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BIO-ACTIVITY GUIDED DETERMINATION OF ACTIVE COMPOUNDS IN THE LEAVES OF *Pithecellobium dulce*

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ABSTRACT
The presence of diverse secondary metabolites has been reported from *Pithecellobium* species. However, there has been not much information available on phytochemical components and biological activity in the aqueous extract of *Pithecellobium dulce* leaf. *Pithecellobium* species belonging to the family Leguminosae, is traditionally used for curing different aliments. This study was designed to determine the phyto components in the aqueous extract of *Pithecellobium dulce* leaf. The present investigation was carried out to determine the possible lead phytochemical components from *Pithecellobium dulce* leaf extract by using Gas Chromatography–Mass Spectrometry, while the mass spectra of the compounds found in the extract was matched with the National Institute of Standards and Technology (NIST) library. This investigation was carried out to determine the possible chemical components from *Pithecellobium dulce* leaf. GC-MS analysis of *Pithecellobium dulce* leaf extract revealed the existence of 13-octadecenl,(Z)-cyclopropane octanal, 2-octyl-cis-11-hexadecenal (39.63 %), Diethyl phthalate (18.58), Octadecadienoic acid ethyl ester(9.12%), justifying the use of this plant to treat many ailments in folk and herbal medicine. From the results, it is evident that *Pithecellobium dulce* leaf contains various bioactive compounds and is recommended as a plant of phytopharmaceutical importance.
Keywords: GC-MS analysis, phytocomponents, *Pithecellobium dulce*, whole plant ethanol extract

INTRODUCTION

The term of medicinal plants include a various types of plants used in herbalism and some of these plants have a medicinal activities Herbal medicines are popular remedies for a number of diseases and used by a vast majority of the world's population. Plants are considered as important source of nutrition and as a result of that these plants recommended for their therapeutic values. Phytochemicals derived from plant sources possess a complex of chemicals with unique biological activity. *Pithecellobium* species belonging to the family Leguminosae and the subfamily Mimosoideae are widely distributed in the tropics, chiefly in Asia and America. The bark of the plant is reported to be used as astringent in dysentery, febrifuge and it is also useful in dermatitis and eye inflammation.

The leaves have been reported to possess astringent, emollient, abortifacient and antidiabetic properties. A steroid saponin, lipids, phospholipids, glycosides, glycolipids and polysaccharides have been reported from the seeds[1-3]. Quercetin kaempferol, dulcitol and afzelin have been reported from the leaves[4.] Roots have been reported to possess estrogenic activity[5]. Studies on alkylated resins from seed oil have been reported[6]. Recently, it is reported *P. dulce* possess antiulcer activity.

The fruits of *P. dulce* have been consumed as a dietary supplement for its high nutritive and medicinal value. Non-toxic in nature. The fruit extract was found to be rich in phenolic compounds and revealed the presence of flavonoids– quercitrin, rutin, kaempferol, naringin and daidzein[7].The edible fruit has been widely used traditionally to combat gastric problems and found to be in the literature.Figure-1 shows the leaves of *Pithecellobium dulce.*

The current study is therefore an attempt to determine the determination of Bioactive compounds present in the leaves of *Pithecellobium dulce* by using *GC-MS.*

EXPERIMENTAL

Collection of plant Material

Fresh leaves of the plant, *Pithecellobium dulce* as shown in figure 1was collected locally during the month of February to April from the local areas of Kanchipuram district of Tamilnadu State. The taxonomic identification of the plant material was authenticated by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, Chennai, India. A voucher specimen is maintained in plant anatomy research Centre, Chennai. The plant material was washed thoroughly, initially with tap water and then with distilled water to remove any debris or dust particles and was shad dried. The dried plant material was ground to a fine powder and stored at room temperature in airtight containers until used further.

Preparation of leaf extract

Leaves were washed with water and 50 g of fresh leaves (kept at 25C for 5 days in absence of sunlight) were extracted in 1 litre of boiling water for 2 h and concentrated to half of the volume by boiling in a water bath. The dark-brown extract thus obtained was cooled, filtered using Whatman No. 1 filter paper, and the filtrate was centrifuged at 10,000 rpm at 25° C. The supernatant was concentrated up to 100 ml on rotavapour under reduced pressure. The lyophilized concentrated crude extract was used for the study.

Fig.-1: Leaves of *Pithecellobium dulce.*

Phytochemical analysis

Preliminary phytochemical analysis of the extracts

To assess the chemical composition of the various extracts qualitatively, a preliminary phytochemical analysis was conducted according to the standard methods 27, 28. Using these methods, the presence of several phytochemicals like sterols, tannins, proteins, sugars, alkaloids, flavonoids, saponins, anthraquinones, terpenoids, and cardiac glycosides was evaluated (Table-1).

