PHYTOCHEMISTRY, GC-MS ANALYSIS AND INVITRO CYTOTOXIC ACTIVITY OF PRUNUS ANGUSTIFOLIA LEAVES AGAINST MCF-7 BREAST CANCER CELL LINE

1 Poongodi T., 2Srikanth R. and 3Lalitha G.

1Assistant Professor, Department of Biochemistry, Rathnavel Subramaniam College of Arts and Science, Coimbatore-641 402, Tamil Nadu, India.

2Research Scholar, Department of Biochemistry, Rathnavel Subramaniam College of Arts and Science, Coimbatore-641 402, Tamil Nadu, India.

3Assistant Professor, Department of Biochemistry, Rathnavel Subramaniam College of Arts and Science, Coimbatore-641 402, Tamil Nadu, India.

ABSTRACT

The current investigation was carried out to study the preliminary phytochemical screening, GC-MS and In vitro cytotoxicity assay of leaf extract of Prunus angustifolia. Phytochemical screening revealed the presence of active plant metabolites like alkaloids, phenol, tannins, glycosides, saponins and diterpenes. It was further continued to identify 8 different compounds of medicinal use in the Prunus angustifolia by GC-MS analysis. The study also investigated about the Invitro cytotoxic nature of Prunus angustifolia leaves in breast cancer cell line (MCF-7) using MTT assay. IC50 value was found to be 99.97µg/ml. 100% cell inhibition occurred at 300 µg/ml.

KEYWORDS: Prunus angustifolia, MCF-7 cell line, GC-MS analysis, Cytotoxicity, MTT assay.

INTRODUCTION

Prunus species have been found to be antipyretic, leucodermatic and effective against leprosy. According to World Health Organisation (WHO), an estimate of 85% – 90% of world’s population consumes traditional medicines. The medicinal plants are used in different countries and they are the source of potent and powerful drugs for many rare and common...
Prunus angustifolia is a colonizing shrub that forms dense thickets, ideal nesting cover for quail and turkeys. This native tree produces fragrant white flowers in early spring followed by lance-shaped, shiny, dark green leaves. Juicy, 1/2" round, yellow to red drupes ripen in late summer to be enjoyed by both humans and wildlife. Prunus angustifolia pronounced, known commonly as Chickasaw Plum, Cherokee plum, Florida sand plum, sandhill plum, or sand plum, is a plum bearing tree native to North America. 

The genus Prunus includes about 430 species of deciduous or evergreen trees and shrubs naturally widespread throughout the temperate regions. It belongs to the Rosaceae family as a subfamily, the Prunoideae. While some species do not yield edible fruits and are used for decoration, others are grown commercially for fruit and "nut" production. The fruit of these species is botanically defined as a drupe.

Studies of the volatile composition of fresh plums found that the esters are qualitatively and quantitatively the most important class of compounds. Other major volatile constituents of fresh plums are alcohols or carbonyl compounds depending on the cultivar and on the methodology used for the extraction of the volatile compounds. Nonanal, 1-hexanol, (Z)-3-hexenol, linalool, benzaldehyde, γ-octalactone, and γ-decalactone were considered as important contributors to the aroma of fresh plums. These phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties including anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral and anti-bacterial activities.

The present investigation was carried out to determine the phytochemistry, invitro cytotoxicity study in breast cancer cell line and to identify the chemical components by GC-MS analysis.

**MATERIALS AND METHODS**

**Collection and preparation of plant material**

Prunus angustifolia plants were collected from in and around Ooty, The Nilgris, Tamil Nadu, India. Then washed, shade dried and powdered. The plant was authenticated as Prunus angustifolia by Botanical Survey of India in Tamil Nadu Agriculture University (TNAU), Coimbatore. The authentication number is BSI/SRC/5/23/2013-14/1746. The leaves were shade dried at room temperature and protected from direct sunlight. The dried leaves were ground into fine powder using mixer grinder, which was then used for the study.
**Preparation of plant extract:** Five different solvent extracts were prepared by soaking 25 g of powdered leaf sample in 250 ml of ethanol, methanol, water, chloroform and petroleum ether separately and agitated manually, and allowed to extract for 48 hours. 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 using the above-mentioned solvents individually according to the standard methods. Presence of metabolites like alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenols, tannins, flavonoids, aminoacids and diterpenes were evaluated.

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

Detection of carbohydrates: Molisch’s Test: To 3 ml of extract, 2 drops of freshly prepared 20% alcoholic solution of alpha napthol was added and mixed. To this solution, 2 ml 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.

