PHYTOCHEMICAL SCREENING AND ANALYSIS OF ACTIVE SECONDARY METABOLITES PRESENT IN THE ETHANOLIC EXTRACT OF CALOTROPIS GIGANTEA LEAVES USING GC-MS TECHNIQUE

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ABSTRACT
Natural products have been a major source of drugs for centuries. In the last few decades there has been an exponential growth in the field of herbal medicines. Medicinal plants and herbs contain substances known to modern and ancient civilizations for their healing properties. Plants produce chemical compounds as part of their normal metabolic activities. These metabolites are the source of active principles capable of curing health ailments. There is an increasing interest in the bioactive potential of compounds derived from plants, which could be relevant in relation to their role in health and disease. The aim of the present study is to analyze the active constituents present in the ethanol extract of *Calotropis gigantea* leaf by phytochemical analysis and GC-MS technique. GC-MS analysis of *C. gigantea* leaf ethanol extract showed a high complexity profile, containing many bioactive components. As these compounds may have multiple pharmacological activities the results confirms that the ethanolic extract of *C. gigantea* may have efficient therapeutic potency. However, isolation of individual phytochemical constituents may proceed to find a novel drug.

KEYWORDS: Phytochemical analysis, GC-MS, *Calotropis gigantea*, plant, drug.
1. INTRODUCTION

Plants, owing to its medicinal value have continued to play a dominant role in the maintenance of human health since ancient times. The World Health Organization estimates that plant extracts or their activity constituents are used as folk medicine in traditional therapies of 80% of the world's population.\textsuperscript{[1]} Over 50% of all modern clinical drugs are of natural product origin.\textsuperscript{[2]} Turkish people have a tradition of using a number of plant species for the treatment of infectious diseases and various ailments.\textsuperscript{[3]} Traditional and folklore medicines play important role in health services around the globe. About three quarter of the world's population rely on plant and plant extracts for health care. India has an extensive forest cover, enriched with plant diversity.\textsuperscript{[4]}

The genus of \textit{Calotropis} (Asclepiadaceae) is comprised of about six species of shrubs distributed in tropical and subtropical Africa and Asia. \textit{Calotropis gigantea} (Crown flower) is a species of \textit{Calotropis} native to Cambodia, Indonesia, Malaysia, Philippines, Thailand, Sri Lanka, India and China. This species is a large shrub or small tree. Its stems are erect, up to 20 cm in diameter. The leaves are broadly elliptical to oblong-ovate in shape, with the size of 9-20 cm x 6-12.5 cm but subsessile. The plant has oval, light green leaves and milky stem. \textit{Calotropis gigantea} are a common weed in open waste ground, roadsides and railway lines, as well as village surroundings. It grows especially on littoral sandy soils and dry uncultivated land, with periodic dry periods.\textsuperscript{[5]} \textit{Calotropis gigantea} are extensively used by Ayurvedic physicians for treatment of disorders such as diabetes mellitus, bronchial asthma, rheumatoid arthritis, and nervous disorders.\textsuperscript{[6,7]}

There is an increasing interest in the phytochemical compounds, which could be relevant to their nutritional incidence and their role in health and disease.\textsuperscript{[8]} In the recent years, the interest for the study of the organic compounds from plants and their activity has increased. A lot of extraction methods and analytical methods as spectrophotometry, high performance liquid chromatography (HPLC), capillary electrophoresis, gas chromatography - mass spectrometry (GC-MS) are developed for the study about plant active compounds.\textsuperscript{[9]} The combination of an ideal separation technique (GC) with the best identification technique (MS) made GC-MS an ideal technique for qualitative and quantitative analysis for volatile and semi-volatile compounds.\textsuperscript{[10]}

Herbal medicine is currently in the lime light and is given more popularity than ever before as sales figures in some countries, for example the USA, have risen beyond the expectations of
some producers. The reason for this change are complex but clearly are connected with what could be described as the greening of medicine.[11] Natural products are an unending source of chemical compounds which are often useful in pharmaceutics, cosmetics and agro ecosystem developments.[12] Herbal remedies have proved successful in folk medicines and Ayurvedic System of medicine and it is now accepted worldwide. Increasing resistance of synthetic antibiotics towards existing as well as newly born species of pathogens and deadly infectious diseases prompts the researchers to investigate the traditional medicinal plants as a source of active principles capable of treating various diseases.[13] In this sense, the main contribution of this investigation relies on the phytochemical screening and GC-MS analysis for the identification of important active chemical constituents present in ethanol extracts of *C. gigantea* leaves that may be useful for the management of many diseases.