Test for sterols (Salkowaski reaction)

A few milligram of the plant extract was dissolved in 2 ml chloroform and then 2 ml of conc. H2SO4 was added from the sides of the test tube. The test tube was shaken for a few minutes. Red color development in the chloroform layer indicated the presence of sterols.

Test for tannins (Ferric chloride reagent test)

The test sample of each extract was taken separately in water, warmed and filtered. To a small volume of this filtrate, a few drops of 5 % w/v solution of ferric chloride prepared in 90 % alcohol were added. Appearance of a dark green or deep blue color indicated the presence of tannins.

Test for proteins (Xanthoproteic test)
The extract (few mg) was dissolved in 2 ml water and then 0.5 ml of conc. HNO3 was added in it. Yellow color indicated the presence of proteins.

Test for sugars (Fehling's test for free reducing sugar)
About 0.5 g of each extract was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars.

Test for flavonoids (Ferric chloride test)
About 0.5g of each extract was boiled with 5 ml of distilled water and then filtered. To 2 ml of this filtrate, a few drops of 10% ferric chloride solution were added. A green-blue or violet coloration indicated the presence of a phenolic hydroxyl group.

Test for Saponins
One gram of each extract was boiled with 5 ml of distilled water and filtered. To the filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins.

Test for anthraquinones (Borntranger's test)
An aliquot of 0.5 g of the extract was boiled with 10 ml of sulphuric acid (H2SO4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for color changes.

Test for terpenoids (Salkowski test)
To 0.5 g of each extract, 2 ml of chloroform was added, followed by a further addition of 3ml of concentrated H2SO4 to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

Test for cardiac glycosides (Keller-Killiani test)
To 0.5 g of the extract diluted to 5 ml in water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Determination of Phytocompounds by Gas Chromatography – Mass Spectrum Analysis (GC-MS)
Instrument : GC-MS (SHIMADZU QP2010)
Software : GC-MS solution ver.2.53
GC-MS technique was used in this study to identify the phyto components present in the ethanolic extract of *Pithecellobium dulce* leaf. GC-MS technique was carried out at BVCPS India PVT LTD, Chennai, Tamil Nadu. GC-MS analysis of this extract was performed using GC SHIMADZU QP2010 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column (Length : 30.0m, Diameter : 0.25 mm, Film thickness : 0.25 µm Composed of 100% Dimethyl poly siloxane). For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.51ml/min and an injection volume of 2µl was employed. Injector temperature was 200°C and Ion-source temperature was 200°C. The oven temperature was programmed from 70°C (isothermal for 2 min.), with an increase of 300°C for 10 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 – 1000 m/z. Total GC running time was 35 min. The relative percentage

amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a GC-MS solution ver. 2.53.

RESULTS AND DISCUSSION

Phytochemical analysis of ethanolic leaf extract of *Pithecellobium dulce*

The ethanolic leaf extract of *Pithecellobium dulce* was found to contain major phytochemicals. Preliminary phytochemical analysis of showed the presence of alkaloids, anthraquinones, cardiac glycosides, proteins, tannins, terpenoids, and sugars (Table 1). Saponins were found to be absent in all the tested extract; while flavonoids and sterols were found to be present in the extract. Moreover, secondary metabolites such as tannins and other compounds of phenolic nature are also classified as antimicrobial compounds.

Table-1: Preliminary phytochemical analysis of Ethanolic Leaf extract of *Pithecellobiumdulce*

Phytochemicals tested	Test Performed	Inference
Alkaloids	Wagner's test	+
Anthraquinones	Borntranger's test:	+++
Cardiac glycosides	Keller-Killiani test	_
Flavonoids	Ferric chloride test	++
Proteins	Xanthoproteic test	+++
Tannins	Ferric chloride reagent test	++
Terpenoids	Salkowski test	++
Saponins	Foam test	_
Sterols	Salkowaski test	++
Sugars	Fehling's solution test	++

(+) Presence of Phytoconstituents (-) Absence of Phytoconstituents

The preliminary phytochemical tests revealed that the leaves of the plant possess alkaloids[8], glycosides, flavonoids[9], tannins etc. The flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving the vascularity. Hence any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibers, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis[10].Flavonoids[11] and triterpenoids[12]are also known to promote the wound healing property which seems to be responsible for wound contraction and increased rate of epithelialization.

Tannins the main component of many plant extract acts as free radical scavenger[13]. *Pithecellobium dulce*has many alkaloids such as glaucin and annonaine in different part of the plants. Terpenoids from this plant have anti-HIV principle and anti-platelet aggregation activity [14]. It has been reported that flavonoids from this plant is responsible for antimicrobial and pesticidal activities[15].

