IN VITRO CYTOTOXICITY, PHYTOCHEMISTRY AND GC-MS ANALYSIS OF PITHECELLOBIUM DULCE LEAVES.

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ABSTRACT
The objective of the study was to investigate the preliminary phytochemical screening, GC-MS and In vitro cytotoxicity assay of leaf extract of *Pithecellobium dulce*. Phytochemical screening revealed the presence of active plant metabolites like alkaloids, flavonoids, glycosides, phenol, tannins, diterpenes, saponins and phytosterols. It was further continued to identify 18 different compounds of medicinal use in the *Pithecellobium dulce* by GC-MS analysis. The study also investigated about the In vitro cytotoxic nature of *Pithecellobium dulce* leaves in breast cancer cell line (MCF-7) using MTT assay. IC50 value was found to be 112µg/ml. 100% cell inhibition occurred at 300 µg/ml.

KEYWORDS: *Pithecellobium dulce*, MCF-7 cell line, GC-MS analysis, Cytotoxicity, MTT assay.

INTRODUCTION
In recent years, the application of plants shows more importance towards treatment of diseases and other illness. According to World Health Organisations (WHO), an estimate of 85 – 90% of world’s population consumes traditional medicines. The medicinal plants are used medicinally in different countries and they are the source of potent and powerful drugs for many rare and common diseases.¹[1]

*Pithecellobium dulce* is one of the familiar species among them, commonly referred as manila tamarind, as its sour taste resembles tamarind. The generic name is derived from the...
Greek word ‘pithekos’ meaning an ape and lobos referring to a pod and the species name ‘dulce’ in Latin means sweet. The vernacular name of this plant known as in Hindi: vilayati babul; Tamil: Kodukkapuli; Kannada: Kottampuli, Seemae Hunase; Bengali: Dekhani Babul; Marathi: Vilayati hinch; Telugu: Simachinta and Malayalam: Korukkapuli etc.\textsuperscript{[2]}

*Pithecellobium* species belonging to the family Leguminosae and the subfamily Mimosoideae are widely distributed in the tropics, chiefly in Asia and America. *Pithecellobium* dulce being one of the familiar species among them, is a small to medium-sized, evergreen spiny tree upto 18m height. The plant is reported to be a folk remedy for earache, leprosy, peptic ulcer and toothache. It also acts as emollient, anodyne and larvicidae in folk medicine. Infusions of different parts have been used traditionally to treat diseases, such as skin of the stem for dysentery, leaves for intestinal disorders, and seeds for ulcer, among others. Leaves can also be used as a plaster to allay pain from veneral sores, and can also relieve convulsions.\textsuperscript{[3, 4, 5]} Chemical investigations of the different parts of the plant have resulted in the isolation of a few novel and interesting metabolites, some of which have been screened for bioactivity.\textsuperscript{[6, 7, 8]}

**MATERIALS AND METHODS**

**Collection and preparation of plant material**

The leaves of *Pithecellobium dulce* was collected from areas of Pappampatti pirivu, Coimbatore. The leaves were authenticated by the Head, Botanical Survey of India, Coimbatore. The authentication number is BSI/SRC/5/23/2013-14/Tech/1748. The leaves were shade dried at room temperature and protected from direct sunlight. The dried leaves were ground into fine powder using mixer grinder. The leaf powder was then used for the study.

**Preparation of plant extract**

Five different solvent extracts were prepared by soaking 25g of powdered leaf sample in 250 ml of ethanol, methanol, water, chloroform and petroleum ether separately and agitated manually, and allowed to extract for 48hours. Extracts were then filtered using Whatman No.1 Filter paper and the filtrates were evaporated. The extracts were stored at 4°C until further processing.
Phytochemical screening

Preliminary phytochemical screening was conducted in 5 solvents according to standard methods. Presence of metabolites like alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenols, tannins, flavonoids, aminoacids and diterpenes were evaluated.\[9, 10, 11, 12]\n
Detection of alkaloids

Mayer’s test: 1.36g of mercuric chloride was dissolved in 60ml of distilled water and 5g of potassium iodide was dissolved in 10ml of distilled water. The two solutions were mixed and diluted to 100ml with distilled water. To 1.0ml of extract, few drops of above prepared reagent was added. Formation of white or pale precipitate showed the presence of alkaloids.

