QUALITATIVE CHARACTERIZATION OF PHYTOCHEMICALS AND \textit{IN VITRO} ANTIMICROBIAL EVALUATION OF LEAF EXTRACT OF \textit{COUROUPITA GUIANENSIS} AUBL. - A THREATENED MEDICINAL TREE

\textbf{REETIKA SINGH}**, \textbf{NISHI KUMARI}**, \textbf{MAYANK GANGWAR}**, \textbf{GOPAL NATH}**

**Department of Botany, Mahila Mahavidyalaya, 2Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, Uttar Pradesh, India

\texttt{Received: 01 Apr 2015 Revised and Accepted: 21 May 2015}

\section*{ABSTRACT}

\textbf{Objective:} Screening of phytochemicals present in aqueous extract and evaluation of the antibacterial and antifungal activities from different organic extracts of leaf of \textit{C. guianensis} Aubl.

\textbf{Methods:} Antimicrobial activity of different extracts was evaluated by using the disc diffusion assay. Methanolic, ethanolic and chloroform extracts of leaf were tested against fungus and representatives of Gram-positive and Gram-negative bacteria.

\textbf{Results:} Presence of alkaloids, flavonoids, glycosides, phlobatannins, steroids, tannins and terpenoids was observed in aqueous extract of leaf. Chloroform extract showed better activity against Gram-positive bacteria in comparison to Gram-negative bacteria. Methanolic extract was more effective on Gram negative bacteria. Leaf extract was also effective against \textit{Candida species}. Minimum inhibitory concentration was 25 mg/ml for ethanolic, 50 mg/ml for methanolic and 100 mg/ml for chloroform extracts against \textit{S. aureus}.

\textbf{Conclusion:} Present study of \textit{C. guianensis} seems to be promising for pharmaceutical industries for making an antimicrobial drug or cream especially against \textit{S. aureus} and provides details of pharmacological investigation, identification, isolation and characterization of novel bioactive compounds.

\textbf{Keywords:} Couroupita guianensis, Phytochemicals, Antibacterial activity, Antifungal activity, Leaf extract, Threatened plant, Medicinal plant.

\section*{INTRODUCTION}

Bacterial and fungal infections are the most common cause for illness of humans, animals and plants. These microorganisms sometimes create very serious problem. The alarming world-wide spread of drug-resistant bacteria and limited access to anti-infective drugs emphasizes the importance of discovering new antimicrobial compounds [1]. Plants have always been an important source of medicines since ancient times and 70% of the worldwide population still relies on one or other forms of traditional plant based medicine [2]. There is a variety of pharmacologically important molecules, but only a small percentage of plants have been explored for their phytochemical constituents and activities [3]. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc. [4]. The trend of using natural products is increasing because of its negligible side effect. The principles of many products in plants are secondary metabolites such as alkaloids, flavonoids and terpenoids which are responsible for antimicrobial activity [5]. Since ancient time, active plant extracts are frequently used in traditional medicine system and screened for new drug discoveries. Couroupita guianensis Aubl. (family-Lecythidaeae) popularly known as the cannon ball tree, is a highly medicinal tree. This tree is commonly known as Nagalingam Pushpam in Tamilnadu because its shape of flower. Almost all parts of this plant like leaf, flower, bark, stem and fruit-shell are used in the treatment of various ailments. People from an Amazonian region and other states of the north region of Brazil use infusions or tea obtained from leaves, flowers, and bark of \textit{Couroupita guianensis} to treat hypertension, tumours, pain and inflammatory processes [6]. Chemical studies of this species have shown the presence of α-amirin, β-amirin, β-sitosterol, nerol, tryptanthrine, indigo, indirubin, isatin, linoleic acid, carotenoids and steroids [7-12]. In leaf, triterpenoid ester of fatty acids such as β-amirin palmitate has been reported [13]. Bark of \textit{C. guianensis} possesses antimicrobial and antitoxin activity [14] and fruit having antibacterial activity [15] have also been reported. Immunomodulatory activity [16] and antioxidant activity [17] in flowers have been also reported. The leaves of \textit{C. guianensis} possess herbal hand wash formulation and yielded an aliphatic triterpene. Methanolic extract of root has been observed with an anti-depressant in mice [18]. The phenolic compounds obtained from extract of \textit{C. guianensis} have been reported having anti-inflammatory activity and curing the kidney and stomach alment [5]. The biological functions of flavonoids, apart from their antioxidant properties, include protection against allergy, inflammation, platelet aggregation, infections, ulcers, heptatoxins and tumors [19]. It is known that one of the active constituents of the medicinal plant \textit{C. guianensis}, namely isatin, is known to exert cytotoxic activity against certain cancer cell lines, being a potential source of new chemotherapeutic agents [20]. Although antibacterial activity in leaf extract was studied [21] but they did not used chloroform as solvent. In the light of above observation, the present work was undertaken to study the phytochemical characterization and antibacterial and antifungal activity of leaf extracts of this plant in different solvents namely methanol, ethanol and chloroform.