The alkaloids reported from this plant belong to different groups such as aporphineand benzoquinazoline which is known for its various medicinal values[16]. As reviewed by Leboeuf *et al.,* (1982) terpenoids, alkaloids from this plant possess antitumour, immunosuppressant, insecticidal antifeedalproperties[17]. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity.

GC-MS analysis of ethanolic leaf extract of *Pithecellobium dulce*

The lead phytocomponents present in the ethanolic leaf extract of *Pithecellobium dulce* was identified by GC-MS analysis. GC-MS chromatogram of ethanolic leaf extract of *Pithecellobium dulce* was shown in Figure-2.

Totally 15 compounds were identified in the ethanolic leaf extract of *Pithecellobium dulce* by GC-MS analysis. The chromatogram obtained by GC-MS analysis was shown in Figure 2. The prevailing compounds were 13-octadecenl,(Z)-cyclopropaneoctanal,2-octyl-cis-11-hexadecenal.,Bicyclo[3.1.1]heptane,2,6,6-trimethyl [1R(1.Alpha.,2.beta.,5.alpha.)],Cyclohexane,1,2,3-

trimeethyl-2-hexadecene,1-mthyl-cis-9,10-epoxy0ctadecan-1-ol.,Phytol, acetate., Hexadecanoic acid, ethyl ester., 2-nonen-1-ol, 2-methyl-., Linoleic acid ethyl ester., Ethyl 9,12,15-octadecatrienoate. The phytochemical composition of ethanolic leaf extract of Pithecellobium dulce with its molecular formula, molecular weight and Peak Area in (%) was shown in Table-2. Table-3 shows the major phytocompounds and its biological activities obtained through the GCMS study of ethanolic leaf extract of *Pithecellobium dulce.*

