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Pharmacognostical, Antibacterial and Antifungal potentials of the leaf extracts of *Pithecellobium dulce* Benth

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ABSTRACT - The hexane, chloroform, alcoholic and total alkaloidal extracts of the leaves of *Pithecellobium dulce* Benth. (Mimosoideae) were studied for their antimycobacterial activity by L J (Lowenstein –Jensen) method. The activity was compared with primary standard drug *Rifamycin*. The antifungal activity was also studied by screening against *Candida albicans* and *Aspergillus niger* by disc diffusion method. The antifungal activity was compared with standard drugs *Fluconazole* and *Nystatin* at the concentration of 10µg /ml. The alcoholic and total alkaloidal extracts showed activity against C*andida albicans* at the concentration of 6.25µg/disc, 12.5µg/disc & 25µg/disc. The hexane, chloroform extracts were found to be inactive. On contrary, all the extracts of *P.dulce* were found to be inactive against *Aspergillus niger*. The Antimicrobial susceptibility testing revealed that *Mycobacterium tuberculosis* is susceptible to the alcoholic & alkaloidal extracts of *P. dulce.* The alkaloidal extract of *P. dulce* showed high susceptibility against *Mycobacterium tuberculosis* at the concentration of 1mg/mL. While the alcohol extract showed activity at 2mg/mL. The concentration of primary standarad drug Rifamycin was 2.5µg/mL.Anatomical, histochemical &chromatographic studies were also carried out to fulfill the botanical quality control standards. The results of the investigation are discussed in detail.

KEY WORDS - *Pithecellobium dulce,* Anatomical, Histochemical and chromatographic studies, *Mycobacterium tuberculosis, Candida albicans, Aspergillus niger*, LJ method.

INTRODUCTION

Pithecellobium dulce Benth. (Leguminosae : *Mimosoideae*) is a small to medium sized, evergreen, spiny tree up to 18m height, native of tropical America and cultivated throughout the plains of India and in the Andamans. It is known as *Vilayatibabul* in Hindi and *Kodukkapuli* in Tamil. (23). 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 (2). The secondary plant metabolites such as saponins and flavanoids from the plant showed analgesic and anti-inflammatory activities (15). The leaves have been reported to possess emollient, abortifiacient, anti-diabetic, anti-leprotic, anti-convulsant properties (7). Steroid, saponin, lipids, phospholipids, glycosides, glycolipids and polysacchrides have been reported from the seeds (11, 12&13).The bark contains 37% of tannins of catechol type. Flavanoids such as quericitin, kaempferol, and afezilin along with dulcitol have been reported from the leaves (1&25). The aqueous and ethanolic extracts of seeds and harvested leaves of *Pithecellobium dulce* were shown to have anti- fungal activity against *Rhizopus stolonifer* and *Pencillium digitatum* (5). The present study attempts to bring out the hitherto unearthed antifungal and antitubercular potentials of the plant coupled with the botanical protocol of the plant.

MATERIALS AND METHODS

Anatomical& Histochemical studies*:* The plant specimens for the proposed study were collected from the forest conservation area, Tamil Nadu Housing Board, Chennai, India. Care was taken to select healthy plants and normal organs. The required samples of leaves were cut and removed from the plant and fixed in FAA for 24 hours. After fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol as per schedule given by Sass, 1940.The paraffin embedded specimens were sectioned using rotary microtome; the sections were stained with Toludine blue (14). Paradermal sections were studied for the stomatal morphology and venation pattern. Sections were stained with safranin for studying the vein termination and vein islet (16). Glycerin mounted temporary preparations were made for macerated/cleared material. Photographs of different magnifications were taken using Nikon lab photo 2- microscopic unit. Histochemical analyses were

also studied with the paraffin embedded sections as well as hand cut sections of the fresh materials (10).

Phytochemical studies*:* The leaves of the plant were collected, air-dried, powdered and passed through a mesh No: 85 sieve. The sieved powder was stored in an airtight container away from sunlight at room temperature and used for study.

Successive solvent extraction*:* The shade- dried and coarsely powdered leaves of the plant material (20g) was extracted with hexane, chloroform and alcohol successively in a soxhlet apparatus.