2. MATERIALS AND METHODS

2.1 COLLECTIONS OF TEST MATERIALS

Fully developed leaves of the plant namely *Calotropis gigantea* were collected from the natural habitat of Coimbatore locale of 11°1’N 76°56’S Longitude.

2.2 PREPARATION OF LEAF POWDER AND EXTRACT

Fresh leaves were collected washed in water and dried at room temperature. After drying for 2 to 3 weeks, the leaves were ground in an electric pulverizer to get the powder. 10 g of the leaf powder was weighed using an electronic balance (Denver XS-210) and made into packets using Zero haze filter paper (A Grade, SD’s). The powder was subjected to extraction with 500 ml of ethanol for 8 h using a Soxhlet apparatus.[14,15] The leaf extracts thus obtained were concentrated by distillation and dried by evaporation in a water bath at 40°C. The residue thus obtained was stored in tightly closed glass vials in the refrigerator for further

2.3 PHYTOCHEMICAL SCREENING

a) Test for alkaloids

Mayer’s test

A fraction of extract was treated with Mayer’s test reagent (1.36 g of mercuric chloride and 5 g of potassium iodide in 100 ml of water) and observed for the formation of cream coloured precipitate.
Wagner’s test
A fraction of extract was treated with Wagner’s reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml water) and observed for the formation of reddish brown colour precipitate.

Hager’s test
A few ml of extract was treated with Hager’s reagent (saturated aqueous solution of picric acid) and observed for the formation of prominent yellow precipitate.

b) Test for tannins

Acetic Acid Test
The extract was treated with acetic acid solutions and observed for the formation of red colour solution.

Dilute HNO₃ Test
The extract was treated with dil. HNO₃. The extract turns from reddish to yellow colour which indicates the presence of tannins.

c) Test for phenols

Ferric chloride test
The fraction of extract was treated with 5% ferric chloride and observed for the formation of deep blue or black colour

Liebermann’s test
The extract was heated with sodium nitrite, added H₂SO₄ solution diluted with water and excess of dilute NaOH was added and observed for the formation of deep red or green or blue colour.

d) Test for flavonoids

NaOH test
A small amount of extract was treated with aqueous NaOH and HCl, observed for the formation of yellow orange colour.

H₂SO₄ test
A fraction of the extract was treated with concentrated H₂SO₄ and observed for the formation of orange colour.
e) Test for sterols

Liebermann-Burchard test
Extract (1ml) was treated with chloroform, acetic anhydride and drops of H₂SO₄ was added and observed for the formation of dark pink or red colour.

f) Test for terpenoids

Liebermann-Burchard test
Extract (1ml) was treated with chloroform, acetic anhydride and drops of H₂SO₄ was added and observed for the formation of dark green colour.

g) Test for saponins

Foam Test
The extract or dry powder was vigourosly shaken with water and observed for the formation of persistent foam.

h) Test for anthraquinones

Borntrager’s test
About 50 mg of powdered extract was heated with 10% ferric chloride solution and 1ml concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia and observed for the formation of pink or deep red colouration of aqueous layer.

i) Test for proteins

Ninhydrin test (Aqueous)
The extract was treated with aqueous ninhydrin and observed for the presence of blue colour, indicating the presence of amino acid or purple colour indicating the presence of protein.

Ninhydrin (acetone)
Ninhydrin was dissolved in acetone; the extract was treated with ninhydrin and observed for the formation of purple colour.

Biuret test
The extract was heated in distilled water and filtered. The filtrate was treated with 2% copper sulphate solution, 95% ethanol and potassium hydroxide and observed for the formation of pink ethanolic layer.
j) Test for quinones

A small amount of extract was treated with concentrated HCl and observed for the formation of yellow colour precipitate.