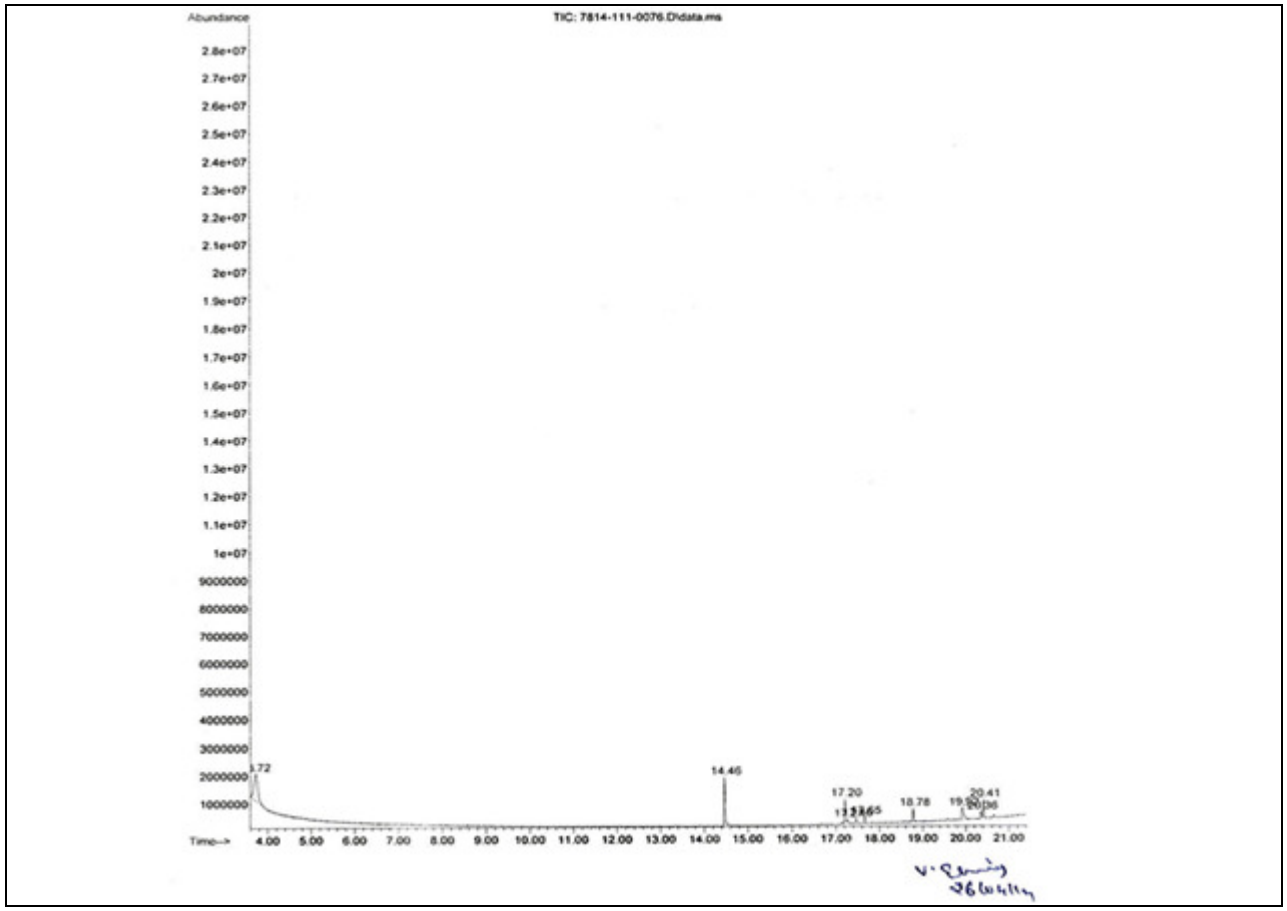

Fig. - 2: GC-MS chromatogram of ethanolic leaf extract of *Pithecellobium dulce*

Table- 2: The phytochemical composition of ethanolic leaf extract of *Pithecellobium dulce* with its molecular formula, molecular weight and Peak Area in (%)

S.No.	RT	Name of the compound	Molecular Formula	Molecular weight	Peak Area (%)
1.	3.720	13 octadecenol	$C_{18}H_{36}O$	266	39.63
2.	3.720	2-octyl-cis-11-headecenal	$C_{16}H_{30}O$	238	39.63
3.	14.466	Diethyl phthalate	$C_{12}H_{14}O_4$	222	18.58
4.	17.208	Bicyclo[3.1.1]heptanes,2,6,6-trimethyl-, (1alpha,2beta,5alpha)-	C_7H_{12}	96	9.59
5.	17.265	Cycloheane	C_6H_{12}	84	1.76
6.	17.265	2-hexadecene, 3,7,11,15-trimethyl-, [R-[R*,R*-(E)]]-	$C_{20}H_{40}$	0	1.76
7.	17.265	Cyclooctane	C_8H_{16}	112	1.76

8.	17.653	Phytol	$C_{20}H_{40}O$	297	3.09
9.	17.653	Acetate camphor (+)-2-bornanone			3.09
10.	18.773	Headecanoic acid	$C_{16}H_{32}O_2$	256	5.07
11.	20.357	Linoleic acid	$C^{18}H_{32}O_2$	280	2.83
12.	20.357	9,12-octadecadienoic acid ethyl ester	$C_{20}H_{36}O_2$		2.83
13.	20.357	9,17-octadecadienal , (Z)-	$C_{18}H_{32}O$	264	2.83
14.	20.414	Ethyl 9,12,15-octadecatrienoate	$C_{20}H_{34}O_2$	306	7.35

Table - 3: Bioactivity of phytocomponents identified in the ethanolic leaf extracts of *Pithecellobium dulce* by GC-MS analysis

S.No.	RT	Name of the compound	Activity
1.	3.720	13 octadecenol (Z)-cyclopropaneoctanal	Not intended for therapeutic purpose
3.	3.720	2-octyl-cis-11-headecenal	Preparation of fatty aldehyde semicarbazone derivatives
4.	14.466	Diethyl phthalate	Used to bind cosmetics and fragrances. plasticizers, detergent bases and aerosol sprays
5.	17.265	cycloheane	Nonpolar solvent, raw material for the industrial production of adipic acid and caprolactam, both of which are intermediates used in the production of nylon.
6.	17.265	2-hexadecene, 3,7,11,15-trimethyl-, [R-[R*,R*-(E)]]- Cyclooctane	Not intended for therapeutic purpose
7.	17.653	Phytol	Manufacture of synthetic forms of vitamin E and vitamin K1
8.	17.653	Acetate camphor (+)-2-bornanone	Its strong scent is believed to be toxic to insects and used as a repellent, ingredient in skincare products.
9.	18.773	Hexadecanoic acid	Used to produce soaps, cosmetics, and release agents, detergents
10.	19.918	Phytol 2-nonen-1-ol, 2 methyl	Not intended for therapeutic purpose
11.	20.357	Linoleic acid	Used in making quick-drying oils - useful in oil paints and varnishes.
12.	20.357	9,12-octadecadienoic acid ethyl ester	Not intended for therapeutic purpose

The mode of action of plants producing therapeutic effects can also be better investigated if the active ingredients are characterized. Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. Higher plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care since ancient times. Over 50% of all modern clinical drugs are of natural product origin and natural products play a vital role in modern drug development in the pharmaceutical industry.

CONCLUSION

It would be worthwhile to further isolate the compounds and determine their specific activity and also to understand the synergistic effect of compounds for therapeutic roles. Thus, this study explores the goodness of the *P. dulce* leaves which has a commendable sense of purpose and can be advised as a plant of phytopharmaceutical importance.

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