Extraction of Total alkaloids (6): The shade- dried and coarsely powdered leaves of the plant material (1kg), was soaked in2% aq. acetic acid at room temperature for 48 hours. The contents of aspirator bottle were filtered in a cloth and then filtered through a whatmann filter paper no: 41.The filtrate was cooled in ice and basified with aqueous ammonia (Sp.gr:0. 9) to PH 10.The pale brown precipitated alkaloidal mixture was extracted with chloroform (4x 500mL). The combined chloroform extracts were washed with little water and then dried over anhydrous sodium sulphate. It was finally distilled on a water bath to about 10mL and then dried in vacuum. Preliminary phytochemical Standardization parameters such as ash value, extractive value, loss on drying etc were carried out to identify the class of compounds present.

Chromatographic analyses: HPTLC and GC-MS analyses were carried out for the different extracts for finger printing purpose. However, since the hexane extract was found to be inactive. no chromatographic analyses were carried out. The HPTLC chromatograms of chloroform and alcohol extracts have already been reported by us (18). Here we report the HPTLC& GC-MS analyses of the total alkaloidal extract.

GC-MS analyses: GC-MS was run on shimadazu 17A/QP5000 model.Column material: Methyl silicone. Column dimensions: 30mx0.3mm.dia. Inj.Temp:150°C. Temp.Program: 70° C -240° C 5 deg/min. Carrier gas and flow rate: Helium – 1mL/min.

Antitubercular screening: LJ (Lowenstein – Jenson) Method.

Antitubercular activity against *Mycobacterium tuberculosis* was studied with tuberculosis infected patients. The positive sputum was collected and confirmed for the presence of *Mycobacterium tuberculli* bacilli by AFB (Zeihl –Neelson) method (21). The alcoholic and alkaloidal extracts of leaf of *P.dulce* were taken at a concentration of 2mg/mL, 1mg/mL & 0.5mg/mL. The drug extracts were incorporated in the LJ medium. The positive specimen was coated on the L J medium. The control and drug vials of the LJ medium were incubated for 2-4 weeks (28 days) at 37°C. The reference control (*Rifampicin* 10µg /mL) was also incubated for 2-4 weeks. The observations were made on 7th, 15th and 30 th day after the incubation.

Antifungal screening –Candida albicans **and** ***Aspergillus niger***

Antifungal activity (3, 9, 17&20) against the *Candida albicans* and *Aspergillus niger* was carried by disc diffusion assay (19). The extracts (hexane, chloroform, alcoholic and total alkaloidal) were taken at concentrations of 25mg/mL, 12.5mg/mL &6.25mg/mL. The antifungal- activities of the extracts were tested by disc diffusion technique using Sabouraud dextrose agar medium against *Candida albicans* and *Aspergillus niger*. *Fluconazole* and *Nystatin* were used as a standard at a concentration of 10µg/mL.

Susceptibility testing:

The respective concentration disc of the extracts of *P.dulce* was prepared by dissolving the extracts in dimethyl formamide. *Fluconazole* and *Nystatin* discs were supplied by Hi-media laboratories, Mumbai. The SD agar plates were incubated at room temperature for 48 hrs. The diameter zone size inhibition (Kirby baur technique) was read after 48 hrs of incubation.

RESULTS

Anatomical and Histochemical studies

Anatomy of the leaflet: The lamina of the leaflet is150 µm thick, dorsiventral, mesomorphic with even and smooth surfaces. The adaxial epidermis is fairly thick with tabular cells and cuticularised walls.The abaxial epidermis has hemispherical outer tangential walls. The mesophyll has palisade cell layer measuring 70µm wide and spongy mesophyll tissue has 4 or5 layers of loosely arranged, aerenchymatous spherical cells (Fig .1). The midrib of the leaflet is spindle shaped in transectional view, projecting equally on adaxial and abaxial sides. The palisade tissue forms horizontally transcurrent zone above the vascular strand The midrib has a prominent arc of collateral vascular bundle surrounded by thick sclerotic bundle sheath (Fig.2-3). The lamina exhibits distinct vein islets formed by thick boundaries. The islets vary in shape and size. Vein terminations are invariably present in all islets. The terminations are repeatedly branched forming dendroid configuration (Fig.8). Stomata are predominantly *paracytic* with two subsidiary cells. The anticlinal walls of epidermal cells are thin and wavy (Fig 9). Calcium oxalate crystals are fairly abduant along the veins of the lamina and around sclerenchyma sheath of the petiole and