Detection of carbohydrates

Molisch’s Test: To 3ml of extract, 2 drops of freshly prepared 20% alcoholic solution of alpha napthol was added and mixed. To this solution, 2ml of concentrated sulphuric acid was added, so as to form a layer below the mixture. Formation of reddish violet colour ring at the junction of the solution and its disappearance on addition of excess solution indicated the presence of carbohydrates.

Fehling’s test: To 2ml of extract, 1ml of equal parts of Fehling solution A and B was added. The contents were boiled for few minutes. Formation of red or brick red precipitate indicated the presence of carbohydrates.

Detection of glycosides

To 2ml of the extract, added 4 drops of chloroform, 2 drops of concentrated sulphuric acid at the side of the test tubes. Then development of a brownish ring at the interface of the two liquids and appearance of violet colour in the supernatant layer indicated the presence of glycosides.

Detection of saponins

Foam Test : 0.5g of leaf extract was shaken with 2ml of water. Persistence of foam for about ten minutes indicated the presence of saponins.

Detection of phytosterols: Salkowski’s Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of concentrated sulphuric acid, shaken and
allowed to stand for few minutes. Appearance of golden yellow colour indicated the presence of phytosterols.

**Detection of phenols**
Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

**Detection of tannins**
About 1-2 ml of the extract was taken. A few drops of 5% ferric chloride was added and observed for brownish green or blue black coloration.

**Detection of flavonoids**
Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicated the presence of flavonoids.

**Detection of amino acids**
Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicated the presence of aminoacids.

**Detection of diterpenes**
Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicated the presence of diterpenes.

**Gas Chromatography-Mass Spectrometry Analysis**
The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the metabolic extract of leaf was performed using a clarus 500 Perkin Elmer gas chromatography equipped with a Elite-5 capillary column. Elite wax (Polyethylene glycol) was the polar column used in the estimation. An insert gas such as Hydrogen or Nitrogen or Helium was used as a carrier gas at a flow rate 1ml/min, split 10:1. The test sample was evaporated in the injection part of the GC equipment and segregated in the column by adsorption and desorption technique with suitable temperature programmes which is controlled by software. Different components are eluted from based on the boiling point of the individual components. 

The GC column is heated in the oven between 110°C to 280 °C. The time at which each component eluted from the GC column is termed as retention time (RT). The total GC running time is 36 min. The eluted component is detected in the mass detector. The spectrum
of the known components stored in the NIST library ascertains the name, molecular weight and structure of the components of the test material in GC-MS study. Identification of components was based on comparison of their mass spectra with NIST Libraries and as well as on comparison of their retention indices with literature.[17, 18, 19, 20, 21]

**In Vitro Cytotoxicity Assay**

About 5g of powdered material of plant was taken in a clean, flat –bottomed glass container and soaked in 25ml of 80% methanol. The container with its content was kept for a period of 4-7 days accompanying occasional stirring. The whole mixture was then underwent a coarse filtration through whatman filter paper. The filtrate obtained from the plant was evaporated under ceiling fan and in a water bath until dried. Then the extract was scrapped from the container was used for the analysis. In vitro cytotoxicity assay was carried out on MCF-7 (breast cancer) cell line.

The human breast cancer cell line (MCF 7) was grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted by tryphan blue exclusion assay using a hemocytometer. The cell suspension was diluted with medium containing 5% FBS to give final density of 1x10^5 cells/ml. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following this, the plates were incubated for an additional 48 h at 37°C, 5% CO2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations. [22, 23, 24]
3-[(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula. [25, 26]

\[
\text{% Cell Inhibition} = 100 - \frac{\text{Abs (sample)}}{\text{Abs (control)}} \times 100.
\]

**RESULTS AND DISCUSSION**

**Table 1: Phytochemical screening**

<table>
<thead>
<tr>
<th>Test</th>
<th>Aqueous</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Petroleum Ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amino acids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+- indicates presence, - indicates absence)

The phytochemical screening of *P. dulce* extracts indicated the presence of carbohydrates, glycosides, saponins, phytosterols, phenols, tannins, flavonoids, aminoacids and Diterpenes. Methanolic extract gave positive result for most of the compounds compared to other extracts. Aqueous extract gave positive result for alkaloids, saponins, phenols, tannins, flavanoids, aminoacids and terpenes. Chloroform and petroleum ether extracts gave positive result for only few compounds.
GC-MS ANALYSIS

Figure 1: Chromatogram of *Pithecellobium dulce*

The GC-MS combines the gas chromatography and mass spectrometry techniques. The result carried out by this technique revealed that, the each peak represents the different compounds present in the methanolic extract of plant. The compounds are separated according to its RT value. Following table gives the list of compounds identified by GC-MS study.