2.4 GC–MS ANALYSIS

Mass experiments were performed on GC (T8000 Top CE) combined with Mass Spectrometer (Md 800 FIS ONS). Sample was dissolved in methanol and introduced into the column TR-5-MS capillary standard non-polar by splitles injection system. Ultra high purity helium was introduced as the buffered collision gas with flow rate of 1.0 ml/min. The source temperature for ionization was set at 250°C. All the experiments were performed on the positive ion ion mode.

3. RESULTS AND DISCUSSION

Herbal medicines are very popular in developing and underdeveloped countries. Therefore, a clear understanding of potential adverse effects of herbs used is necessary for implementing safety measures. This present study tends to investigate the phytochemical content of the ethanol extract of C. gigantea. The phytochemical analysis showed the presence of some bioactive compounds in the plant. In the two forms of the extract, ten bioactive constituents were tested for, out of which seven were present. Preliminary phytochemical screening of the ethanol extract of C. gigantea leaf showed the presence of alkaloids, phenols, flavonoids, sterols, saponins, proteins and quinines. Tannins, terpenoids and anthroquinones were absent. The results of phytochemical analysis are depicted in Table 1. Similar study was carried out by Devendran and Balasubramanian[16] in which the qualitative analysis of the extracts from the leaf sample of Ocimum sanctum showed the presence of phytochemical constituents such as tannins, saponin, flavonoids, steroid, terpenoids and cardiac glycerides. In accordance with the results of the present study Kavit et al[17] has reported the presence of medicinally active constituents like tannins, alkaloid, terpenoids, steroids and saponins in the leaves of Phyllanthus fraternus.

The phytochemicals may be related with its ethno-medicinal use in the treatment of various diseases. Generally, alkaloids are known to have antimicrobial, antifungal and anti-inflammatory effects (Okwu and Okwu[18] and also act as anti-hypertensive agents.[19] The present study showed the presence of alkaloids in the leaf extract of C.gigantea. Onike[20] has reported that alkaloids are used for the treatment of tumors, nocturnal leg cramps caused by vascular spasms and diarrhoea. These compounds possess anti-microbial activity and sedative
effects. Many alkaloids are anaesthetics and have calming effects on psychotic or hypertensive patients without inducing sleep. Alkaloids can also be used to treat psychiatric and palpitation.

Phenols act as primary antioxidants. Antioxidants are widely consumed in the diet, naturally and as food additives and also consciously taken as therapeutic agents in the form of supplements such as herbs. They ultimately have high redox potentials which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. This attribute is possibly due to the delocalization of electrons over the phenolics and stabilization by the resonance effect of the aromatic nucleus that prevents the continuation of the free radical chain reaction. Phenolic antioxidants are potent water soluble antioxidants which prevent oxidative cell damage suggesting their possible implication in the protection of various diseases.

As reported by Singh and Kumar flavonoids are recognized as having beneficial properties to human health. These compounds possess antioxidant elements and ensure healthy circulation. Flavonoids help to strengthen capillary walls. These compounds, at times, are referred to as phytoestrogens. Phytoestrogens are associated with relief of menopausal symptoms, reduction of osteoporosis, improvement of blood cholesterol levels and lowering the risk of certain factors related to cancer and coronary heart diseases.

In the present investigation sterols were found to be present in the ethanol extract of C. gigantea leaf. Steroidal compounds have been used to reduce stress, reduce cholesterol levels, activate immune system, enhance memory and learning and to treat tumor cells in cancer cases.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Constituents</th>
<th>C. gigantea Ethanol extract of leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Tannins</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Sterols</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Terpenoids</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Anthroquinones</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Proteins</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Quinones</td>
<td>+</td>
</tr>
</tbody>
</table>
Saponins are generally regarded as antinutrients but are also believed to be useful in human diet for controlling cholesterol. Saponins were found to be present in ethanolic leaf extract of _C. gigantea_. Its presence in this plant therefore could suggest that the plant is of medicinal value. There is evidence of the presence of saponins in traditional medicine preparations.\cite{29,30} Saponins extracted from plants show biological and pharmacological activities such as anti-inflammatory, anti-hepatotonic, wound healing, veinotonic, expectorant, spasmolytic, hypoglycemic, antimicrobial and antiviral.\cite{20}

