritis and the activity can be attributed to the presence of terpenoid and flavonoids present in hydroalcoholic extract, as well ethyl acetate fraction [8].

Boerhaavia diffusa
- Common name: Hog weed, Pigweed
- Tamil name: Mookirattai
- Family: Nyctaginaceae
- Parts used: Whole plant
- Distribution: Throughout India, as a weed in waste lands and road sides.

It is a perennial diffuse herb with stout root stock and many procumbent branches. It has bitter taste and astringent, coolant, cardiac stimulant, expectorant and anti-inflammatory properties [9].

It is useful in all types of Vadha diseases [10].

Leaves are immunosuppressive due to the presence of chemical principles. The properties of flavonoids are present in leaves (Bd I and II eupalitin) [11] which inhibit the inflammation [12].

Caesalpinia sappan
- Common name: Sappan red wood
- Tamil name: Pathimugam
- Family: Fabaceae
- Distribution: Cultivated in south India and Bengal
- Parts used: Wood.

A small thorny tree, 6-9 m in height and 15-25 cm in diameter with a few prickly branches. It contains protosappanins A, B, C, taxaxerol, homoisoflavonoids [13,14].

It inhibits the expression of pro-inflammatory cytokines and tumor necrosis factor alpha (TNF-alpha) [12].

Butea monosperma
- Common name: Flame of the forest
- Tamil name: Palasu
- Family: Fabaceae
- Distribution: Throughout India, is a deciduous tree, very conspicuous when in flowers, 12-15 m in height with gum containing gray bark exfoliating in irregular pieces.
- Parts used: Leaves, flower, seeds, bark, and gum.

It has tonic, diurectic, astringrent action. It cures all Vadha, Pittha and Kappa diseases and also relieves body pain [15].

Plant contains flavonoids and glycosides, such as butin, butrin, isobutrin, coreopsin. Flower contains butrin, ceropsin, monospermoside, sulfurein [16].

It increases the levels of hemoglobin, red blood cell, and white blood cell. Erythrocyte sedimentation rate levels were suppressed.

Costus speciosus
- Common name: Costus root
- Tamil name: Kosttam
- Family: Zingiberaceae
- Distribution: Throughout India in moist localities
- Part used: Rhizome.

A succulent herb with long leaf spirally twisted stems 2-3 m in height with horizontal rhizomes. The rhizomes are bitter, astringent and are useful in inflammations. It has tonic, diaphoretic action. It cures Vadha disease [17].

Rhizomes are rich in ascorbic acid, beta-carotene, alpha-tocopherol, glutathione, phenols, steroids, and terpenoids. It also contains diosgenin, prosapogenin B of dioscin, dioginone, cycloartenol, and octacosanoic acid [18].

It suppresses the inflammatory mediators.

Euphorbia antiquorum
- Common name: Quadrangular Spurge
- Tamil name: Sadhurakalli
- Family: Euphorbiaceae
- Distribution: Throughout the hotter parts of India.

A small armed tree with whorled fleshy branches, thick and broad, 3-5 winged, having sharp stipular spines.
- Parts used: Latex, juice

Latex of this plant cures Vadha disease. It is useful in rheumatism, dropsy, and gout.

Phytochemicals, such as diterpenes and triterpenes were isolated and characterized from latex of *E. antiquorum*. Moreover, these substances reported for anti-inflammatory and antiarthritic activities [19].

Withania somnifera
- Common name: Winter cherry
- Tamil name: Amukkura kizhangu
- Family: Solanaceae
- Distribution: Throughout the drier parts of India. In waste places also cultivated.

An erect branching undershrub reaching about 150 cm in height, usually clothed with minutely stellate tomentum, leaves ovate up to 10 cm long.
- Parts used: Leaves, root.

It acts as febrifuge, diuretic, deobstruent, tonic, and sedative. It cures all types of joint disorders. The leaves are bitter and are recommended in fever, painful swellings. A paste of roots and bruised leaves are applied to ulcers and painful swellings [20].

The main constituents are alkaloids and steroidal lactones. The leaves contain steroidal lactone which are commonly called as "Withanolides." Withaferin A isolated from the root have strong anti-inflammatory activity [21].

Semicarpus anacardium
- Common name: Marking nut tree
- Tamil name: Cherangkottai

- Family: Anacardiaceae
- Distribution: Throughout India, in semi-evergreen and moist deciduous forests.

A medium sized to large tree, 15-25 m in height with gray bark exfoliating in small irregular flakes.

- Parts used: Fruits and nuts

It cures Vadha disease (joint disorders). The fruits are bitter, astringent liver tonic, antiarthritic, anti-inflammatory, and tonic [22].

Chemical and phytochemical analysis of the nut revealed the presence of flavonoids, phenolic compounds, minerals, vitamins, and amino acids [23].

The substances which are present in this plant inhibit the cytokine production. Protective effect of *Semecarpus anacardium* was also observed by the decrease in the level of TNF-alpha. Besides it showed strong antiarthritic property by regulating bone turnover [24].

Vitex negundo

- Common name: Five leaved chaste tree
- Tamil name: Notchi
- Family: Verbenaceae
- Distribution: Throughout India, on waste land. An aromatic large shrub or small tree of about 3 m in height with quadrangular branches.
- Parts used: Whole plant.

The plant is bitter, thermogenic. It has anti-inflammatory, expectorant, and diuretic action. The roots are useful in vitiated conditions of Vadha [25].

Seeds contain hydrocarbons, beta-sitosterol, benzoic acid, and phthalic acid.

Anti-inflammatory diterpene, flavonoids, artemetin, and triterpenoids were also found to be present [26].

Smilax china

- Common name: China root
- Tamil name: Parankippattai
- Family: Liliaceae
- Distribution: In China and Japan

A hard tendril climber with sparsely prickled or unarmed stems and thick tuberous rhizomes.

- Part used: Rhizome.

The rhizomes are bitter, thermogenic, anti-inflammatory, and diuretic. It cures all types of Vadha diseases [27].

Steroidal saponins 1, 2, 3 and 4 were isolated from *Smilax china* L. These compounds showed

inhibition of cyclooxygenase-2 enzyme activity and mild inhibition of TNF-alpha production [28].

CONCLUSION

RA is a chronic, degenerative disease that is characterized by the inflammations of the synovial membrane of joints and surrounding tissues. If the treatment fails to control the disease or not properly treated, it may lead to destruction of the bones, ligaments, cartilages, and tendons around the affected joint leading to joint deformity and resulting in disability to perform day today activities and physical movements. This will affect the quality of life of patients. Even though many drugs are available in the allopathic medical system to control the symptoms of RA effectively, most of the drugs produced adverse side effects. Hence, the majority of the RA patients have turned toward Siddha system of medicine because of little or no side effects of these drugs. In this study, systematic review of important herbs mentioned in the classical Siddha literature for managing RA was discussed. However, further research is needed to find out not only the efficacy of the herbs presented but also to evaluate the safety as well as herbal drug interactions which can help to develop potent and safe drug from traditional sources for treating degenerative and inflammatory joint diseases like RA.

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Review Article

BEAUTY CARE THROUGH SIDDHA SYSTEM OF MEDICINE: A REVIEW

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ABSTRACT

Historical record of usage of natural cosmetics can be traced back since ancient times among various civilizations. Traditional use of beauty products is believed to have originated in India. Natural cosmetics refer to preparations of natural or plant products using herbs popularly used in the Siddha system of medicine. Many cosmetics, perfumes, and soaps contain molecules extracted from aromatic herbs and spices. Plant and animal kingdom are enriched with countless treasures to be use by mankind in health and beauty care. Plants, flowers, and fruits belonging Siddha system of can be regarded as for beauty care especially for skin and hair care. In this paper, few herbal and their usage in connection with cosmetics applications are discussed so as to understand the efforts taken by the "Siddhars" to evolve the science of cosmetics. Some of the natural ingredients originally mentioned in ancestral beauty rituals of Siddha system of Medicine include skin tonics, sun screen, skin lightning and exfoliating scrub, pimple cure, lip balm, antidandruff agent and hair care product, dental cares, depilatory, breast developers, cure for lice and nits and antiaging. These herbs are discussed as per the classical references and available scientific validations. It is concluded that the whole range of modern cosmetic usage is conceived by the ancient Siddha system and was exercised from the raw materials available in both land and sea.

Keywords: Cosmetic, Siddha, Natural cosmetics, Sun screen, Skin lightning, Antiaging herbs.

INTRODUCTION

The art of cosmetics is a very ancient practice, dating back 10,000 years as we learn the discoveries of makeup palettes or pots care products on some archaeological sites which are closely linked to the history of civilization.

Historical record of cosmetic usage can be seen in ancient people civilization's practical concerns, such as protection from the ultraviolet rays A and B.

Cosmetics were used even in ancient age. Already, during prehistory, the men realized the body paint of mineral, mixed with fat.

Cosmetology defined as the study of skin, hair, and nails, wherein the priorities are given to hair-shaping, manicure, pedicure, occasional hairstyling, shampooing of hair, cosmetic applications, body hair removal, hair relaxers, coloring and highlighting of hair and hair extensions or wig treatments. There is evidence of beautification by both races in ancient India, but the earliest records of products and their application dates back to Circa 2500 and 1550 B.C, to the Indus valley civilization [1].

Natural cosmetics we refer to the preparations of cosmetic ingredients from natural sources especially from medicinal plants popularly used in ancient Siddha system of medicine.

According to the Drugs and Cosmetics Act (India), 1940, "cosmetic" means any article intended to be rubbed, poured, sprinkled or sprayed on, introduced into, or otherwise applied to the human body or any part thereof for cleansing, beautifying, promoting attractiveness, or altering the appearance, and includes any article intended for use as a component of cosmetic [2].

In general, cosmetics may be defined as any substance or preparation other than drugs to be in contact with the various external region of the human body or with the teeth and inner lining membranes to clean, protect or maintain them in good condition, changing their appearance, flavor or to correct the odor.

Many *Kalimbu* (ointment), *Anjanam* (eye shadow) and oil are described in various Siddha literature in context of *Sarma Noigal* (Skin disorders). The very common medicine is *Parangipattai chooranam* (*Simalax china* L. powder), *Tripala* powder, *Sivanar vembu kuzhi thylam* (*Indigofera aspalathoides* DC. oil), *Vetpalai thylam* (*Wrightia tinctoria* Roxb.), *Aruganthylam* (*Cynodon dactylon* (L.) Pers.), *Karbogipasai* (*Psoralea corylifolia* L. ointment), etc., are very well-established medicine in Siddha for various skin disorders.

The use of fats, milk, honey, wax, and resins in variable concentration are allowed them to adjust the texture of a product to be used as powder, eye shadow, facial mask, lotion, cream or ointment. Oils and vegetable fats are very similar to fatty acids in the skin. These vegetable oils and fats are derived from fruits or seeds (sun flower oil, peanut oil, sesame oil, corn oil, cottonseed oil, nuts and seeds) and are well accepted by the body.

Herbs and spices are a group of plant products and are characterized present by their content of aromatic components in different parts such as fruits, seeds, roots, leaves, flowers or inflorescences.

Many cosmetics, perfumes, and soaps contain molecules extracted from aromatic herbs and spices.

Plant and animal kingdom provide countless treasures to human for their health and beauty care. Plants, flowers, and fruits from the treasure of Siddha system are unparalleled wealth for beautification and for skin and hair care.

Advances in chemistry help in the creation of many synthetic substances which can improve. Natural cosmetics are increasingly booming, which is equivalent to traditional cosmetics, because it is made mostly by components of plant origin. More and more laboratories prepare natural cosmetics such as creams, lotions, and shampoo with herbal ingredients.

The demand of herbal products is increasing rapidly due to their lack of side effects [3]. Some of the natural ingredients originally used in beauty rituals according to Siddha system of medicine.

PLANTS USED AS SKIN TONICS

***Samai* (*Panicum sumatrense* Roth ex. Roem. & Schult.)**
It is called as "little millet." It has been grown in many parts of India, and belongs to the family of Poaceae. Several types are chiefly cultivated in Madras, Mysore, and Maharashtra. Little millet is considered to have great cosmetic potential. Whole grains contain protein, fat, carbohydrates, fiber, and minerals. Essential aminoacids present are arginine, histidine, isoleucine, leucine, lysine, methionine, etc. The people take this as food. Plant contains phenol, flavonoids, and antioxidants. It has sweet taste. It tones the skin.

Chandanam (*Santalum album* L.)
Sandalwood is one of the finest woods for carving and is employed for making various types of exquisite beauty products and used for fragrance. The finest tree grows in dry regions. The trees are distributed in India. Botanical name is *S. album* and belongs to the family Santalaceae. Both wood and oil are used in incenses and perfumes. It contains terpenoic acids and volatile oil. The tree and sandalwood oil are used for many skin disorders. It tones and blemishes the skin.

***Panneer poo*/rosa (*Rosa damascene* Mill.)**
It is an important plant belongs to the genus *Rosa* (*R. damascene*). It is cultivated in gardens throughout India. It contains essential oil, tanning matter, cyanin, and yellow glycoside of quercetin, yellow crystalline dyestuff and proline. It has antioxidant and antimicrobial properties. The petals of this plant have toning and cooling effect.

***Atimadhuram* (*Glycyrrhiza glabra* L.)**
The root of this plant grows in hilly areas. Roots vary in size and yellow in color. The botanical name is *G. glabra* belongs to the family Fabaceae. It is well known for its sweet taste. The main components are polyphenols and flavonoids. It has the property of whitening the skin [4].

PLANTS USED AS SUN SCREENS

***Kathalai* (*Aloe vera* (L.) Burm. f.)**
A. vera is succulent plant and many varieties are found in a semi-wild state in all parts of India. This plant belongs to the family Liliaceae. There are many types but all are similar in characters and the red variety is rare. Chemical contents are aloe-emodin and resins. This is a rejuvenating herb and is a good moisturizer [4].

***Vendhayam* (*Trigonella foenum-graecum* L.)**
It is an annual plant with botanical name *T. foenum-graecum*, belongs to the family Fabaceae. It grows in Kashmir, Punjab and Tamil Nadu. In Tamil Nadu, it is cultivated in kitchen garden and backyard. It is an antioxidant.

Plants used as sun tan

Korai kizhangu (Cyperus rotundus L.)

Rhizomatus herbs, botanically called as *C. Rotundus,* belong to the family Cyperaceae. In India, it grows in all type of lands. Rhizome contains flavonoid glycoside, cyperene 1 and 2, sesquiterpene ketone and cyperolone. The composition is used for promoting the pigmentation of skin.