Detection of glycosides: To 2 ml 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: About 0.5 g of leaf extract was shaken with 2 ml 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 a 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 to 4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

Detection of tannins
About 1 to 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 to 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 inert gas such as Hydrogen or Nitrogen or Helium was used as a carrier gas at a flow rate 1 ml/min, split 10:1. The test sample was evaporated in the injection port 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 were eluted based on the boiling point of the individual components.\[^{13, 14, 15}\]
The GC column was 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 was 36 min. The eluted component was detected in the mass detector. The spectrum of the known components stored in the NIST library ascertained 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 as well as on comparison of their retention indices with literature.[16-20]

**In Vitro Cytotoxicity Assay**

About 5 g of powdered material of plant was taken in a clean, flat-bottomed glass container and soaked in 25 ml of 80% methanol. The container with its content was kept for a period of 4 to 7 days accompanying occasional stirring. Coarse filtration of the whole mixture was carried out 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 and 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 1x10⁵ 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 hours, 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, which resulted in the required final sample concentrations. Following this, the plates were incubated for an additional 48 hours at 37 °C, 5% CO₂, 95% air and 100% relative humidity. The
medium without samples served as control and triplicate was maintained for all concentrations.\textsuperscript{[21, 22, 23]}

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 48 hours of incubation, 15\(\mu l\) of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 hours. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100\(\mu l\) of DMSO and measured the absorbance at 570 nm using micro plate reader. The percentage cell inhibition was determined using the following formula.\textsuperscript{[24, 25]}

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

RESULTS AND DISCUSSION

Table 1: Phytochemical screening

<table>
<thead>
<tr>
<th>Test</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</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>Steroids</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>Carbohydrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Protein/Amino acids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diterpines</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+ indicates presence, - indicates absence)

Phytochemical screening of \textit{Prunus angustifolia} indicated the presence of alkaloids, phenols, tannins, glycosides, saponins, carbohydrates, proteins and diterpenes. Ethanol extract of \textit{Prunus angustifolia} revealed the presence of alkaloids, phenols, tannins, carbohydrates, protein and diterpenes. Methanolic extract of \textit{Prunus angustifolia} signified the presence of phenols, carbohydrates, proteins and diterpenes. Petroleum ether extract showed the presence of alkaloids and carbohydrates. Chloroform extract hinted the presence of alkaloids, tannins, glycosides and proteins. Aqueous extract indicated the presence of alkaloids, phenols, tannins, glycosides, saponins, carbohydrates and aminoacids.
GC-MS ANALYSIS

The GC-MS combines the gas chromatography and mass spectrometry techniques. The result carried out by this technique revealed that 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 Prunus angustifolia plant carried out by GC-MS analysis

<table>
<thead>
<tr>
<th>S.No</th>
<th>RT</th>
<th>Name of the Compounds</th>
<th>Formula</th>
<th>M.W</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.99</td>
<td>Heptadecanoic acid</td>
<td>C\textsubscript{17}H\textsubscript{34}O\textsubscript{2}</td>
<td>270.45</td>
</tr>
<tr>
<td>2</td>
<td>14.33</td>
<td>2,3-Epoxy-3-methylcyclohexanone</td>
<td>C\textsubscript{7}H\textsubscript{10}O\textsubscript{2}</td>
<td>126.15</td>
</tr>
<tr>
<td>3</td>
<td>18.34</td>
<td>1-ethyl-adamantane</td>
<td>C\textsubscript{12}H\textsubscript{20}</td>
<td>164.28</td>
</tr>
<tr>
<td>4</td>
<td>22.68</td>
<td>Cleomeolide</td>
<td>C\textsubscript{20}H\textsubscript{30}O\textsubscript{3}</td>
<td>318.45</td>
</tr>
<tr>
<td>5</td>
<td>26.00</td>
<td>Thunbergol</td>
<td>C\textsubscript{20}H\textsubscript{34}</td>
<td>290.48</td>
</tr>
<tr>
<td>6</td>
<td>30.87</td>
<td>3-(Benzhydryl)-2,6-dimethyl-4-pyrene</td>
<td>C\textsubscript{20}H\textsubscript{18}O\textsubscript{2}</td>
<td>290.35</td>
</tr>
<tr>
<td>7</td>
<td>34.38</td>
<td>4,9-Diphenyl-10-methyl-3,5-dithiatricyclo[4.4.0.0(2,6)] deca-7,9-diene</td>
<td>C\textsubscript{21}H\textsubscript{18}S\textsubscript{2}</td>
<td>334.49</td>
</tr>
<tr>
<td>8</td>
<td>37.47</td>
<td>2,2'-dithiodibenoic acid</td>
<td>C\textsubscript{14}H\textsubscript{10}O\textsubscript{4}S\textsubscript{2}</td>
<td>306.35</td>
</tr>
</tbody>
</table>

Figure 1: Chromatogram of Prunus angustifolia
The cytotoxicity assay was carried out in MCF-7 (Breast cancer) cell line with five different concentration of plant extract (methanol). From figure 3 to 7, there is an increase in cytotoxicity. It was found that as plant concentration was increased, the cell viability...
decreased and anticancer activity increased. A 100% cytotoxicity was achieved at 300 µg concentration.

![Graphical representation of cytotoxicity assay](image)

**Figure 8: Graphical representation of cytotoxicity assay**

The figure 8 shows the graphical representation of cell inhibition. As the concentration increases the percentage of cell inhibition also increased and cell viability got decreased. IC50 value was found to be 99.97µg/ml.

**CONCLUSION**

This study is concluded by the findings that the *Prunus angustifolia* leaves showed the presence of phytochemicals like carbohydrates, saponins, phytosterols, tannins, glycosides, diterpenes, flavonoids and aminoacids. GC-MS analysis showed the presence of 8 different compounds. Also, cytotoxicity assay of *Prunus angustifolia* 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 99.97µg/ml. Henceforth, it is justified that *Prunus angustifolia* is a medicinal plant and its leaves are with anti-cancer activity against breast cancer.

**ACKNOWLEDGEMENT**

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REFERENCES