Table 2: List of compounds present in *P. dulce* plant carried out by GC-MS analysis:

<table>
<thead>
<tr>
<th>S.No</th>
<th>RT</th>
<th>Name of the Compound</th>
<th>Formula</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.637</td>
<td>4h-pyran-4-one 3-hydroxy-2-methyl-4H-pyran-4-one</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>126.11</td>
</tr>
<tr>
<td>2</td>
<td>9.757</td>
<td>2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>144.12</td>
</tr>
<tr>
<td>3</td>
<td>10.455</td>
<td>Azulene</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;</td>
<td>128.17</td>
</tr>
<tr>
<td>4</td>
<td>14.211</td>
<td>Methyleneugenol</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>178.22</td>
</tr>
<tr>
<td>5</td>
<td>15.268</td>
<td>Cedranone 5-</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O</td>
<td>220.35</td>
</tr>
<tr>
<td>6</td>
<td>15.666</td>
<td>1,2,4-Trimethoxybenzene</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>168.18</td>
</tr>
<tr>
<td>7</td>
<td>16.325</td>
<td>1 2 4- trimethylbenzene</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;</td>
<td>120.19</td>
</tr>
<tr>
<td>8</td>
<td>16.916</td>
<td>1 2 4- trimethylbenzene</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;</td>
<td>120.19</td>
</tr>
<tr>
<td>9</td>
<td>17.743</td>
<td>Tetradecanoic acid</td>
<td>C&lt;sub&gt;14&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>228.37</td>
</tr>
<tr>
<td>10</td>
<td>17.862</td>
<td>Dehydroxy-isocalamendiol</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O</td>
<td>220.35</td>
</tr>
<tr>
<td>11</td>
<td>19.571</td>
<td>3-methyl-3-(4-methylpent-3-en-1-yl)oxirane-2-carboxylic acid</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>184.23</td>
</tr>
<tr>
<td>12</td>
<td>19.635</td>
<td>Naphthalene, 1,2,3,4-tetrachloro-</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;Cl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>265.95</td>
</tr>
<tr>
<td>13</td>
<td>19.736</td>
<td>Methyl ester of 3-hydroxydecanoic acid</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>202.90</td>
</tr>
<tr>
<td>14</td>
<td>19.866</td>
<td>L-Ascorbyl 6-palmitate</td>
<td>C&lt;sub&gt;22&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O&lt;sub&gt;7&lt;/sub&gt;</td>
<td>414.53</td>
</tr>
<tr>
<td>15</td>
<td>21.141</td>
<td>5,9,12-octadecatrienoic acid</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>278.42</td>
</tr>
<tr>
<td>16</td>
<td>21.563</td>
<td>5,9,12-octadecatrienoic acid</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>278.42</td>
</tr>
<tr>
<td>17</td>
<td>23.571</td>
<td>3-Phenyl-2-propenoic acid methyl ester</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>162.18</td>
</tr>
<tr>
<td>18</td>
<td>26.492</td>
<td>Isoamylcinnamate</td>
<td>C&lt;sub&gt;14&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>218 29</td>
</tr>
</tbody>
</table>
The cytotoxicity assay was carried out in MCF-7 (Breast cancer) cell line with five different concentration of plant extract (methanol). The figure 3 shows that in 18.5 µg concentration the cancer cells was not affected and was seen viable, in figure 4, 37.5 µg concentration only few cells was dead, as the concentration increases the cell viability decreases. Finally at 300 µg concentration 100% cell inhibition was occurred.
CONCLUSION
This study is concluded by the findings that the *Pithecellobium dulce* leaves showed the presence of phytochemicals like carbohydrates, saponins, phytosterols, tannins, glycosides, diterpenes, flavonoids and aminoacids. GC-MS analysis showed the presence of 18 different compounds. Also cytotoxicity assay of *Pithecellobium dulce* leaves against proliferation of MCF-7 breast cancer cell line confirmed that the plant has anti-cancer activity. As the concentration of plant extract was increased, the cell viability decreased. IC50 value was found to be 112.5%. So, *Pithecellobium dulce* is a medicinal plant with anti-cancer activity against breast cancer.

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REFERENCES