Astringentare used to improve skin appearance

Kadukkai (Terminalia chebula Retz.)

The tree is grown mainly in Northern India. Grows in various heights and it belongs to the family Combretaceae. This tree nearby Narmada grows up to 100 feet. Fruit rind used as medicine. It is used as an astringent, antibacterial, antifungal and antiseptic could be used as skin nourisher.

Sirunagapoo (Mesua ferrea L.)

M. ferrea belongs to the family Calophyllaceae. It is found in Himalaya, West Bengal, Assam and Burma. Flower, seed, root and bark used as medicine. It is a strong astringent and is also aromatic.

***Kadukkaipoo* (leaf galls of *T. chebula* Retz.)**
The fruit of this tree is similar to goats horn. It is broad at the base and shrunken at the tip. It is formed by the latex which is secreted by an insect invading the *T. chebula* leaves. This gall used as an astringent, rubefacient, and antibacterial agent.

Siddha herbal ingredients as dental care products

Vetrilai (Piper betle L.)

The climbing vine *P. betle* belongs to the family Piperaceae. It is cultivated in tropical regions of India. It is a climber and mainly cultivated for its leaf. Based on color it is of three types. The leaves possess antibacterial and antimicrobial effects [5]. In night times when the leaves are used to prevents halitosis and gives aromatic flavor [6].

***Karungali* (*Senegalia catechu* L.)**
The tree called as "cutch tree" the botanical name is *S. catechu*. It is grown in India and Burma. The catechu extract from the wood due to its astringent property can be used for bleeding gums and other gum related problems.

***Kudasappalai* (*Holarrhena pubescens*)**
It is called as Kurchi the botanical name is *H. pubescens*, family Apocynaceae. It grows in the forests of India. The bark has astringent property. The alkaloid present in it has antimicrobial and antidiarrheal activity. The bark made into decoction is used for toothache.

***Birama-dandu* (*Argemone mexicana* L.)**
This plant also called as "The yellow thistle." This plant is grown allover India. The botanical name is *A. mexicana* Linn., family: Papaveraceae. The part used is seed, latex, and whole plant. The latex of this plant is used for glaucoma and trachoma. The seeds of this plant is powdered and used for toothache and dental caries.

Koththamalli (*Coriandrum sativum* L.)
The plant is mainly grown for its seeds. The botanical name is *C. sativum* belongs to the family Apiaceae. It is a small herb cultivated in many parts of India. It has antibacterial activity, anti-inflammatory. The seeds of this plant chewed for halitosis [5].

Dermatological applications

Sarakkontrai (Cassia fistula L.)

It is otherwise called "Indian laburnum." It is a tree grown in India and Burma. The flowers are yellow in color, belongs to the family Caesalpiniaceae. The parts used are leaf, flower, seed, stem bark, root bark. Its phytochemical constituents are oxalic acids, tannins, anthraquinones, and glycosides. The leaf is crushed and made into paste used for ringworm [7].

***Vellaikunkiliyam* (resin of *Boswellia serrata* Roxb.)**
It is obtained from Frankincense tree. This tree is found in India and West coastal countries. The exudate resin from the tree is collected. The botanical name is *B. serrate* and it belongs to family Burseraceae. The part used is resin and oil. It contains monoterpene, diterpenoids, triterpenes and pentacyclic triterpenic acids (boswellic acids). The resin mixed with sesame oil and white wax made into oil is used for leukoderma [8].

***Vembu* (*Azadirachtaindica* A. Juss.)**
Traditionally, it is grown nearby temples and houses. It is commonly called as Neem tree. It belongs to the family Meliaceae. The chemical constituents are nimbidin, margolone, gallic acid, and polysaccharides. It is a potent antibacterial, antifungal and can be used for many of the skin disorders. The leaves can be used separately or it can be made into paste with turmeric used for chickenpox ulcers [9].

***Cenkonrai* (*Cassia marginata* Roxb.)**
The cassia species are widely distributed in the World but abundantly in India. It is called as Red shower tree and belongs to the family Caesalpiniaceae. The seed contains chrysophanol,

physcion, 1,3-dihydroxy-2-methylanthroquinone, flower contains 1,8-dihydroxyanthroquinone, roxburginol, wood contains roxburghinol, chrysophanol. Bark paste can be applied for leukoderma [10].

PLANTS USED FOR HAIR CARE

***Neerbrahmi* (*Bacopa monnieri* (L.) pennell)**
Decumbent or creeping herbs rooting at nodes. Flowers are bluish and solitary. This plant belongs to the family Scrophulariaceae. Plant contains nicotine, betulinic acid, glycoside, saponins and stigmasterol. It possesses air growth activity [11].

***Karisalai* (*Eclipta prostrata* L.)**
The plant grows more in mountain regions. There are four types blue, yellow, red, and white. White plant is seen abundantly. The plant belongs to the family Asteraceae. It is used as dying agent for hair in Siddha. The oil prepared with this leaf juice can be used hair growth.

***Korattai* (*Trichosanthes tricuspidata* Lour.)**
It is a climber can grow above 30 feet in height. It has a strong smell and for this it is used in many hair oils [5].

Plants used as lip balm/lip salve
Cracked lips, besides being painful, spoil the beauty of the face. The following remedy is recommended in such cases. The rind of *Vilvam* (*Aegle marmelos* Corr.) fruit is powdered and mixed in mother's milk and the paste is prepared which is applied to the cracked lips, within 10 days it will heal [12].

Plants used for skin lightning and exfoliating scrub

Pungam (Pongamia pinnata (L.) Pierre.)

It belongs to the family Fabaceae. The chemical constituents of the seeds are beta-sitosterol acetate, galactoside, stigmasterol, fatty acids like oleic acid, stearic acid and palmitic acid. It has the skin lightening property.

***Ellu* (*Sesamum indicum* L.)**
Oil of the seed called as Elluennai or Nallaennai. This plant is grown abundantly in India. This plant belongs to the family Pedaliaceae. It contains momor-cerebroside, soya-cerebroside 2, beta-sitosterol, daucosterol, and D-galactitol. It can be used as scrubbing agent.

***Devadaru* (*Cedrus deodara* (Roxb.) Loud.)**
Heartwood of the tree used in Indian medicine for various ailments including cosmetics. The tree belongs to the family Pinaceae (Gymnosperm). The constituent present in it are P-methylacetophenone, atlantone, sesquiterpenes, and toxifilin. It reduces the pigmentation of the skin [4].

Plant used to treat dry skin

Chemparattai (Hibiscus rosa-sinensis L.)

It is plant of China and North India. This plant is also cultivated in Southern parts of India. It is classified according to the colors and 12 types are found in Mumbai. It is grown as garden plant. It has the power of softening and soothing the skin [5].

Ilanthei (Ziziphus mauritiana Lam.)

Z. mauritiana belongs to the family Rhamnaceae. It is grown in all parts of India. The chemical constituents are betulinic acid, ceanothic acid, frangufoline and spinosin. It possesses antioxidant activity, scavenging activity, and emollient action [13].

PLANTS USED TO CURE DANDRUFF

***Kumil* (*Gmelina arborea* L.)**
Kumil belongs to the family Verbenaceae. It is planted in gardens and agricultural lands. The unriped fruit after ripening becomes yellow in color. The oil prepared from using the fruit can be used as an antidandruff agent and in controlling itching [5].

***Kasa-kasa* (*Papaver somniferum* L.)**
The plant *Kasa-kasa* is erect, unbranched herb. Leaves lobed dentate or serrate and flowers are large bluish-white belongs to the family Papaveraceae. Latex of the unriped fruits contains meconic acid and stepholidine. Seeds contain alkaloids such as morphine, codeine, and papaverine [4]. *Kasa-kasa* seeds in milk and to applied the scalp.

Plants used as depilatory
The presence of hair on arms, face, legs and pubic area was considered an eyesore, and certain formulae were used to remove them. *Nellimulli* (dried fruits of *Emblica officinalis* Gaetrn.) and *Tippili* (dried fruits of *Piper longum* L.) are pound together. Soak this mixture in the milky latex of *Ilaikkalli* (*Euphorbia nivulia* Buch. Ham.). If this compound is applied to the desired place, the hair from that area will fall off.

Plant used as breast developer
Powdered drugs of *Amukkiran-kilzhangu* (root tuber of *Withania somnifera* Dunal.), *Aanaithippili* (dried fruit of *Scindapsus officinalis* Schott.) and *Koshtam* (dried root of *Saussurea lalappa* C.B.CL.) and *Vasambu* (the dried rhizomes of *Acorus calamus* L.) mixed with butter made from buffalo's milk and massage the bust with this medicated butter. This will increase the bust line and make it firm and beautiful breast [14].

PLANTS FOR PIMPLE CURE

Oil prepared from, *Nallennai* (*S. indicum* seed oil) and *Thuththi* (A*butilon indicum* root) can be applied over pimples twice a day and pimple can be cured within a week. Paste prepared by mixing the powdered ingredients of *Kothamalli* (*C. sativum*), *Vasambu* (*Acorus calamus*), V*ellilothram* (*Symplocos racemosa*) and *Kostam* (*Saussurea lappa*) and applied to cure pimples [15].

PLANTS TO CONTROL LICE AND MITES

Kakkay-k-kolli (*Anamirta cocculus* (L.) Wight. & Arn)
Kakkay-k-kolli seed contains a toxic substance called Picrotoxin. The seed is powdered and made into paste applied over the head [5].

On tying the head with a piece of cloth dipped in the juice of *Vetriliai* (*P. betle*) to which has been added with mercury and used to control lice and mites [16].

GENERAL HAIR REMEDY AND CURE FOR THE PREMATURE GRAYING

"Juice of *Karisalai* (*Eclipta alba*) together with *Logamanduram* (Iron-rust), *Triphala* (*T. chebula*, *Terminalia bellerica*, and *E. officinalis*) collection of the above three fruits boiled in oil and applied to the scalp, it would cure dandruff, itching, alopecia and would also darken the hair, which have become gray prematurely [17].

ANTIAGING HERBS

***Nellikkai* (*E. officinalis* Gaertn.)**
This belongs to the family Euphorbiaceae and this fruit contains triacontanoic acid, betulonic acid, gallic acid, daucosterol, and quercetin. It is proven antioxidant and hence could be used for delaying aging symptoms [18].

***Mancitti* (*Rubia cordifolia* L.)**
This plant belongs to the family Rubiaceae. The following chemical constituents are presents such as rubimalin, β-sitosterol, and anthraquinone. It possesses wound healing and antiaging properties [19].

***Thulasi* (*Ocimum tenuiflorum* L.)**
This plant belongs to the family Lamiaceae. The plant contains phenolic acids especially rosmarinic acid which is known for the activity of scavenging superoxide anion radicals, hydrogen peroxide, and

Ferric ion-reducing potential. It possesses antiseptic and antimicrobial properties. Due to its antioxidant property it prevents aging [20].

CONCLUSION

Herbs are not only known for their curative properties but are also effective cosmetics. Preparations made of single herb are used for many skin ailments, skin toning, and beauty products.

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Review Article

BHASMAPARIKSHA, THE CLASSICAL PARAMETERS FOR STANDARDIZATION OF HERBO-METALLIC DRUGS - A REVIEW

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ABSTRACT

Ayurveda, the most ancient medical system, has faced many debates currently. The science that has been envisaged in the Ayurvedic text will stand valid even today. There is no other system of medicine other than Ayurveda that has taken this much care in manufacturing drugs. However, still it received various statement regarding safety and the quality by some of the vested interests. To counter such arguments and revalidate the claims, the present topic has been selected specially taking *Bhasma* of heavy metals. *Rasaushadhis* are made into medicine by various pharmaceutical processing. These organometallic drugs are known as *Bhasma.* Our ancient *Acharyas* had their own parameters which guaranteed quality, safety, and efficacy of prepared *Bhasma*. These include tests that assess the physical and chemical properties of *Bhasma.* Present paper is a review of various tests mentioned by *Acharyas* for assessing the quality of *Bhasmas.*

Keywords: *Rasashastra, Bhasma pariksha,* Nanoparticle.

INTRODUCTION

Ayurveda, the most ancient medical system, has faced many debates currently. The science that has been envisaged in the Ayurvedic text will stand valid even today. In the manufacturing of medicines neither Ayurveda nor *Rasashastra* left no stones unturned and they have taken utmost care in maintaining the quality and have also documented the variety, possible impurities, purification processes the methods of standardization of prepared medicine and the proper administration of dose and the antidotes to any adverse effect, if any. There is no other system of medicine other than Ayurveda that has taken this much care in manufacturing drugs. But still, it received various statement regarding safety and the quality by some of the vested interests. To counter such arguments and revalidate the claims, the present topic has been selected specially taking *Bhasma* of heavy metals.

Rasaushadhis are made into medicine by various pharmaceutical processing. These organometallic drugs are known as *Bhasma*. Pharmacological efficacy of *Bhasma* preparation is largely attributed to the number and type of *Puta* used in its making. *Puta* can be defined as the quantum of heat required to convert the metals and minerals to their *Bhasma* state [1]. Different types of metals, minerals, gems, and jewels need application of different amount of heat to be reduced to *Bhasma* form. They are digested in a specially designed fire-place which is called *Puta.* Depending on the heat requirement, some of them are arranged in pits of different sizes drugs in the earth and some others are done over the ground. Cow-dung cake is generally used as fuel. For *Putana*, pelletized drug is enclosed in a *Sharava Samputa*, in which two similar *Sharavas* are attached to each other by mud smeared cloth, which is subjected to a specific heating pattern. The whole process of *Puta* involves levigating the purified drugs with herbal extracts and then molding into small rounded pellets. These pellets are then dried in sun light and then exposed to heat.

Increased incinerations are there for able to reduce particle size and subsequently give rise to increased efficacy of the *Bhasma*. Nanoparticle size of ancient *Bhasma* has been confirmed in studies where it is proposed that nanoparticles are responsible for its fast and targeted action [2].

Our ancient *Acharyas* had their own parameters which guaranteed quality, safety, and efficacy of prepared *Bhasma*. The heavy metal salts are currently topics of discussion from the toxicological point of view. The *Acharyas* have told about the adverse effects of improperly prepared metallic preparations and their standard operating procedure to control the adverse effects. *Bhasma pariksha* is one of the evidence of such references. The scholars have mentioned some parameters that deal with some tests that can help in checking proper preparation of *Bhasma*. These include tests that assess the physical and chemical properties of *Bhasma.*

PHYSICAL PARAMETERS

These parameters evaluate the physical characters of incinerated material.