These bioactive agents may contribute to the medicinal efficacy of the plant. Furthermore, the presence of phenols and flavonoids as detected from the ethanolic extract shows that the ethanolic extract of _C. gigantea_ might be able to manage oxidative stress. The present study also showed the presence of proteins and quinones in the ethanolic leaf extract of plant. Previous studies on the phytochemical screening of tuber _Dioscorea bulbifera_ also revealed the presence of alkaloids, steroids, fats and fixed oil, flavonoids, tannins, proteins and carbohydrates. This phytochemical screening aids as an initial step for future determination of its activity like antioxidant, anticancer, anti-inflammatory, antimutagenic etc.\cite{31} Sampathkumar and Ramakrishnan\cite{32} showed the presence of carbohydrates, proteins, lipids, phenols, flavonoids, saponins, alkaloids and quinones in the plant _Narinji crenulata_.

The present study was carried out to identify the phytoconstituents present in _C. gigantea_ leaf ethanol extracts using GC-MS. The results pertaining to GC-MS analysis leads to the identification of number of compounds from the ethanolic extract of _C. gigantea_ leaves. These compounds were identified through mass spectrometry attached with GC. The retention time, compound name, molecular formula and molecular weight of various components present in the leaf of _C. gigantea_ that were detected by the GC-MS are shown in Table 2. The GC-MS spectrum confirmed the presence of various components with different retention times as illustrated in Fig 1 and 2.
Fig 1: GC-MS Chromatogram of the ethanolic leaf extract of *C. gigantea*

Fig 2: Mass spectrum at (RT: 43.86)
Table 2: Phytocomponents identified in the ethanolic extract of the flower of *Calotropis gigantea* by GC-MS analysis

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>RT</th>
<th>Compound name</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.34</td>
<td>1,1,6,6-Tetramethylcyclodecane</td>
<td>C(<em>{14})H(</em>{28})</td>
<td>196</td>
</tr>
<tr>
<td>2</td>
<td>11.17</td>
<td>1-Octene</td>
<td>C(<em>{8})H(</em>{16})</td>
<td>112</td>
</tr>
<tr>
<td>3</td>
<td>14.27</td>
<td>1-Eicosanol</td>
<td>C(<em>{20})H(</em>{35})O</td>
<td>298</td>
</tr>
<tr>
<td>4</td>
<td>14.74</td>
<td>1-Nonadecene</td>
<td>C(<em>{19})H(</em>{38})</td>
<td>266</td>
</tr>
<tr>
<td>5</td>
<td>20.32</td>
<td>8-Pentadecanone</td>
<td>C(<em>{15})H(</em>{30})O</td>
<td>226</td>
</tr>
<tr>
<td>6</td>
<td>22.93</td>
<td>1-Hexadecanol</td>
<td>C(<em>{16})H(</em>{34})O</td>
<td>242</td>
</tr>
<tr>
<td>7</td>
<td>23.25</td>
<td>3-Propoxyphthalide</td>
<td>C(<em>{11})H(</em>{12})O(_3)</td>
<td>192</td>
</tr>
<tr>
<td>8</td>
<td>27.37</td>
<td>1-Nonadecene</td>
<td>C(<em>{19})H(</em>{38})</td>
<td>266</td>
</tr>
<tr>
<td>9</td>
<td>27.37</td>
<td>n-Heptadecanol-1</td>
<td>C(<em>{17})H(</em>{36})O</td>
<td>256</td>
</tr>
<tr>
<td>10</td>
<td>30.86</td>
<td>Ethyl Oleate</td>
<td>C(<em>{20})H(</em>{38})O(_2)</td>
<td>310</td>
</tr>
<tr>
<td>11</td>
<td>30.86</td>
<td>Ethyl 9-octadecanoate</td>
<td>C(<em>{20})H(</em>{38})O(_2)</td>
<td>310</td>
</tr>
<tr>
<td>12</td>
<td>30.86</td>
<td>9-Octadecenoic acid (Z)-, ethyl ester</td>
<td>C(<em>{20})H(</em>{38})O(_2)</td>
<td>310</td>
</tr>
<tr>
<td>13</td>
<td>35.50</td>
<td>(cis)-2-nonadecene</td>
<td>C(<em>{19})H(</em>{38})</td>
<td>266</td>
</tr>
<tr>
<td>14</td>
<td>38.26</td>
<td>1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester diisooctyl-phthalate</td>
<td>C(<em>{24})H(</em>{38})O(_4)</td>
<td>390</td>
</tr>
<tr>
<td>15</td>
<td>38.26</td>
<td>1,2-Benzenedicarboxylic acid, diisooctyl ester</td>
<td>C(<em>{24})H(</em>{38})O(_4)</td>
<td>390</td>
</tr>
<tr>
<td>16</td>
<td>38.26</td>
<td>Di-(2-ethylhexyl)phthalate</td>
<td>C(<em>{24})H(</em>{38})O(_4)</td>
<td>390</td>
</tr>
<tr>
<td>17</td>
<td>38.26</td>
<td>Di-n-octyl phthalate</td>
<td>C(<em>{24})H(</em>{38})O(_4)</td>
<td>390</td>
</tr>
<tr>
<td>18</td>
<td>43.86</td>
<td>Nonacosane</td>
<td>C(<em>{29})H(</em>{60})</td>
<td>408</td>
</tr>
<tr>
<td>19</td>
<td>43.86</td>
<td>Dotriacontane</td>
<td>C(<em>{32})H(</em>{66})</td>
<td>450</td>
</tr>
<tr>
<td>20</td>
<td>43.86</td>
<td>Heptacosane</td>
<td>C(<em>{27})H(</em>{56})</td>
<td>380</td>
</tr>
<tr>
<td>21</td>
<td>43.86</td>
<td>Docosane</td>
<td>C(<em>{22})H(</em>{46})</td>
<td>310</td>
</tr>
<tr>
<td>22</td>
<td>45.80</td>
<td>[(E)-6,7-Dihydroxy-3,7-dimethyl-2-octenyl]ester of acetic acid</td>
<td>C(<em>{12})H(</em>{22})O(_4)</td>
<td>230</td>
</tr>
</tbody>
</table>