petiolule. The crystals are different morphological categories of prismatic type (fig.7). The petiolule (stalk of the leaf let) is circular with two thick lateral wings, which are directed adaxially. The vascular tissue comprises of a wide abaxial bowl shaped strand and adaxial plate of flat strand. A thick sclerenchymatic sheath surrounds the vascular strands. There is a lateral accessory vascular strand one on each wing, which also has sclerotic bundle sheath (fig 4). The rachis (petiole) is circular in sectional view. It has homogenous parenchymatous ground tissue. The vascular tissues occur in an abaxial system of bowl shaped main strand (fig 5). A thin, internodal part of the stem was studied. It exhibits frequent lenticels, intact epidermis and cortex. The vascular cylinder has prominent boundary of cortical (pericyclic) fibres followed by thin, continuous zone of secondary phloem and dense cylinder of secondary xylem. The vessels are wide, thin- walled and diffusely distributed. The pith is wide and parenchymatous xylem rays are prominent (fig 6).

***Histochemical studies**:* The results of localization of alkaloids, proteins, phenols, lipids, starch and tannins in different tissues of the leaflet, petiolule, petiole and stem are shown in the figures 10-13 tabulated in the Table. No: 1.

***Analytical standards of the leaves of Pithecellobium dulce**:* Ash value –10.54%, Watersoluble ash-5.35%, Acid insoluble ash –1.20%, Loss on drying –10.85% at 110° C. The extractive values of N-Hexane-2.0%, Benzene-0.84%, Chloroform-0.90% and Methanol –7.20% respectively. Solubility (B.P.method) on the dried leaves of *P. dulce* revealed that it has more water solubility (21.67%) compared to alcohol (7.5%). The results of the phytochemical qualitative analyses of various extracts have already been reported (18).

HPTLC analyses: The HPTLC chromatograms of the total alkaloidal mixture with developing system of Chloroform: Methanol: Acetic acid (45:4:1) respectively. The total alkaloidal extracts showed 9 peaks (chromatogram no: 1).

GC-MS analyses: GC-MS showed six major compounds, (2.71,19.16,14.82,16.47,13.8,17.73). The GC-MS chromatogram and the mass spectrum of the major individual compounds are identified.

Anti mycobacterial screening (LJ method):

The sputum samples were collected from the TB- HIV infected persons. Sputum sample positive for *Mycobacterium tuberculli* complex was identified by AFB staining *(Zehil-Neelson method*). The sample was coated on the LJ medium with the aid of sterile swab and incubated at 37°C for 2-4 weeks (30 days). Medium with out any inhibitory substance was used as a control. The colonies were been observed on 7th, 15th and 30th days. Observation studies revealed that there were no growths of *M.tb* colonies on positive control *Rifampicin* (10µg/mL). There was positive growth inhibition in the LJ medium (Drug – *P. dulce*- alcoholic extracts incorporated) 2mg/mL. Total alkaloidal extract of *P. dulce* showed a positive inhibition against *M.TB* colonies at a concentration of 1mg/mL in drug-incorporated medium. (Table.No: 2)

Antifungal activity: (Disc diffusion method)

Sterile discs punched out of whatmann No: 1 filter paper containing 25µg/disc, 12.5µg/disc, 62.5µg/disc were overlaid on lawn cultures of *Candida albicans* and *Aspergillus niger* on sauboraud's dextrose agar medium. Then the plates were inoculated for 48 hours at room temperature. The diameter of the zone of inhibition was measured. Susceptibility discs of *Nystatin* and *fluconazole* by Himedia was used as a positive control. Discs containing alcoholic extract and total alkaloidal mixture of *P. dulce* (6.25µg, 125µg & 250µg) showed inhibition zone similar to that of the positive control disc. Studies with *Aspergillus niger* showed no marked zone of inhibition, but the intensity of growth is very minimal around the discs.