Varitara

This character indicates the lightness and fineness of *Bhasma* [3]. "*Varitara*" is floating character of *Bhasma* on stagnant water surface. Clear pure water is taken in a transparent glass beaker and a little amount of *Bhasma* is sprinkled over the surface of the water. The floating character of the *Bhasma* indicates that the incineration process adopted was proper. *Varitara* tests can be considered based on the law of surface tension. Here, the particles of *Bhasma* attain so much fineness and light character that surface area of the particles increases as they cannot break surface tension of stagnant water.

Unam

A rice grain is to be placed gently over floating *Bhasma*. If the rice grain floats over the layer of *Bhasma*, it indicates that the process of incineration is proper. This test is further confirmation of *Varitara* test [4].

Rekhapurnata

This character indicates micro fineness of the prepared *Bhasma* [5]. The *Bhasma* particles should be very fine for the easy absorption and assimilation. The prepared *Bhasma* is taken in thumb and index finger, rubbed, it will fills the furrows of finger tips, indicates very small particle size. Finer the particle, fastest the absorption and quickest the action is the fundamental principle of pharmacology, i.e., if the medicine is reduced to nanoparticles, better therapeutic activities can be expected.

Slaksnatwa

Slaksna is smooth. If *Bhasma* is touched with fingers, a smooth tactile sensation is produced; it is called *Slaksnata* [6]. The properly of

incinerated *Bhasma* attain this quality. The *Slaksna Bhasma* never produces irritation on the mucus membrane of gastrointestinal tract.

Suksmatwa
It is also used for testing micro fineness property of a *Bhasma* [7]. *Suksma* means micro fine. It helps the *Bhasma* to circulate in the body very quickly.

Anjansannibha
Properly prepared *Bhasma* should be soft and fine like *Anjana* or collyrium, which does not produce any irritation when applied in eyes [8].

CHEMICAL PARAMETERS

Along with physical parameters, chemical parameters are also mentioned in the ancient classics. Few of them are as follows:

Apunarbhava
This *apunarbhava* test means the irreversible states of the *Bhasma* [9]. *Apunarbhava* means incapability to regain metallic form properly incinerated *Bhasma* never regains its metallic form. The metallic particles having particles size 100 nm do not show the metallic character. The incinerated metal is to be mixed with equal quantity of *Guda* (jaggery), *Gunja* (abrus seeds), *Madhu* (honey), *Ghrta* (ghee) and *Tankana* (borax), kept in *Musha* (crucible), sealed with another *Musha,* and is to be subjected to intense heat. After self-cooling the product from the *Musha* is to be collected and analyzed. The presence of free metal in the product indicates that the metal is not incinerated properly.

Niruttha
This test is also similar to that of *Apunarbhava* test and is applicable for all the metals [10]. Incinerated metal is to be added with equal quantity of silver and this complex is heated in a *Musha* under intense heat. After self-cooling, the silver is to be examined for changes in weight and color. If the metal is properly incinerated there will not be any change in the weight of the silver.

Varna
It indicates the color of *Bhasma*. Specific color is mentioned for each *Bhasma* and alteration in this specific color, indicates the *Bhasma* is not prepared properly. A particular metallic compound is formed during *Bhasma* preparation and every chemical compound has specific color, example – *Tamra bhasma* is black, *Abhrak bhasma* is red in color.

Gatarastwa
Every metal has specific taste. When a *Bhasma* is prepared a metal should be tasteless. It denotes transformation of particular metallic taste to tasteless compound.

Nischandratwa
This taste indicates there is no presence of metallic luster [11]. *Chandratwa* is a feature of metal. After proper incineration, the luster of the metal completely gets lost. It indicates there is absence of free metal.

BIOLOGICAL PARAMETERS/PHARMACOKINETICS

Laghu
The *Laghu* word indicates the easily digestible and absorbable nature of the *Bhasma* [12]. After proper processing, the body can digest the inorganic preparation.

Shighra Vyapti
The "*Shighra Vyapti*" indicates quick acting/quick circulation inside the body [13]. The *Bhasma* can circulate in the body very quickly and spreads in the blood if it is properly prepared.

Deepan
It denotes the increase of enzymatic factor (*Agni*) in the body, properly prepared *Bhasma* after entering the body, can reach inside the cell and stimulate the functions of the enzyme and acts as catalysts [14].

Rasibhavan
The properly prepared *Bhasma* should be available in the body fluid. This concept is known as *Rasibhavan* [15].

Yogvahi
Yogvahi means to carry of medicines in to the site of action. The *Bhasmas* have quality to carry the properties of the other drugs along with retaining its own qualities. It may carry specific drugs to its specific site of action. Hence, these may act as a targeted drug delivery system inside the body.

Rasayan
In Ayurveda, *Rasayan* means the drug which can prevent aging and diseases. The properly prepared *Bhasma* claim to have antioxidant properties and can act as a *Rasayana* drug.

CONCLUSION

The thermal treatment to which the metals and minerals are subjected for reducing them to ashes, help not only in attaching organic ligands but also in reducing the metallic particle size to such a fine state that they can be compared with the current trend in the study of particle size – the nanotechnology. Thus, the science of Ayurvedic Pharmaceuticals itself specifies above tests to discover the quality of metals in *Mritalauha*/*Bhasma* and follows similar scientific processes that are available today for the chemical synthesis of nanoparticles. While the advancement of modern science and technology has enabled us to create objective and verifiable standards for the *Bhasma* preparations the traditional *Bhasma, Bhasma Pariksha* remains distinctive in the standardization of *Bhasma* preparations.

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Review Article

THE ROLE OF SIDDHA SYSTEM OF MEDICINE IN THE DEVELOPMENT OF NUTRACEUTICALS AND COSMECEUTICALS

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ABSTRACT

Siddha system of medicine strongly emphasises on the fact that "unavae marunthu, marunthae unavu" which means "food is medicine and medicine is food." Since the ancient days, Siddha system of medicine has been a pride to India and is a rich source of enormous health-care supplements, Kayakarpam (preventive/rejuvenative medicines) and cosmeceutical products. The health scenario is positioned at unique crossroads as the world is facing a "triple burden of diseases" constituted by the unfinished agenda of communicable diseases, newly emerging, and re-emerging diseases as well as the unprecedented rise of noncommunicable diseases. All the Siddha formulations are known to contribute to health promotion and longevity of human race, such as urai mathirai for child care, health-care formulations, and skin care formulations. Thus, overall promotion of well-being, prevention of premature ageing of skin, and protection against possible skin ailments may be managed through Siddha formulations such as *Amukkara choornam* and *Ponnanganni chooranam* as internal medications and *Nalangu maavu* as external therapy. The concept of Siddha and other traditional system of medicines are widely accepted worldwide, and there has been an increased interest for medicinal plant based formulations. This paper deals about the nutraceuticals and cosmeceuticals derived from various Siddha medicinal plant based formulations, which are currently in practice.

Keywords: Siddha medicine, Nutraceuticals, Cosmeceuticals.

INTRODUCTION

Herbal medicines are very familiar globally from the prehistoric period. Longevity and maintaining good health is the basic aim of Siddha system of medicine. Selected foods/recipes can improve the physical/mental performance and also decreases the disease risks. The said concept is the core principle of Siddha medicine, which emphasizes "Food is Medicine and Medicine is Food." Specific food recipes/grains of food crops are prescribed to the patients according to their udaliyal, i.e., constitution of body. However, this scenario is directly/indirectly forcing us to give more emphasis to our basic nutrition such as balanced diet, and organic foods which in turn throws light on the traditional systems of medicine [1]. Various Siddha formulations are easily available, which are mentioned in Siddha literatures as coded/non-coded forms. Especially, *Kayakalpa* is a medicine for longevity. "*Kayam*" means "body" and "*Kalpa*" means "transformation"/"transmutation." *Kayakalpa* treatment delays aging and regenerates the entire body and mind [2]. When the body is not in harmony, it causes distress, disease, and discomfort. *Kayakalpa* therapy focuses on curing degenerative diseases and prolonging life by harmonizing the mind and body. The *kayakalpa* medicines are broadly classified as *Pothu karpam* (medicines aimed at general health and well-being) and *Sirappu karpam* [1] (medicines targeted to fight a specific illness).

Herbal health promoters - Siddha formulations

Among *Pothu karpam*, certain common nutraceuticals used are discussed below:

Ingithen (Zingiber officinale Rosc.)

Processing of Ginger in honey for specified days is called *Ingithenkarpam* consumption of this *karpam* helps to prevent the changes related to ageing and offers rejuvenation [3].

Ponnanganni karpam (Alternanthera sessilis Linn.) [4]

Fresh *ponnanganni* leaves cooked with bell peppers and salt helps to maintain good skin complexion.

Karisalai karpam (Eclipta prostrate Roxb.) [5]

Consumption of dried *karisalai* with tender coconut for a month followed in honey, for the successive month helps in attaining good health with adequate immunity.

Katrazhai karpam (Aloe vera Linn.)

Intake of aloe pulp in any form helps to acquire masculinity and vigor.

Nellikai legium (Emblica officinalis Linn.)

It is a polyherbal Siddha formulation with nelli, ghee, tender coconut, ginger juice and sugar enriched with Vitamin C, and various other nutrients. It is a health tonic and renowned immune modulator.

Amukkara legium (Withania sominifera dunal)

It is a very famous herbal jam. *Amukkara* is its main ingredient. It is a natural Siddha body building supplement and helps to relieve emaciation, blood impurities, and brings about rejuvenation.

Panchadeepagnilegium

It is a polyherbal combination of *Thirikadugu* (*chukku, milagu,* and *thippili*) *cardamom* and *jeeragam* processed with cow's milk, ghee, and honey. On consumption, it helps in healthy weight gain, good appetite, and offers immunity too.

Madhulai manapagu

It is pomegranate juice processed with rose water, sugar candy, and honey. It helps to increase hemoglobin content of the body and settles down diseases related to gastrointestinal tract.

Apart from above said health promoters, following certain health life style modifications also pave a better way to achieve healthy life.

***Pancha karpam* [6]**

As written in Siddha literature, taking bath according to "*Pancha Karpa Vidhi*" ensures perfect health. *Kasthuri manjal* (*Curcuma zedoaria* Rosc.), *milagu* (*Piper nigrum* Linn.), *vembu* (*Azadirachta indica* A. Juss.), *kadukkai* (*Terminalia chebula* Retz.), and *nellikai* (*E. officinalis* Linn.) are blended with cow's milk and heated. Application of this paste in the head for bathing once in a week renders strength and immunity to the body.

Role of cosmeceuticals in Siddha system of medicine [3]

Indian herbs and its significance are reckoned worldwide. With the advancement of human civilization, various synthetic cosmetics have been a keen area of interest, since marketing of cosmetic products has a good profit margin. Recently, herbal cosmetics, herbal facial therapies, etc., have gained importance, due to the skin-friendliness, and safety aspects. Traditional use of *kasthuri manjal* (*C. zedoaria* Rosc.), are in practice among Indian women for improving skin tone and complexion.

On that account, Siddha system of medicine focuses on a balanced diet, stress-free lifestyle, and healthy practices, which would pave way for a bright and healthy complexion. A few tips for healthy complexion according to Siddha system of medicine are mentioned below:

1. Consumption of 1-2 teaspoon of pure honey with Luke warm water, during early morning, helps to detoxify and also nourishes your skin.
2. Natural aloe facial pack: In Siddha system of medicine, *A. vera* had been used for beauty and glowing skin since the ancient times.

   - This herb, which is known as "plant of immortality," is one of the greatest gifts of nature to solve many problems of skin. A face pack with the aloe pulp, honey and few drops of almond oil helps in reducing wrinkles and improves skin tone.
3. Application of Pindathylam (A Siddha formulation) as an external application helps in reducing dryness and pimples.
4. Drinking plenty of water helps to flush out the toxins in stomach, relieves constipation and maintains a good skin tone.
5. Regular use of *Nalangu maavu* (Siddha herbal bathing powder) for bath renders a healthy and glowing skin.

   - Preparation of *Nalangumaavu* [7]: *Pachai Payaru* (*Vigna radiata*) *Kasthuri manjal* (*Curcuma aromatica salisb*), *Chandanam* (*Santalum album* Linn.), *Korai kilangu* (*Cyperus rotundus* L.), *Kichili kilangu* (*C. zedoaria Rosc*), *Karunjeeragam* (*Nigella sativa*), *Karboga* (*Psoralea Corylifolia*), *Vetiver* (*Vetiveria zizanioides* L.), *Vilamichuver* (*plectranthus vettiveroides*) and rose petals are dried in shade and are ground together to a dry powder. It is mixed with rose water and used for bath.
6. Washing the face with *Sangu pushpam* reduces the incidence of acne.
7. *Chirattai Thylam*: This Siddha formulation is prepared from pure coconut shells and has in-depth potential for curing of warts, corns, black spots, white spots, eczema, and ringworm. For these problems, *Chirattai thylam* is one of the best remedies.
8. Neem leaves: Neem leaves have the power to treat skin allergy and problems of skin. Neem leaves are mashed with water to form a paste and applied.
9. Application of *Arugampul thailam* (*Cynodondactylon* L. (Pers)) in the areas of itch, and allergic rashes or hypersensitivity gives good results.
10. Application of *kungiliya vennai* in areas of suntan helps in reduction of hyper pigmentation.

CONCLUSION

Keeping in view of this scenario and trend, the authors have taken efforts to record the observations of Siddha system of medicine in the management of treating various skin related and other common ailments, which we are facing in daily life.

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Research Article

STANDARDIZATION OF *NEERKOVAI MATHIRAI:* A SIDDHA COMPOUND FORMULATION

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ABSTRACT

Objective: Standardization aspect involves confirmation of its identity, quality, and purity by determining certain nationally or internationally accepted properties. Standardization ensures the quality of medicines, authenticates the same and ensures the prescriptions of physicians. Also directly/indirectly gives relief to the end users, i.e., general public. This paper deals with the standardization of a Siddha compound formulation - *Neerkovai mathirai*. *Neerkovai mathirai*, an important compound formulation in Siddha system of medicine is used for upper respiratory infections such as common cold, head ache, sinusitis. It is to be mixed with lukewarm water to make a paste and used externally on the forehead/affected area.