The gas chromatogram shows the relative concentrations of various compounds getting eluted as a function of retention time. The heights of the peak indicate the relative concentrations of the components present in *C. gigantea*. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different m/z ratios.

The spectral analysis of the ethanol extract of *C. gigantea* revealed the presence of compounds by forming fifteen major peaks indicating the presence of phytochemical compounds with different therapeutic activities. The mass spectrum displayed the characteristic peaks at (M-13), (M-14), (M-15), (M-18) (M-27), (M-28) which indicated the presence of hydrocarbon, methyl, hydroxyl, nitrogen and carbonyl functional groups in the extract (Fig 1 and 2). In concordance with the present study Vadivel (2011)\(^\text{[32]}\) reported that
the ethanolic extract of *Mussaenda frondosa* subjected to GC-MS analysis showed twenty major peaks thereby revealing twenty chemical constituents. Similar observations were recorded by Derwich *et al.*[33] in which essential oil of the leaves of *Mentha pulegium* showed the presence of 28 compounds. Previous studies on the active principles in the *Indigofera aspalathoides* whole plant methanolic extract by GC-MS analysis clearly showed the presence of ten compounds.[34]

The ethanolic solvent extract of *C. gigantea* leaves is a very good source of phytochemicals and can offer remedies to some of the common ailments. Secondary metabolites in plants are responsible for several biological activities in man and animals. There is a growing awareness in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity. Screening active compounds from plants has lead to the invention of new medicinal drugs which have efficient protection and treatment roles against various diseases. Therefore, further research on toxicological aspects of the compound is in need to develop safe drug.

4. CONCLUSION

A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials. A majority of the rich diversity of Indian medicinal plants is yet to be scientifically evaluated for such properties. In the present study phytochemical screening and GC-MS analysis showed the existence of various compounds which may have different bioactivities. Therefore, the presence of various bioactive compounds confirms the application of *C. gigantea* leaves for various ailments by traditional practitioners. However, isolation of individual phytochemical constituents may proceed to find a novel drug.

5. REFERENCES