DISCUSSION

Macroscopic as well as microscopic studies of any phyto drug are the primary steps to establish its botanical quality control before going to other studies. As per WHO norms, botanical standards are to be proposed as a protocol for the diagnosis of the herbal drug. *P. dulce* is characterized by certain specific anatomical features of the lamina, petiole, petiolule and stem. The stomatal type, venation pattern and crystal habits were also found to be characteristic for the plant.These microscopic parameters are proposed as protocol for the botanical standardization of *P. dulce*. Histochemical localization of certain important compounds enables to get a preliminary idea of type of compounds and their accumulation in the plant tissues. Based upon this study, one can choose the organ or tissue where the required compounds are located. In the present study, alkaloids are found to be located more in the stem tissues than in the leaves. Tannins were found to be more in leaf tissues. Tannins being important astringent compound, the leaf tissues were given importance for bioactive studies. Protiens were found in all most all tissues of the leaf. Proteins being basic units of enzymes seem to play a vital role in

Table.No. 1 - Histochemical Analysis

Compound	*Reagents employed*	*Tissues localized*	*Figures*
Alkaloids	Dragendroff's reagent & Mayer's reagent	Phloem parenchyma of the vascular bundles, Xylem ray cells of the stem, Phloem parenchyma of the petiolule, Hypodermis of the petiole& parenchymatous cells of bundle sheath.	10
Protiens	Coumassin blue & Ninhydrin reagent	Parenchymatous cells ,palisade cells of the vascular bundle & epidermis of the stem.	11
Phenols	Vanillin reagent	Radial cells of xylem ray the stem	-
Lipids	Sudan black	Petiole wall	12
Starch	Lugol's Solution (Iodine+Potassium iodide)	It is absent in the entire leaf- Petiole,Petiolule &stem.	-
Tannins	Ferric chloride solution	Phloem parenchyma of the Petiolule,midrib , palisade and mesophyll tissue.	13

Table No. 2- Susceptibility of Mycobacterium tuberculosis of alcohol and alkaloidal extracts of Pithecellobium dulce by LJ method.

Drug	*7th day*	*15th day*	*28th day*
Solvent control	+++	+++	+++
Rifampicin 10µg/mL	No growth	No growth	No growth
ALC- 1-0.5mg/mL	+++	+++	+++
ALC- 2- 1mg/mL	++	++	++
ALC- 3 -2mg/mL	+	+	+
ALK- 1 -0.5mg/mL	+++	+++	+++
ALK- 2 -1mg/mL	No growth	No growth	No growth
ALK- 3 - 2mg/mL	No growth	No growth	No growth

+++: Intensive growth of M.TB complex (Media color changes from sky blue to parrot green)
++: Growth of M.TB complex (Media changes from sky blue to pale blue)
+: Few traces of M.TB complex (slightly change in color) No Growth: No change in color.

pharmacological activities. Preliminary phyytochemical studies are complementary to microscopic studies. These studies are not only diagnostic potentials, but also offer an insight in to the biochemical constituents. HPTLC analyses of the various extracts are useful as fingerprints in identifying the phyto constituents of the plant material. In the present investigation, results of HPTLC and GC-MS studies of the total alkaloidal mixture were analysed. The former showed 9 peaks, while the latter, 6 major components. The antimycobacterial studies (LJ method) revealed that the alcoholic extract showed moderate inhibition comparable to total alkaloidal mixture of *P.dulce* .The alkaloidal mixture of *P. dulce* has good inhibitory action against *Mycobacterium tuberculosis* complex in LJ Method at the concentration 1mg/mL and *Candida albicans*, an endogenous fungus that cause lateral respiratory infection along with tuberculosis (6.25µg/ disc).*P. dulce,* a common tropical tree has been enjoying the folklore claim as a potential source of drug against tuberculosis. The present findings fill up the lacuna in scientific authentification of the plant for its antitubercular activity

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