Methods: The formulation used for the study was prepared according to the method described in Siddha Formulary of India. The organoleptic properties and physicochemical parameters of the formulation were determined by standard methods. High performance thin layer chromatographic (HPTLC) photo documentation was carried out and HPTLC fingerprinting profiles were determined. To ensure the quality of the formulation, the experiments such as elemental analysis, microbial analysis and determination of organochlorine pesticides, organophosphorus pesticides and aflatoxins were carried out.

Results: The organoleptic features such as color, odor, and touch were evaluated and are a qualitative evaluation technique based on the morphology and sensory profile of the sample. Physicochemical parameters of the formulation were determined and are an important diagnostic tool for laying down pharmacopoeial standards. HPTLC photo documentation and fingerprinting profiles, which have a wide application in herbal drug analysis, contribute to a great extent for standardization. The results showed that toxic/heavy metals and microbial contaminations were within the permissible limit/not detected. Pesticide residues were below detectable limit and their levels were insignificant.

Conclusion: The results obtained from this study will help to determine the genuineness and can be considered as pharmacopoeial standards of *Neerkovai mathirai*.

Keywords: *Neerkovai mathirai,* Organoleptic, Physicochemical, Elemental analysis.

INTRODUCTION

For herbal drugs, no uniformity is observed in the aspects of quality, efficacy, and drug safety in the manufacturing practices. Moreover physical, chemical, and biological variations affect the drug's efficacy. It is observed that there is no uniformity in the aspects of quality, efficacy, and safety with respect to the single drugs as well as compound preparations manufactured by various pharmaceuticals due to various reasons.

One of the major bottlenecks in the wider acceptance of herbal drugs in developing countries is the inadequacy or lack of standardization for the raw materials and for the finished products. It necessitates the need for standardization of herbal drugs and medicinal preparations. Standardization embodies total information and controls that are necessary to guarantee consistency of composition of the product ensuring their quality. Therefore, pharmacopoeial standardization of Ayurveda, Siddha and Unani (ASU) drugs, both for single and compound drugs are essential. Macroscopic and microscopic studies, physicochemical analysis, microbial analysis, determination, and use of thin layer chromatographic (TLC)/high performance TLC (HPTLC) fingerprinting profiles can be all good tools for standardization and validation of herbal drugs. Quality control of herbal drugs has an impact on their safety and efficacy, which needs to be evaluated by modern techniques for global acceptance.

Internationally, several pharmacopoeia has provided monographs stating quality parameters and standards of many herbal drugs and products there from. No pharmacopoeia of the world is comprehensive enough to cover all the medicinal herbs. Government of India set up the Pharmacopoeia Committee for laying down pharmacopoeial standards. The Pharmacopoeia Committee is following international norms for preparing pharmacopoeial monographs of single and compound ASU drugs.

In this paper, an attempt has been made to standardize an important Siddha formulation, *Neerkovai mathirai* following the WHO and FDA guidelines [1]. Mathirai is prepared using one or more drugs of plant, animal, or mineral origin. The ingredients are powdered, sieved, mixed with prescribed liquids and triturated till they attain the consistency suitable for making pills. These are dried under shade or sun and stored in air tight containers. The preparation containing herbal drugs can be used for 2 years while those containing minerals and metals can be used for indefinite period. *Neerkovai mathirai* is used for upper respiratory infections such as common cold, head ache, sinusitis, etc. In this study, different parameters like physicochemical parameters, HPTLC profile, elemental analysis, microbial analysis and determination of organochlorine pesticides, organophosphorus pesticides and aflatoxins were carried out to develop globally accepted standards for the selected formulation.

METHODS

Standard operating procedure of *Neerkovai mathirai*

Neerkovai mathirai is a compound formulation having 11 ingredients and was prepared by solids fat index method [2]. The ingredients of the preparation are given in Table 1.

Organoleptic characters
The organoleptic characters such as color, touch, taste, and odor were noted.

Physicochemical parameters
The physicochemical parameters such as loss on drying at 105°C, hardness, disintegration time, uniformity of weight, total ash, acid insoluble ash, water soluble ash, extractable matter in alcohol, and water and pH value of water extract were determined by standard methods [3]. The chemical tests for terpenoids, phenolics alkaloids, and sugars were carried out by standard methods [4].

Development of HPTLC profile
HPTLC is a micro analytical separation and determination method which has a wide application in herbal drug analysis.

Preparation of chloroform extract

1 g of the powdered sample was soaked in 10 ml chloroform and kept overnight. The solution was boiled and filtered. The filtrate was concentrated to 2 ml. This extract was used for chromatographic analysis.

Application of extract on TLC plates and development of plates

The extract was spotted in the form of bands with Camag microliter syringe attached with Camag ATS4 instrument on a Merck Aluminum plate precoated with silica gel 60 F_{254} of 0.2 mm thickness. The plate was developed in toluene:ethyl acetate:formic acid (5:1:2 drops). The developed plate was air dried, visualized under ultraviolet (UV) 254, UV 366 and the images were documented. The plate was derivatized using vanillin-sulfuric acid reagent, heated at 105°C until the development of colored spots, the spots were viewed in white light and the chromatograms were documented. The plates were scanned in UV 254 nm, 366 nm and in white light (575 nm) to obtain the densitometric profiles and R_f values [5].

Assay of inorganic constituents
The inorganic constituents such as calcium, sodium, potassium, zinc, iron, magnesium, mercury, cadmium, manganese were estimated by standard methods [6].

Microbial contamination
Microbial contamination such as total viable aerobic count, total *Enterobacteriaceae*, total fungal count and specific pathogens *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Pseudomonas aeruginosa,* etc., were determined by standard methods.

Determination of pesticide residue
Neerkovai mathirai was tested for organochlorine pesticides such as Benzene Hexachloride (BHC) like beta-BHC, gamma-BHC, delta-BHC, aldrin, dieldrin, endrin, 44'DDD, 44'DDE, 44'DDT, Endosulfan I, Endosulfan II, heptachlor and heptachlorepoxide; and organophosphorus pesticides such as dichlorvos, disulfoton, parathion methyl, fenchlorphos, chlorpyrifos, prothiofos, ethoprofos, guthion, and malathion by the AOAC method [6].

Determination of arsenic and heavy metals
The heavy/toxic metals, lead, cadmium, arsenic, and mercury were determined by the AOAC method [6].

RESULTS AND DISCUSSION

The organoleptic features such as color, odor, and touch were evaluated and the results were found to be: Color - Deep orange; touch – Smooth and odor - Turmeric smell. This method is a qualitative evaluation technique based on the morphology and sensory profile of the sample. The physicochemical parameters of *Neerkovai mathirai* are given in Table 2.

The test for loss on drying determines moisture content and volatile oil in the drug. Hardness was determined using a hardness tester. Hardness of a tablet means how much force is needed to break it. Disintegration test was conducted in a disintegration test apparatus in water medium. If the time duration at which the tablet or capsule disintegrates is within 15-30 minutes, the tablet is considered as standard. Uniformity of weight, (%) variation observed was ±0.5578. If the deviation is within the permissible limit (±5%), the tablets are uniform. Friability was determined using friability apparatus. Friability test gives information about how much mechanical stress, tablets are able to withstand during their manufacturing, distributing and handling.

Total ash includes both physiological ash and nonphysiological ash. Acid insoluble ash measures mainly the amount of silica present as sand. Water soluble ash contains the physiological ash which is soluble in water. Alcohol soluble extractive determines the amount of organic constituents which can be extracted with alcohol from a given amount of sample. Water soluble extract of the drug represents the percentage of organic constituents such as tannin, sugar, plant acid, mucilage, and glycosides which are soluble in water. pH value was determined by means of a digital pH meter. It is the quantitative indication of the acidity or alkalinity of a solution. If the pH value obtained is <7 the extract is acidic, if 7 neutral and more than 7, it is alkaline.

HPTLC is particularly valuable for the qualitative determination of different compounds present in the extracts. HPTLC photo

Table 1: Ingredients of *Neerkovai mathirai*

S.No.	Tamil name	Scientific name	Parts used	Quantity (ratio)
1	Kappumancal	*Curcuma longa* L.	Rhizome	4 part
2	Kasturimanjal	*Curcuma aromatica* Salisb.	Rhizome	4 part
3	Venkaram (purified)	Borax (disodium tetraborate)	-	2 part
4	Campirani	*Styrax benzoin* Dryand.	Oleo resin	2 part
5	Milaku	*Piper nigrum* L	Fruit	2 part
6	Cukku	*Zingiber officinale* Rosc.	Rhizome (Dried)	2 part
7	Catikkay	*Myristica fragrans* Houtt.	Kernel	2 part
8	Omam	*Trachyspermum ammi* (L.) Sprague	Fruit	2 part
9	Ilavankam	*Syzygium aromaticum* (L.) Merr. & L.M. Perry Syn. *Eugenia caryophyllata* Thunb.	Flower bud	1 part
10	Karpuram (purified)	*Cinnamomum camphora* (L.) J. Presl	-	1 part
11	Elumiccam pazaccharu	*Citrus aurantiifolia* (Christm.) Swingle	Fruit	Sufficient quantity

documentation profiles of the chloroform extract of *Neerkovai mathirai* at 254 nm, 366 nm and after derivatization under white light are given in Fig. 1. The solvent system, toluene:ethyl acetate:formic acid (5:1:2 drops) effectively resolved the components present in the crude extract.

Table 2: Physicochemical parameters of *Neerkovai mathirai*

S.No.	Parameter	Result*
1.	Foreign matter (%)	Nil
2.	Loss on drying at 105°C (%)	10.26
3.	Total ash content (%)	13.82
4.	Acid insoluble ash (%)	3.38
5.	Alcohol soluble extractive (%)	15.14
6.	Water soluble extractive (%)	35.12
7.	Volatile oil (%)	Nil
8.	Friability (%)	0.96
9.	Hardness (kg/cm^2)	2.50
10.	Uniformity of weight, (%) Variation	±0.5578

*Result gives the mean value of three samples each

Table 3: Elemental analysis of *Neerkovai mathirai*

S.No.	Parameter	Result (mg/kg)
1	Calcium	408
2	Sodium	15,481
3	Potassium	1283
4	Zinc	60.3
5	Iron	674
6	Magnesium	362
7	Manganese	119

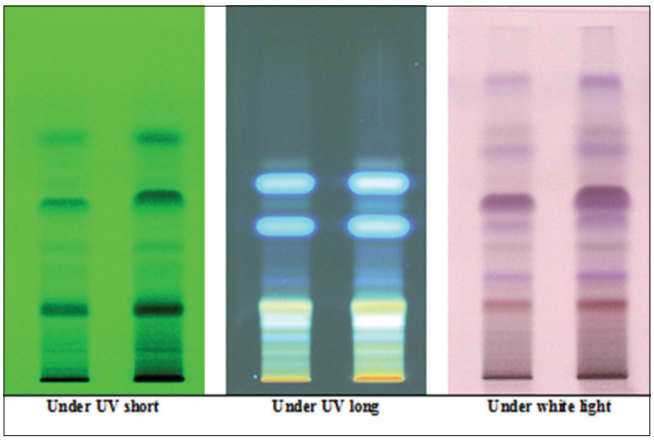


Fig. 1: Thin layer chromatographic photo documentation of chloroform extract of *Neerkovai mathirai*; viewed in ultraviolet (UV) short; viewed in UV long; viewed in visible light after derivatization using vanillin-sulfuric acid; Solvent system – Toluene:ethyl acetate:formic acid (5:1:2 drops)

The HPTLC fingerprinting profiles of the chloroform extract of *Neerkovai mathirai* were recorded at 254 nm, 366 nm and after derivatization with vanillin-sulfuric acid at 575 nm and their finger printing profiles and R_f tables are given in Figs. 2-4, respectively.

The inorganic constituents such as calcium, sodium, potassium, zinc, iron, magnesium, and manganese were found to be present in the formulation and are estimated using standard methods and the results are given in Table 3.

The microbial analysis of the formulation was carried out and the results are given in Table 4. The total viable aerobic count, *Enterobacteriaceae*, total fungal count and specific pathogens were found to be below the permissible limits as per the WHO or found to be absent.

The pesticide residues such as organochlorine pesticides and organophosphorus pesticides were found to be below the detectable limit and the results are given in Tables 5 and 6.

Lead (Pb), mercury (Hg), arsenic (As), and cadmium (Cd) are the four heavy/toxic metals which are of concern. The quantitative analyses of these metals were carried out and the values are given below.

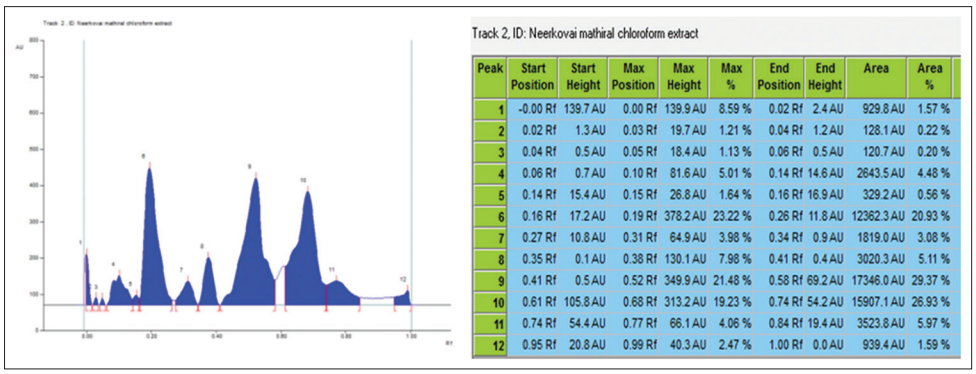
a. Lead - 2.20 ppm
b. Cadmium - 0.09 ppm
c. Mercury - Not detected (Det. limit 0.5)
d. Arsenic - Not detected (Det. limit 0.5).

Consumption of these metals is known to be toxic to human health. The limits for these metals as per the WHO/FDA (Permissible limit) are lead - 10.0 ppm; cadmium - 0.30 ppm; mercury - 1.00 ppm and arsenic - 10.0 ppm.

All the results of the different experiments were considered together for fixing standards and for the test dug *Neerkovai mathirai*, the values obtained for different samples are taken and the standards are fixed at a particular range.

CONCLUSION

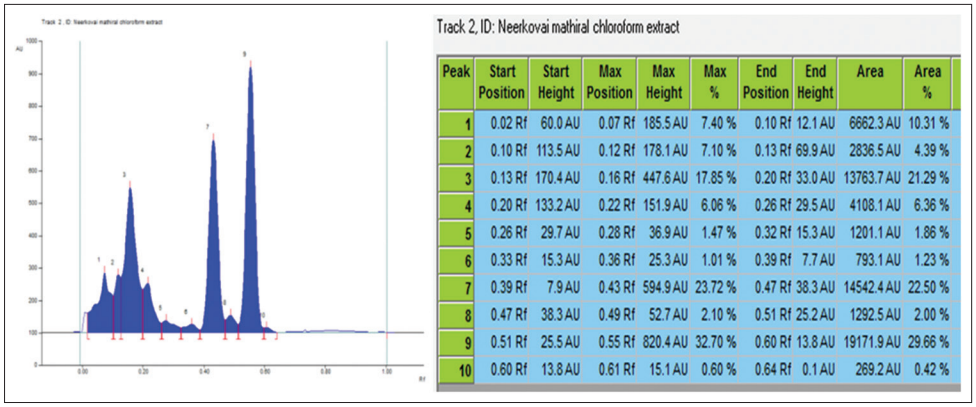
The results obtained from this study will help to determine the genuineness and can be considered as pharmacopoeial standards of *Neerkovai mathirai*. By these types of advance standardization studies, the quality and safety of Siddha drugs may be proved which in turn can contribute toward the propagation of Siddha systems nationally and internationally.



Track 2, ID: Neerkovai mathiral chloroform extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.00 Rf	139.7 AU	0.00 Rf	139.9 AU	8.59 %	0.02 Rf	2.4 AU	929.8 AU	1.57 %
2	0.02 Rf	1.3 AU	0.03 Rf	19.7 AU	1.21 %	0.04 Rf	1.2 AU	128.1 AU	0.22 %
3	0.04 Rf	0.5 AU	0.05 Rf	18.4 AU	1.13 %	0.06 Rf	0.5 AU	120.7 AU	0.20 %
4	0.06 Rf	0.7 AU	0.10 Rf	81.6 AU	5.01 %	0.14 Rf	14.6 AU	2643.5 AU	4.48 %
5	0.14 Rf	15.4 AU	0.15 Rf	26.8 AU	1.64 %	0.16 Rf	16.9 AU	329.2 AU	0.56 %
6	0.16 Rf	17.2 AU	0.19 Rf	378.2 AU	23.22 %	0.26 Rf	11.8 AU	12362.3 AU	20.93 %
7	0.27 Rf	10.8 AU	0.31 Rf	64.9 AU	3.98 %	0.34 Rf	0.9 AU	1819.0 AU	3.08 %
8	0.35 Rf	0.1 AU	0.38 Rf	130.1 AU	7.98 %	0.41 Rf	0.4 AU	3020.3 AU	5.11 %
9	0.41 Rf	0.5 AU	0.52 Rf	349.9 AU	21.48 %	0.58 Rf	69.2 AU	17346.0 AU	29.37 %
10	0.61 Rf	105.8 AU	0.68 Rf	313.2 AU	19.23 %	0.74 Rf	54.2 AU	15907.1 AU	26.93 %
11	0.74 Rf	54.4 AU	0.77 Rf	66.1 AU	4.06 %	0.84 Rf	19.4 AU	3523.8 AU	5.97 %
12	0.95 Rf	20.8 AU	0.99 Rf	40.3 AU	2.47 %	1.00 Rf	0.0 AU	939.4 AU	1.59 %

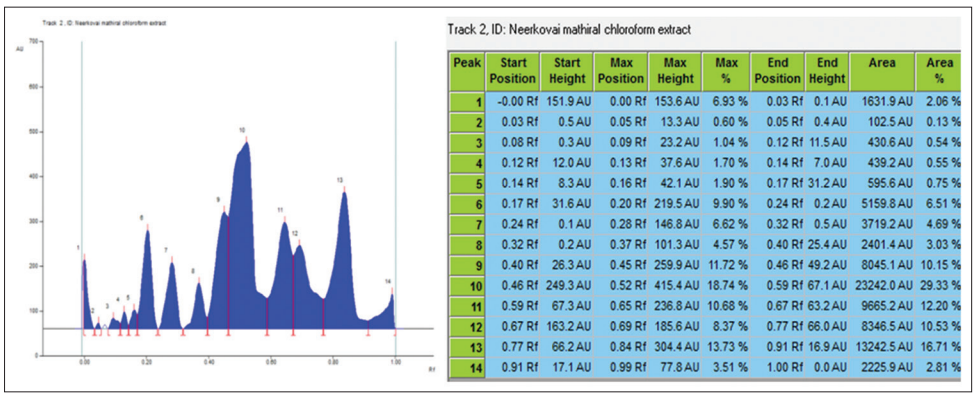
Fig. 2: High performance thin layer chromatographic finger print profile and R_f table of 10 µl of chloroform extract of *Neerkovai mathirai* at 254 nm



Track 2, ID: Neerkovai mathiral chloroform extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.02 Rf	60.0 AU	0.07 Rf	185.5 AU	7.40 %	0.10 Rf	12.1 AU	6662.3 AU	10.31 %
2	0.10 Rf	113.5 AU	0.12 Rf	178.1 AU	7.10 %	0.13 Rf	69.9 AU	2836.5 AU	4.39 %
3	0.13 Rf	170.4 AU	0.16 Rf	447.6 AU	17.85 %	0.20 Rf	33.0 AU	13763.7 AU	21.29 %
4	0.20 Rf	133.2 AU	0.22 Rf	151.9 AU	6.06 %	0.26 Rf	29.5 AU	4108.1 AU	6.36 %
5	0.26 Rf	29.7 AU	0.28 Rf	36.9 AU	1.47 %	0.32 Rf	15.3 AU	1201.1 AU	1.86 %
6	0.33 Rf	15.3 AU	0.36 Rf	25.3 AU	1.01 %	0.39 Rf	7.7 AU	793.1 AU	1.23 %
7	0.39 Rf	7.9 AU	0.43 Rf	594.9 AU	23.72 %	0.47 Rf	38.3 AU	14542.4 AU	22.50 %
8	0.47 Rf	38.3 AU	0.49 Rf	52.7 AU	2.10 %	0.51 Rf	25.2 AU	1292.5 AU	2.00 %
9	0.51 Rf	25.5 AU	0.55 Rf	820.4 AU	32.70 %	0.60 Rf	13.8 AU	19171.9 AU	29.66 %
10	0.60 Rf	13.8 AU	0.61 Rf	15.1 AU	0.60 %	0.64 Rf	0.1 AU	269.2 AU	0.42 %

Fig. 3: High performance thin layer chromatographic finger print profile and R_f table of 10 µl of chloroform extract of *Neerkovai mathirai* at 366 nm



Track 2, ID: Neerkovai mathiral chloroform extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.00 Rf	151.9 AU	0.00 Rf	153.6 AU	6.93 %	0.03 Rf	0.1 AU	1631.9 AU	2.06 %
2	0.03 Rf	0.5 AU	0.05 Rf	13.3 AU	0.60 %	0.05 Rf	0.4 AU	102.5 AU	0.13 %
3	0.08 Rf	0.3 AU	0.09 Rf	23.2 AU	1.04 %	0.12 Rf	11.5 AU	430.6 AU	0.54 %
4	0.12 Rf	12.0 AU	0.13 Rf	37.6 AU	1.70 %	0.14 Rf	7.0 AU	439.2 AU	0.55 %
5	0.14 Rf	8.3 AU	0.16 Rf	42.1 AU	1.90 %	0.17 Rf	31.2 AU	595.6 AU	0.75 %
6	0.17 Rf	31.6 AU	0.20 Rf	219.5 AU	9.90 %	0.24 Rf	0.2 AU	5159.8 AU	6.51 %
7	0.24 Rf	0.1 AU	0.28 Rf	146.8 AU	6.62 %	0.32 Rf	0.5 AU	3719.2 AU	4.69 %
8	0.32 Rf	0.2 AU	0.37 Rf	101.3 AU	4.57 %	0.40 Rf	25.4 AU	2401.4 AU	3.03 %
9	0.40 Rf	26.3 AU	0.45 Rf	259.9 AU	11.72 %	0.46 Rf	49.2 AU	8045.1 AU	10.15 %
10	0.46 Rf	249.3 AU	0.52 Rf	415.4 AU	18.74 %	0.59 Rf	67.1 AU	23242.0 AU	29.33 %
11	0.59 Rf	67.3 AU	0.65 Rf	236.8 AU	10.68 %	0.67 Rf	63.2 AU	9665.2 AU	12.20 %
12	0.67 Rf	163.2 AU	0.69 Rf	185.6 AU	8.37 %	0.77 Rf	66.0 AU	8346.5 AU	10.53 %
13	0.77 Rf	66.2 AU	0.84 Rf	304.4 AU	13.73 %	0.91 Rf	16.9 AU	13242.5 AU	16.71 %
14	0.91 Rf	17.1 AU	0.99 Rf	77.8 AU	3.51 %	1.00 Rf	0.0 AU	2225.9 AU	2.81 %

Fig. 4: High performance thin layer chromatographic finger print profile and R_f table of 10 µl of chloroform extract of *Neerkovai mathirai* (after derivatization using vanillin-sulfuric acid) at 575 nm

Table 4: Microbial analysis of *Neerkovai mathirai*

S.No.	Parameter	Result (cfu/g)	Permissible limits as per WHO	Test method
1	Total viable aerobic count	80	<107 CFU/g	USFDA – BAM Online, Jan 2001, Ch. 3
2	*Enterobacteriaceae*	<10	104 g^{-1}	IS/ISO 7402:1993
3	Total fungal count	<10	<10	USFDA – BAM Online, April 2001, Ch. 18
4.	Specific pathogens			
4.1	*Escherichia coli*	<10	102 g^{-1}	IS: 5887 (part I) 1976 Reaffirmed 1995
4.2	*Salmonella* sp.	Absent	None	USFDA – BAM Online, Nov 2011, Ch. 5
4.3	*Pseudomonas aeruginosa*	Absent	None	IS 14648:2011
4.4	*Staphylococcus aureus*	Absent	None	USFDA – BAM Online, Jan 2001, Ch. 12

Table 5: Organochlorine pesticides of *Neerkovai mathirai*

S.No.	Parameter	Result (ppb)
1	Alpha-BHC	Not detected (Detectable limit 50)
2	Beta-BHC	Not detected (Detectable limit 50)
3	Gamma-BHC	Not detected (Detectable limit 50)
4	Delta-BHC	Not detected (Detectable limit 50)
5	Aldrin	Not detected (Detectable limit 50)
6	Dieldrin	Not detected (Detectable limit 50)
7	Endrin	Not detected (Detectable limit 50)
8	44'DDD	Not detected (Detectable limit 50)
9	44'DDE	Not detected (Detectable limit 50)
10	44'DDT	Not detected (Detectable limit 50)
11	Endosulfan I	Not detected (Detectable limit 50)
12	Endosulfan II	Not detected (Detectable limit 50)
13	Heptachlor	Not detected (Detectable limit 50)
14	Heptachlorepoxide	Not detected (Detectable limit 50)

Table 6: Organophosphorus pesticides of *Neerkovai mathirai*

S.No.	Parameter	Result
1	Dichlorvos	Not detected (Detectable limit 50)
2	Endosulfan II	Not detected (Detectable limit 50)
3	Disulfoton	Not detected (Detectable limit 50)
4	Parathion methyl	Not detected (Detectable limit 50)
5	Fenchlorphos	Not detected (Detectable limit 50)
6	Chlorpyrifos	Not detected (Detectable limit 50)
7	Prothiofos	Not detected (Detectable limit 50)
8	Ethoprofos	Not detected (Detectable limit 50)
9	Guthion	Not detected (Detectable limit 50)
10	Malathion	Not detected (Detectable limit 50)
11	Fenitrothion	Not detected (Detectable limit 50)

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Research Article

ISOLATION OF FLAVONOIDS FROM *PSILANTHUS TRAVANCORENSIS* (WT. & ARN.) LEROY - AN UNEXPLORED TAXON OF RUBIACEAE

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ABSTRACT

Objective: *Psilanthus travancorensis* (Wt. & Arn.) Leroy belonging to the Rubiaceae family is a medicinal plant used for the treatment of a wide range of diseases. The root of the plant is a major ingredient of various formulations used in Indian Systems of Medicine. Little seems to be known about the chemical components of this plant. The evaluation of this plant for biological activity is necessary; to scientifically substantiate the use of this plant by traditional healers to develop the new drugs. Preliminary phytochemical studies revealed the presence of various major secondary components such as alkaloids, flavonoids, glycosides, phenolic compounds, saponins, tannins, steroids, terpenoids, and coumarins.

Methods: This study focuses on flavonoids because the quantitative estimation showed that the root of the plant is rich in flavonoids. The root was extracted with 85% ethyl alcohol and concentrated. The aqueous concentrate was extracted successively with petroleum ether and ethyl acetate. The fractionation of the extract using petroleum ether was to get rid of sterols, carotenoids, chlorophyll, etc.

Results: Only the ethyl acetate extract gave positive Shinoda test for flavonoids and was used for further phytochemical analysis. The ethyl acetate fraction obtained was subjected to column chromatography and the fractions, which showed a positive test for flavonoids were pooled together. Four main fractions were obtained. Characterization of these compounds was carried out using various spectroscopic techniques and high-performance liquid chromatography analysis.

Conclusion: The compounds identified were quercetin, rutin, catechin, and a quercetin glycoside. This paper reports the phytochemical screening and isolation of flavonoids from *P. travancorensis* for the first time.

Keywords: Column chromatography, High-performance liquid chromatography, Quercetin, Rutin, Catechin.

INTRODUCTION

Psilanthus travancorensis (Wt. & Arn.) Leroy belonging to the Rubiaceae family, Rubiaceae is a medicinal plant used for the treatment of a wide range of diseases. This study focuses on flavonoids because the quantitative estimation showed that the root of the plant is rich in flavonoids. This paper reports the isolation of flavonoids from *P. travancorensis* for the first time.

METHODS

Reagents and chemicals

All the reagents and chemicals were of GPR grade. Standards of flavonoids quercetin, catechin, and rutin were purchased from Merck.

Sample preparation for flavonoids

Sample preparation for flavonoids was carried out by the methods of Ivanauskas *et al*., 2008.

Preparation of plant extract

The root of *P. travancorensis* was washed, dried under shade, powdered and used for extraction. The powdered root is extracted using 85% ethyl alcohol (85:15, ethyl alcohol:water) in a Soxhlet extraction apparatus. The extract is concentrated using a rotary evaporator under reduced pressure to obtain the aqueous extract.

Preparation of flavonoid fraction

The aqueous layer was extracted successively with petroleum ether and ethyl acetate. The ethyl acetate fraction gave positive Shinoda test for flavonoids and was subjected to further phytochemical analysis.

Identification of flavonoids by high-performance liquid chromatography (HPLC)

Three flavonoids namely quercetin, rutin, and catechin were analyzed for their presence through HPLC in the root of *P. travancorensis* [1].

Isolation of flavonoids from ethyl acetate fraction

The ethyl acetate fraction was subjected to column chromatography on silica gel G (60-120 mesh). Elution was carried out using different solvents of increasing polarity in the following order: Petroleum ether, a mixture of petroleum ether and ethyl acetate in different ratios, ethyl acetate, a mixture of ethyl acetate and methanol in different ratios, and methanol. Based on thin-layer chromatography (TLC) analysis, the fractions were pooled into 11 groups. 4 fractions showed positive results for flavonoids and also exhibited very good antioxidant activity in 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay. The compounds were purified using preparative TLC. The compounds were recrystallized using appropriate solvents. Then, the compounds were characterized by color reactions and spectral properties.

RESULTS AND DISCUSSION

Identification of flavonoids by HPLC

The retention times of standards were 17.27 for quercetin, 2.73 for rutin, and 2.97 for catechin (Figs. 1-3). Ethyl acetate fraction of the root extract showed the presence of these three flavonoids that were comparable to the retention time of standards.

Characterization of Compound I as quercetin

The compound was crystallized from acetone as yellow needles with melting point 316-317°C. It developed yellow color with alkali, pink

Table 1: Color reactions in ethanolic solution (Compound I)

Color reaction			
Aq. NaOH	**Con. H_2SO_4**	**Mg-HCl (Shinoda test)**	**Na/Hg and HCl**
Yellow to orange	Intense yellow	Magenta	Yellow to pale red

Table 2: UV (λ_{max}, nm) of Compound I

Reagents	UV (λ_{max}, nm)
MeOH	253, 370
MeOH+$AlCl_3$	272, 329, 450
MeOH+$AlCl_3$+HCl	272, 326, 431
MeOH+NaOAc	274, 330, 390

color with Mg-HCl, and olive green with ferric chloride (Table 1). It was yellow under UV/NH_3 and had λ_{max} (MeOH): 253, 370 nm (Table 2).

The results obtained for color reactions in ethanol and color developed on chromatogram suggested that the compound was a flavonol (Table 3). Elemental analysis (Table 4) showed that the molecular formula was $C_{15}H_{10}O_7$. From the mass spectrum, molecular weight of the compound was obtained as 302 (Fig. 4). From the analysis of the data obtained for ^1H nuclear magnetic resonance (NMR) spectrum (Fig. 5), the compound was identified as 3,3',4',5,7-pentahydroxyflavone (quercetin) [2].

Characterization of Compound II as catechin

The compound was recrystallized from acetone. It developed yellow color changing to red and brown with alkali, no change with Mg HCl, and olive green with ferric chloride (Table 5). It was colorless under UV/NH_3 (Table 6).

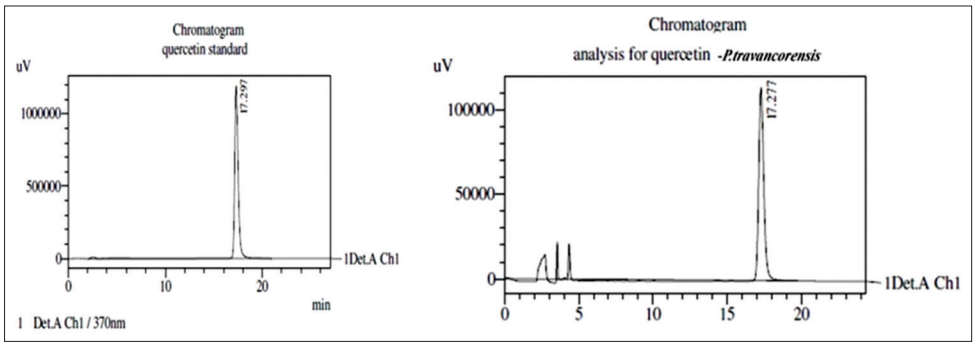

Fig. 1: Chromatogram of standard solution of quercetin and chromatogram of ethyl acetate fraction of *Psilanthus travancorensis* root extract

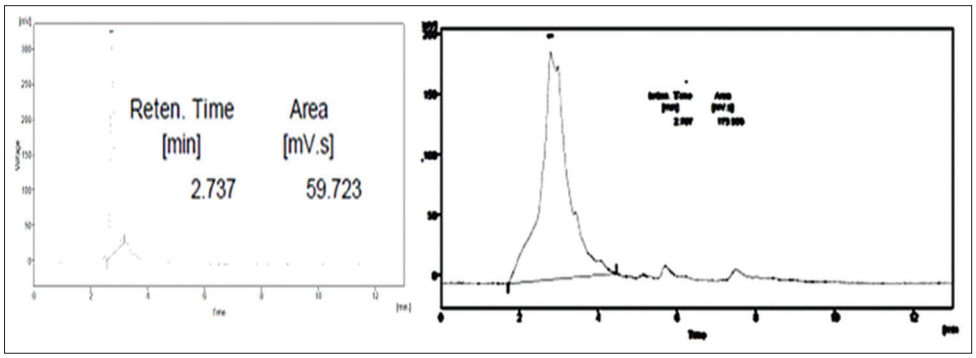

Fig. 2: Chromatogram of standard solution of rutin and chromatogram of ethyl acetate fraction of *Psilanthus travancorensis* root extract

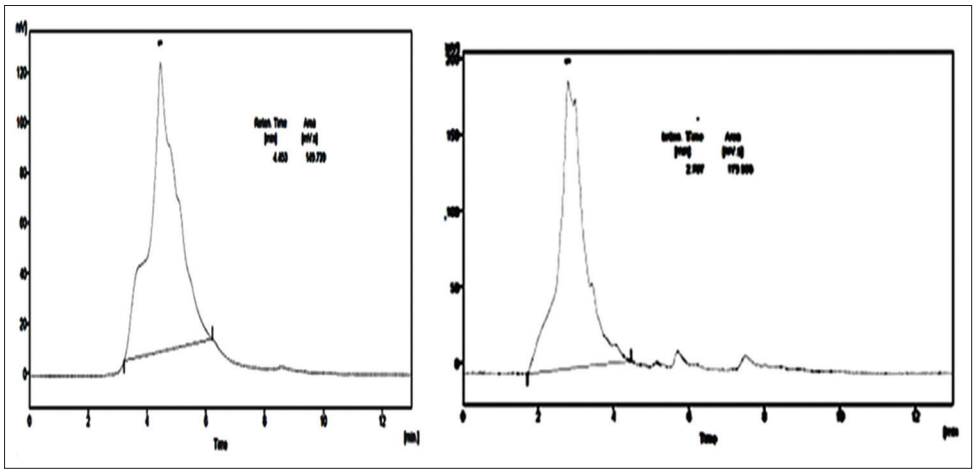

Fig. 3: Chromatogram of standard solution of catechin and chromatogram of ethyl acetate fraction of *Psilanthus travancorensis* root extract

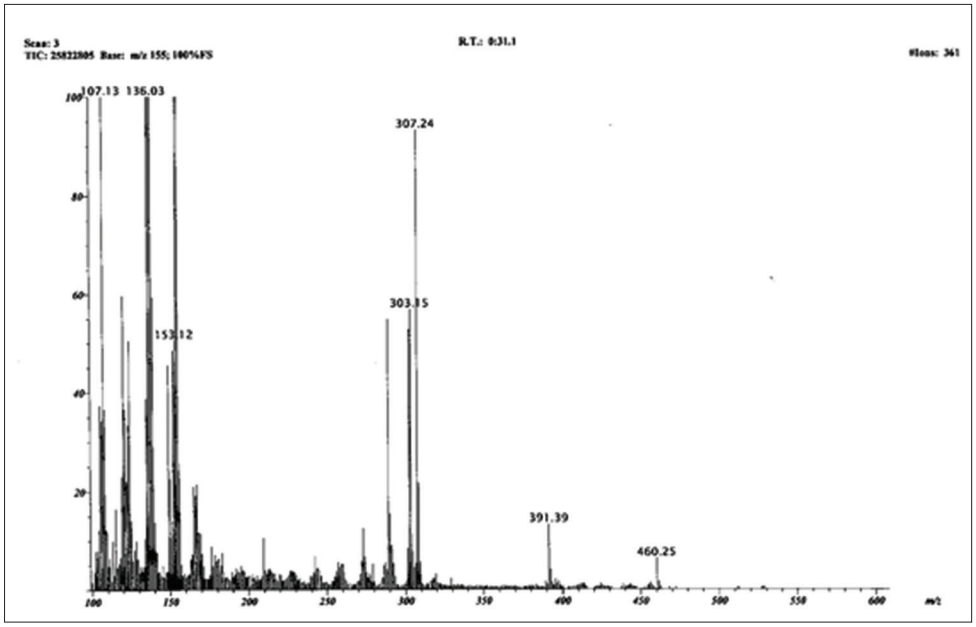

Fig. 4: Mass spectrum of Compound I

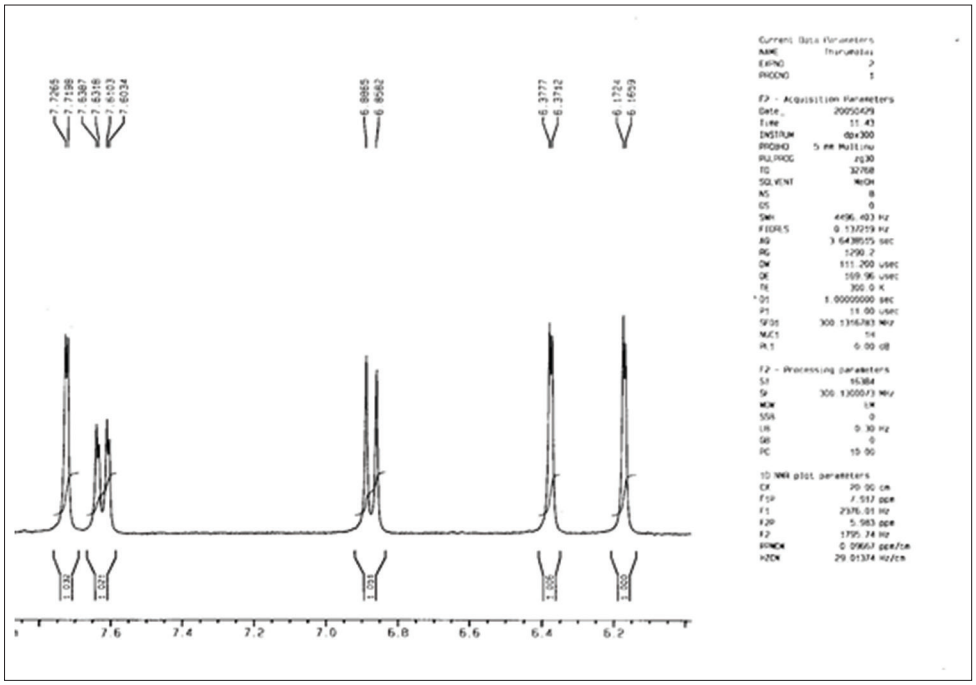

Fig. 5: ^{1}H nuclear magnetic resonance spectrum of Compound I

Table 3: Color reactions on paper

Reagent light	None visible	None UV	NH_3 visible	NH_3 UV	$AlCl_3$ visible	$AlCl_3$ UV	Na_2CO_3 visible	$NaHBO_4$ visible	$ArSO_3H$ visible
Compound I	Pale yellow	Bright yellow	Yellow	Bright yellow	Yellow	Fluorescent yellow	Yellow	Colorless	Yellow

Table 4: Elemental analysis (CHNS Analysis) of Compound I

Element	Percentage (%)
Carbon (C)	59.43
Hydrogen (H)	3.44
Oxygen (O)	37.13
Nitrogen (N)	Not detected
Sulfur (S)	Not detected

UV spectrum

UV (λ_{max}, nm) in methanol showed maxima at 220 nm, and 277 nm showed that the Compound II catechin.

Color reactions in ethanolic solution and color reactions on paper showed that Compound II was a catechin. Elemental analysis showed that the molecular formula is $C_{21}H_{20}O_{12}$ (Table 7) and molecular mass calculated as 464. UV (λ_{max}, nm) in methanol showed maxima

Table 5: Color reactions in ethanolic solution

Color reaction			
Aq. NaOH	**Con. H_2SO_4**	**Mg-HCl (Shinoda test)**	**Na/Hg and HCl**
Yellow changing to red and brown	Red	None	None

Table 6: Color reactions on paper

Reagent light	None visible	None UV	NH_3 visible	NH_3 UV	$AlCl_3$ visible	$AlCl_3$ UV	Na_2CO_3 visible	$NaHBO_4$ visible	$ArSO_3H$ visible
Catechin	Colorless	Colorless	Colorless	Fluorescent pale blue black	Colorless	Colorless	_	_	Brown

Table 7: Elemental analysis (CHNS Analysis) of Compound II

Element	Percentage (%)
Carbon (C)	54.31
Hydrogen (H)	4.32
Oxygen (O)	41.37
Nitrogen (N)	Not detected

Table 8: ^{1}H-NMR spectrum

^{1}H-NMR signals	Position of hydrogen
d, 4.56	H-2
ddd, 4.00	H-3
dd, 2.54	H-4a,
dd, 2.90	H-4e
d, 5.87	H-6
d, 6.01	H-8
d, 6.89	H-2′
d, 6.79	H-5′
dd, 6.73	H-2′
8.00	Phenolic protons

NMR: Nuclear magnetic resonance

Table 9: Color reactions of Compound III in ethanolic solution

Reagent	Color reaction			
	Aq. NaOH	**Con. H_2SO_4**	**Mg-HCl (Shinoda test)**	**Na/Hg and HCl**
Compound III	Yellow to orange	Intense yellow to fluorescent orange	Red to magenta	Yellow to pale red

at 220 nm, and 277 nm shows that Compound II is catechin (Table 8). The proton NMR spectrum further confirms that Compound II is catechin. The HPLC analysis confirms the presence of catechin in *P. travancorensis.*

Characterization of Compound III as quercetin-3-O-β-D-glucoside

Compound III crystallized from MeOH as a yellow solid, with m.p. 224-226°C. It gave yellow color with alkali, pink color with Mg-HCl, and olive green color with Fe^{3+} (Table 9). It was purple under UV and yellow under UV/NH_3 (Table 10).

UV spectrum

The color reactions discussed above indicated that Compound III was a flavonoid glycoside. The analysis of UV spectra indicated that the compound was a quercetin glycoside. The compound was hydrolyzed, and the aglycone was identified as quercetin (Tables 11 and 12). The sugar was identified as glucose by the brown color spot developed when

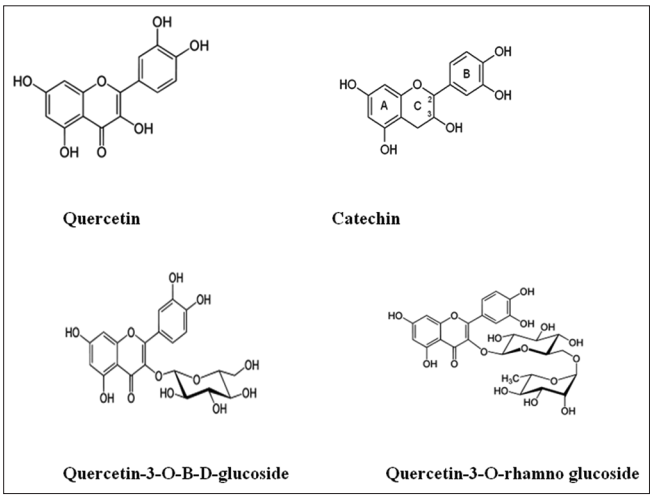

Fig. 6: Flavonoids isolated from *Psilanthus travancorensis*

the chromatogram was sprayed with aniline hydrogen phthalate. The identity of quercetin and glucose were further confirmed by co-IR, co-PC, and m.m.p with authentic quercetin and D-glucose. The 3-O-glucosyl linkage was evidenced from the color of the compound under UV and UV/NH_3. Hence, the compound was identified as quercetin-3-O-β-D-glucoside.

Characterization of Compound IV as rutin

Compound IV was crystallized from methanol acetone (1:1) as pale yellow needles, melting point 182-185°C. The compound gave yellow color with alkali, pink color with Mg-HCl, greenish brown color with ferric chloride, and positive Molisch's test (Table 13). It was purple under UV and yellow under UV/NH_3.

UV spectrum

The color reactions indicated it to be a flavonoid glycoside (Table 14). Bathochromaic shift of band I and II on addition of sodium acetate indicated free OH at C-4' and C-7 (Table 15). IN the UV spectrum, bathochromic shift of band I of the NaOAc/H_3BO_3 spectrum showed the O-dihydroxy system in B-ring (Table 16). Stability of NaOMe spectrum suggested that 3-OH was not free. The analysis of UV spectra indicated that the compound was a quercetin 3-glycoside. The compound was hydrolyzed and the aglycone was identified as querctin. The sugars were identified as glucose and rhamnose sugars in an equimolar ratio. The compound was unaffected by β-glucosidase on enzyme hydrolysis indicating that glucose was not the terminal sugar. From these data, the flavonol glycoside was identified as quercetin-3-O-rhamnoglucoside (rutin) and its identity were confirmed by direct comparison and co-PC with an authentic sample. The presence of rutin was also confirmed by HPLC analysis (Fig. 6).

Table 10: Color reactions of Compound III on paper

Reagent light	None Visible	None UV	NH_3 visible	NH_3 UV	$AlCl_3$ visible	$AlCl_3$ UV	Na_2CO_3 visible	$NaHBO_4$ visible	$ArSO_3H$ visible
Flavonol	Pale yellow	Brown	Yellow	Yellow-green	Yellow	Fluorescent yellow	Yellow	Colorless	Yellow

Table 11: Elemental analysis (CHNS analysis) of Compound III

Element	Percentage (%)
Carbon (C)	54.42
Hydrogen (H)	4.31
Oxygen (O)	41.27
Nitrogen (N)	Not detected
Sulfur (S)	Not detected

Table 12: UV (λ_{max}, nm) of Compound III

Reagents	UV (λ_{max}, nm)
MeOH	259, 350
MeOH+$AlCl_3$	274, 337, 428
MeOH+$AlCl_3$+HCl	269, 357, 389
MeOH+NaOAc	271, 378
MeOH+NaOAc+H_3BO_3	264, 360

Table 13: Color reactions of Compound IV in ethanolic solution

Reagent	Color reaction			
	Aq. NaOH	Con. H_2SO_4	Mg-HCl (Shinoda test)	Na/Hg and HCl
Compound III	Yellow to orange	Intense yellow to fluorescent orange	Red to magenta	Yellow to pale red

Table 14: Color reactions of Compound IV on paper

Reagent light	None visible	None UV	NH_3 visible	NH_3 UV	$AlCl_3$ visible	$AlCl_3$ UV	Na_2CO_3 visible	$NaHBO_4$ visible	$ArSO_3H$ visible
Flavonol	Pale yellow	Brown	Yellow	Yellow-green	Yellow	Fluorescent yellow	Yellow	Colorless	Yellow

Table 15: Elemental analysis (CHNS analysis) of Compound IV

Element	Percentage (%)
Carbon (C)	53.14
Hydrogen (H)	4.89
Oxygen (O)	41.97
Nitrogen (N)	Not detected
Sulfur (S)	Not detected

Table 16: UV (λ_{max}, nm) of Compound III

Reagents	UV (λ_{max}, nm)
MeOH	256, 359
MeOH+$AlCl_3$	275, 434
MeOH+$AlCl_3$+HCl	275, 412
MeOH+NaOAc	270, 325, 394
MeOH+NaOAc+H_3BO_3	262, 298, 387
MeOH+NaOMe	272, 326, 410

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Research Article

DETERMINATION OF PHYSICOCHEMICAL PARAMETERS AND HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHIC PROFILE OF *AERVA LANATA* (LINN.) JUSS EX SCHULT (ROOT)

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ABSTRACT

Objective: *Aerva lanata* (Linn.) Juss. ex Schult, family Amaranthaceae, is a common wayside weed found throughout tropical India. The current work was undertaken with a view to determine the physicochemical parameters, to detect the presence of phytochemicals, and to carry out high-performance thin-layer chromatographic (HPTLC) study of the root of *A. lanata*, and based on the data obtained, pharmacopeial standards were laid out.

Methods: The physicochemical parameters such as loss on drying at 105°C, solubility in water and alcohol, ash content, and acid-insoluble ash were determined by standard methods. Phytochemical study was carried out using different extracts of the plant material to identify the class of compounds. HPTLC study of the chloroform extract of the plant material was carried out under ultraviolet (UV) 254 nm, UV 366 nm, and in daylight after derivatization using vanillin-sulfuric acid reagent.

Results: The physicochemical parameters were studied, which are important diagnostic parameters of the plant. Preliminary phytochemical investigations revealed the presence of steroids, flavonoids, alkaloids, sugar, and tannins. TLC pattern showed 7 visible bands under UV at 254 nm, 12 bands at 366 nm, and 7 bands under white light after derivatization. The observed R_f values and colors were noted. There were several peaks in the HPTLC fingerprinting profiles obtained at different wavelengths. This indicates the presence of various phytochemicals in the plant drug.

Conclusion: The above-mentioned quality assessment profiles obtained for the root of *A. lanata* in the present study could be very much useful in the identification and authentication of this plant drug, which may help in preventing the adulteration.

Keywords: *Aerva lanata*, Amaranthaceae, Physicochemical parameters, High-performance thin layer chromatographic analysis.

INTRODUCTION

Herbal medicines have played great significant role in the primary healthcare of individual and communities. The high degree of efficacy and safety of herbal medicines makes them more acceptable as compared to other therapeutic invention [1]. Plants have served as human's novel weapons against different ailments. However, the scarcity of the medicinal plant materials leads to the problem of adulteration or substitution of drugs. To prevent this, proper standardization of the drugs using approved parameters is necessary. In the present work, a detailed study on *Aerva lanata* (root) based on its physicochemical analysis and high-performance thin-layer chromatographic (HPTLC) studies was carried out and based on the data pharmacopeial standards was laid out.

A. lanata (Linn.) Juss. ex Schult is a perennial and annual herb, commonly known as mountain knotgrass (Fig. 1). It belongs to the family Amaranthaceae. It is one of the prominent 10 sacred flowers of Kerala, named Dasapushpam. It is abundantly seen in India. It is also found in Asia, Africa, and Australia. It grows all over the year (no, it is not seasonal.) on plain lands [2]. This plant is used as food for humans and animals. In traditional medicine, the plant is used in cough, sore throat, indigestion, wounds, diabetes, diarrhea, headache, skin diseases, and urolithiasis [3]. The plant is used as a traditional medicine for snakebite [4]. The root is demulcent, diuretic, and useful in strangury (slow and painful discharge of urine). The roots are used in the treatment of headache [5]. The Meena tribals of the Sawai Madhopur district of Rajasthan give orally the juice of the roots to patients suffering from liver congestion, jaundice, biliousness, and dyspepsia. They also give decoction of the whole plant to cure pneumonia, typhoid, and other prolonged fevers [6]. This plant drug has been reported to possess several pharmacological properties such as anti-inflammatory, anti-diabetic, antioxidant, antimicrobial, antidiuretic, anti-HIV, anti-urolithiasis, anti-ulcer, and anti-asthmatic [7].

A. lanata is an annual branching herb with woody root system (Fig. 2). Roots of the plant smell like camphor. It grows in the tropical climate and grows up to 60 cm. It has a bitter taste. Herb: Erect or prostrate with a long taproot, branched from near the base; branches: Many, pubescent or wholly-tomentose, striate. Leaves: Alternate, 2-2×1-1.6 cm on the main stem, 6-10×5-6 mm on the branches, elliptic or obovate, or suborbicular, obtuse or acute, entire, pubescent above, more or less white with hairs beneath; petioles: 3-6 mm long, often obscure. Flowers: Greenish white, very small, sessile, often bisexual, in small dense subsessile axillary heads or spikes 6-13 mm long, often closely crowded forming globose clusters; bracteoles: 1.25 mm, long, membranous, broadly ovate, concave, apiculate. Perianth: 1.5-1.25 mm long; sepals: Oblong, obtuse, sometimes apiculate, silky-hairy on the back. Utricle: Broadly ovoid, acute; stigmas: Two, seed 0.85 mm in diameter, smooth and polished, black. Fruits of this plant are small and egg-shaped. Fruits have black seeds which are bean-shaped [8]. The vernacular names [9] of the plant are Sanskrit - Bhadra; Hindi - Gorkhabundi, Kapurijadi, Chaya; Bengali - Chaya; Telugu - Pindi-Kumda; Tamil - Sirupulai; Malayalam - Cherula; Rajasthani - Bhui; Marathi - Apurmadhura, Kapurimadhuri, Kapurphuti, Kumra; Punjabi - Bui-kaltan.

Medicinal properties of the plant depend on the presence of secondary metabolites. *A. lanata* was reported to contain β-sitosterol, β-sitosteryl palmitate, α-amyrin, tannins, steroids, flavonoids, alkaloids and their glycosides, polysaccharides, saponins, and minerals [10].

METHODS

Collection of the plant material

Roots of *A. lanata* were collected from Mettur Botanical Garden. The plant material was dried in the shade, cut, crushed, and kept in airtight bottle for experimental purpose.

Physicochemical study

Physicochemical study of the sample was carried out for determining various physicochemical parameters such as ash value, acid-insoluble ash, extractable matter in water and alcohol, and loss on drying at 105°C, following the methods mentioned in the standard books [11,12]. The plant material was subjected to successive Soxhlet extractions using hexane, ethyl acetate, and alcohol.

Chromatographic study

Preparation of the extract

Four grams of the plant material was soaked in 40 ml chloroform and kept overnight. The solution was boiled and filtered. The filtrate was concentrated on a water bath to 10 ml. This extract was used for chromatographic studies [13].

HPTLC analysis

The extract was applied as bands on the plate with a Camag microliter syringe attached with Automatic TLC Sampler 4. The TLC plate used was aluminum sheet precoated with silica gel 60 F_{254}. The Camag twin trough chamber was used for developing the plate. Camag visualizer was used for photo documentation. Camag TLC scanner installed with WINCATS software was used for fingerprint development in the ultraviolet (UV) and visible region.

5 µl and 15 µl of the extract were applied on the TLC plate as 10 mm bands in two tracks of 10 mm distance. The plate was developed in the solvent system, toluene: ethyl acetate: formic acid (5:2:4 drops). The developed plate was air-dried and visualized under UV 254 and 366 nm. The TLC chromatograms were documented. Then, the plate was scanned at UV 254 nm. The fingerprint profile was recorded. The plate was then derivatized using vanillin-sulfuric acid reagent. Photographs under white light were taken and scanned at 575 nm. The chromatograms under white light and fingerprint profile at 575 nm were documented [13].

Preliminary phytochemical study

The qualitative chemical tests were carried out by standard methods for the identification of the class of different phytoconstituents present in the single drug [14].

RESULTS

The physicochemical parameters of the root of *A. lanata* are given in Table 1, which are important diagnostic parameters of the plant.

Preliminary phytochemical investigations revealed the presence of steroids, flavonoids, alkaloids, sugar, and tannins.

TLC photo documentation profiles of the chloroform extract of the plant material at 254 nm, 366 nm, and after derivatization under white light are given in Fig. 3. The observed R_f values and their colors are given in Table 2.

HPTLC fingerprinting profiles of the chloroform extract of the root of *A. lanata* were recorded at 254 nm and 575 nm after derivatization

Fig. 1: *Aerva lanata* plant

Fig. 2: *Aerva lanata* root

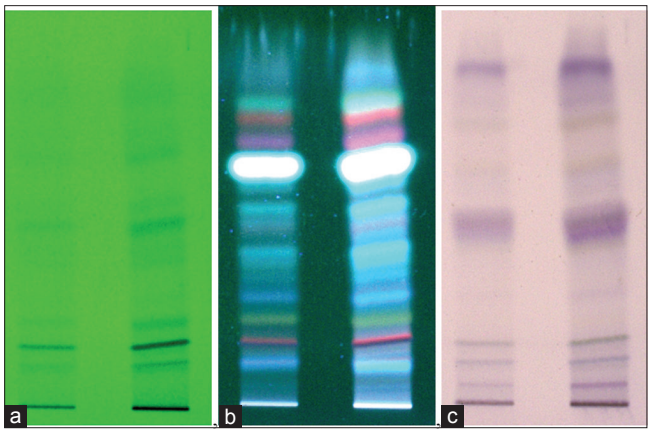


Fig. 3: High-performance thin layer chromatographic profiles of the chloroform extract of *A. lanata* (root). (a) Under ultraviolet (UV) short, (b) under UV long, (c) under white light

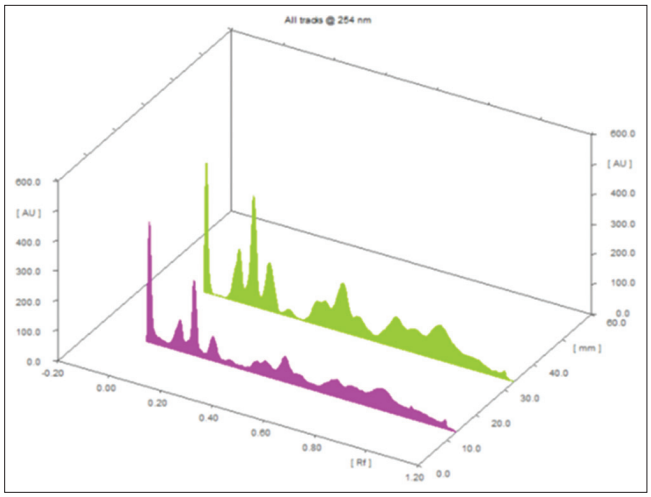


Fig. 4: Three-dimensional densitometric chromatogram of 5 and 15 µl of chloroform extract of *Aerva lanata* at 254 nm

with vanillin-sulfuric acid reagent. The three-dimensional densitometric chromatogram of the chloroform extract of the plant material at 254 nm and 575 nm is given in Figs. 4 and 5, respectively. The HPTLC fingerprinting profile, R_f values, and percentage area of the peaks at 254 nm are given in Fig. 6 and that at 575 nm are given in Fig. 7.

Table 1: Physicochemical parameters of *A. lanata* (root)

S. No.	Parameter	I	II	III	Mean
1.	Foreign matter %	>2	>2	>2	>2
2.	Loss on drying at 105°C %	12.57	11.90	12.83	12.43
3.	Total ash content %	3.98	3.62	4.12	3.91
4.	Acid-insoluble ash %	0.55	0.65	0.72	0.64
5.	Water-soluble extractive %	16.30	16.36	16.12	16.26
6.	Alcohol-soluble extractive %	3.08	2.99	4.13	3.40
7.	Volatile oil %	Nil	Nil	Nil	Nil
8.	Successive extraction %				
	a. Hexane, 0.36, 0.32, 0.52, 0.40				
	b. Ethyl acetate, 0.86, 0.81, 0.88, 0.85				
	c. Alcohol, 2.42, 2.85, 3.01, 2.76				

A. lanata: Aerva lanata

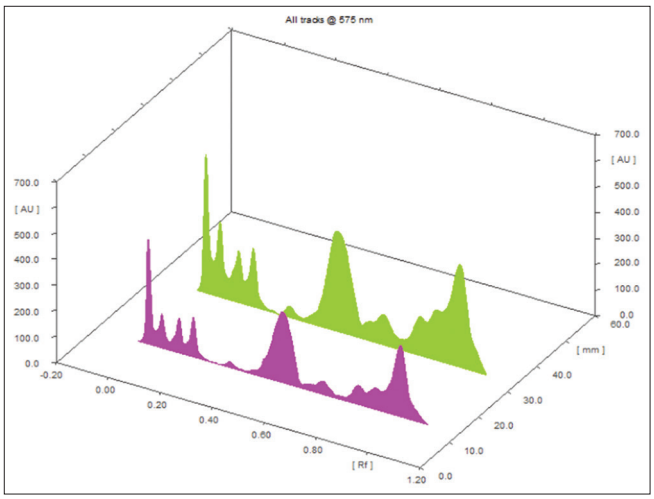


Fig. 5: Three-dimensional densitometric chromatogram of 5 µl and 15 µl of chloroform extract of *Aerva lanata* at 575 nm after derivatization

DISCUSSION

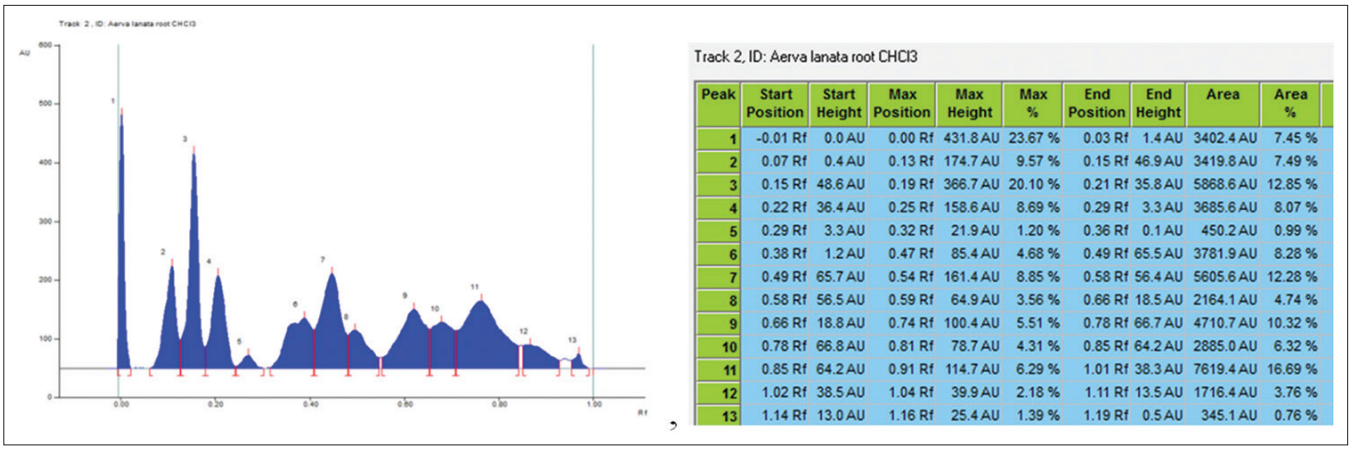
Physicochemical study

The loss on drying of the drug was found to be 12.43%, which is only due to the presence of moisture. The total ash is the total amount of material remaining after ignition. Acid insoluble ash measures the amount of silica present, especially as sand and siliceous earth. Alcohol- and water-soluble extractives determine the amount of active constituents extracted with alcohol and water, respectively, from the drug. The water-soluble extractive was found to be 16.26%; this value indicates the presence of sugar, acids, and inorganic compounds present in the drug. The alcohol-soluble extractive value indicates the presence of polar constituents such as phenols, alkaloids, steroids, glycosides, and flavonoids. The alcohol-soluble extractive was found to be 3.40%, which signifies the presence of constituents which were soluble in alcohol is less. The plant material was subjected to successive Soxhlet extractions. The hexane soluble extractive value was 0.40%. The ethyl acetate-soluble extractive value was determined as 0.85%, whereas the ethanol-soluble extractive is 2.76%.

Table 2: Rf values and color of major bands of chloroform extract of *A. lanata* (root)

Under UV 254 nm		Under UV 366 nm		Under white light after derivatization	
R_f values	Color	R_f values	Color	R_f values	Color
0.13	Green	0.09	Green	0.06	Purple
0.19	Dark green	0.15	Dark blue	0.12	Blue
0.25	Green	0.17	Pink	0.17	Green
0.47	Green	0.21	Greenish yellow	0.48	Purple
0.54	Dark green	0.28	Light blue	0.73	Brown
0.74	Green	0.38	Light blue	0.83	Purple
0.91	Green	0.46	Pink	0.89	Purple
		0.50	Light blue		
		0.62	Fluorescent blue		
		0.67	Pink		
		0.72	Red		
		0.76	Green		

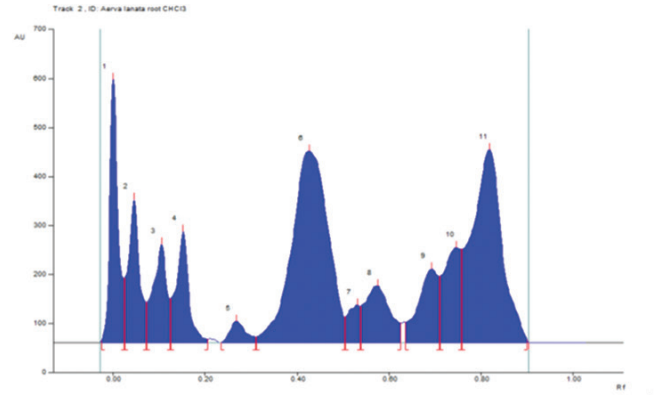
A. lanata: Aerva lanata, UV: Ultraviolet



Track 2, ID: Aerva lanata root CHCl3

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.01 Rf	0.0 AU	0.00 Rf	431.8 AU	23.67 %	0.03 Rf	1.4 AU	3402.4 AU	7.45 %
2	0.07 Rf	0.4 AU	0.13 Rf	174.7 AU	9.57 %	0.15 Rf	46.9 AU	3419.8 AU	7.49 %
3	0.15 Rf	48.6 AU	0.19 Rf	366.7 AU	20.10 %	0.21 Rf	35.8 AU	5868.6 AU	12.85 %
4	0.22 Rf	36.4 AU	0.25 Rf	158.6 AU	8.69 %	0.29 Rf	3.3 AU	3685.6 AU	8.07 %
5	0.29 Rf	3.3 AU	0.32 Rf	21.9 AU	1.20 %	0.36 Rf	0.1 AU	450.2 AU	0.99 %
6	0.38 Rf	1.2 AU	0.47 Rf	85.4 AU	4.68 %	0.49 Rf	65.5 AU	3781.9 AU	8.28 %
7	0.49 Rf	65.7 AU	0.54 Rf	161.4 AU	8.85 %	0.58 Rf	56.4 AU	5605.6 AU	12.28 %
8	0.58 Rf	56.5 AU	0.59 Rf	64.9 AU	3.56 %	0.66 Rf	18.5 AU	2164.1 AU	4.74 %
9	0.66 Rf	18.8 AU	0.74 Rf	100.4 AU	5.51 %	0.78 Rf	66.7 AU	4710.7 AU	10.32 %
10	0.78 Rf	66.8 AU	0.81 Rf	78.7 AU	4.31 %	0.85 Rf	64.2 AU	2885.0 AU	6.32 %
11	0.85 Rf	64.2 AU	0.91 Rf	114.7 AU	6.29 %	1.01 Rf	38.3 AU	7619.4 AU	16.69 %
12	1.02 Rf	38.5 AU	1.04 Rf	39.9 AU	2.18 %	1.11 Rf	13.5 AU	1716.4 AU	3.76 %
13	1.14 Rf	13.0 AU	1.16 Rf	25.4 AU	1.39 %	1.19 Rf	0.5 AU	345.1 AU	0.76 %

Fig. 6: High-performance thin layer chromatographic fingerprint profile and R_f table of 15 µl of chloroform extract of *Aerva lanata* (L.) at 254 nm



Track 2, ID: Aerva lanata root CHCl3

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.03 Rf	4.9 AU	0.00 Rf	536.9 AU	20.48 %	0.03 Rf	31.8 AU	8036.2 AU	8.85 %
2	0.03 Rf	132.2 AU	0.06 Rf	292.7 AU	11.16 %	0.09 Rf	82.1 AU	5928.3 AU	6.53 %
3	0.09 Rf	82.5 AU	0.13 Rf	200.3 AU	7.64 %	0.15 Rf	89.2 AU	4857.5 AU	5.35 %
4	0.15 Rf	89.7 AU	0.18 Rf	227.2 AU	8.67 %	0.25 Rf	7.5 AU	5175.8 AU	5.70 %
5	0.28 Rf	0.3 AU	0.32 Rf	44.1 AU	1.68 %	0.37 Rf	11.8 AU	1252.8 AU	1.38 %
6	0.37 Rf	11.9 AU	0.51 Rf	390.5 AU	14.89 %	0.60 Rf	52.3 AU	25938.2 AU	28.55 %
7	0.61 Rf	52.8 AU	0.64 Rf	77.0 AU	2.94 %	0.64 Rf	73.9 AU	1634.0 AU	1.80 %
8	0.65 Rf	74.2 AU	0.69 Rf	115.9 AU	4.42 %	0.75 Rf	40.3 AU	5276.0 AU	5.81 %
9	0.76 Rf	42.0 AU	0.83 Rf	149.8 AU	5.71 %	0.85 Rf	35.1 AU	5425.3 AU	5.97 %
10	0.85 Rf	135.5 AU	0.89 Rf	193.7 AU	7.39 %	0.91 Rf	90.3 AU	6003.3 AU	6.61 %
11	0.91 Rf	190.6 AU	0.98 Rf	393.7 AU	15.02 %	1.08 Rf	5.2 AU	21316.4 AU	23.46 %

Fig. 7: High-performance thin layer chromatographic fingerprint profile and R_f table of 15 µl of chloroform extract of *Aerva lanata* at 575 nm after derivatization using vanillin-sulfuric acid reagent

Preliminary phytochemical study

Preliminary phytochemical investigations of the material showed the presence of major secondary metabolites which possess the potent therapeutic activity.

Chromatographic study

The solvent system, toluene: ethyl acetate: formic acid (5:2:4 drops) efficiently resolved the components present in the crude extract. TLC pattern showed 7 visible bands under UV at 254 nm, 12 bands at 366 nm, and 7 bands under white light after derivatization.

Out of the 13 peaks observed at 254 nm, seven peaks, and out of the 11 peaks at 575 nm, six peaks were comparatively higher than others, indicating that these chemical constituents are present in significant quantity in the crude extract.

CONCLUSION

The results obtained from the physicochemical parameter determination studies and HPTLC fingerprint images will be helpful in the identification and quality control assessment of the root of *A. lanata*. It can be used as a diagnostic tool to identify and to check the quality and purity of the drug.

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