\section*{MATERIAL AND METHODS}

\textbf{Collection of plant material}

Young leaves of \textit{Couroupita guianensis} Aubl. were collected from the campus of Banaras Hindu University (BHU), Varanasi, India in the month of April. Leaves were dried in shade condition at room temperature for 4-5 d and then dried at 40-45 °C for 2 h. Leaves were crushed to coarse powder using mechanical grinder. Powder was stored at room temperature in air tight container.

\textbf{Preparation of organic solvent extract}

Extracts were prepared by taking 20 g leaf powder for extraction process. Extraction was done in 200 ml of different solvents for 8 h using soxhlet apparatus. Ethanol, methanol and chloroform were used as extraction solvents. Extracts were dried in vacuum.
Percentage yield (w/w) of crude extract was calculated by using following formula:

\[ \text{PY} = \frac{\text{Wt of crude extract recovered (g)}}{\text{Wt of powder used (g)}} \]

Where, PY is percentage yield of extract.

**Aqueous extraction of plant material for phytochemical screening**

Aqueous extract was used for phytochemical screening. For preparation of aqueous extract, five gram of leaf powder was soaked in double distilled water for 50 h in air tight bottle and left at room temperature and filtered with eight layers of muslin cloth. Extract was stored at 20 °C till use.

**Preparation of aqueous extract**

For the preparation of aqueous extract, five gram of leaf powder was soaked in double distilled water for 50 h in air tight bottle and left at room temperature and filtered with eight layers of muslin cloth. Extract was stored at -20 °C till use.

**Preliminary Phytochemical screening**

Preliminary phytochemical screening was carried out by using aqueous extract to identify various constitutes using standard methods [22-24].

**Screening of antimicrobial activity**

**Preparation of sample extract for microbiological assay**

For screening of antimicrobial activity, sample extract was prepared in following manner. In brief, stock solution of extract was prepared in concentration of 100 mg/ml in dimethyl sulphoxide (DMSO). From which, about 5 µl extracts was dispensed onto sterile disc for susceptibility test. Standard drugs were prepared in concentration of 1 µg/µl.

**Test microorganisms**

Some selected Gram positive, Gram negative bacteria and fungi were used for screening anti microbial activity. Four Gram positive bacteria (Staphylococcus aureus ATCC 25323, Enterobacter aerogenes, E. fecalis, S. faecalis), three Gram negative bacteria (Salmonella Typhimurium, Klebsiella pneumoniae, Escherichia coli ATCC 35218) and three fungal strains (Candida albicans ATCC 90028, Candida tropicalis ATCC 750, Candida parapsilosis ATCC 22019) were used for investigation. All microbial cultures were obtained from the Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India. The young bacterial broth cultures were prepared for screening experiments.

**Media used**

Media was prepared by dissolving Mueller Hinton agar 38 g/l and 10 g/l in double distilled water and saline was prepared by dissolving 8.5 g/l in double distilled water. LB broth (25 g/l) media was prepared in double distilled water and autoclaved for 15 min at 15 psi and 121 °C. The plates were prepared with 20 ml of sterile Mueller Hinton Agar (MHA).

**Determination of minimum inhibitory concentration (MIC)**

Minimum inhibitory concentration (MIC) was determined by using 200 mg/ml extract on 96 well U-bottom microtitre plates (Tarson, Mumbai, India). LB broth medium in the volume of 100 µl was added to each well and testing extract was added in serial dilution manner. Finally, inoculum of S. aureus bacterium was added to each well for determination of MIC. Plate was incubated at 37 °C for 24 h.

**Statistical analysis**

All experiments were done in triplicate and repeated thrice in independent manner. Data was analysed using SPSS software (version 16, Chicago, USA). Analysed data was represented as mean±SE.

**RESULTS AND DISCUSSION**

After extraction, extraction yield was calculated. In different solvents, maximum percentage (18.04 %) was found in methanol followed by ethanol (16.02 %) and minimum in chloroform (12.03 %). Percentage yield depends on the nature of solvent used for the extraction and the temperature of extraction. Extraction yield was also calculated in leaf and fruit extract of Sapindus mukorossi by other author [26].

**Antibacterial and antifungal sensitivity test**

Antibacterial activity was tested using disc diffusion method [25]. The test cultures were swabbed on the top of the solidified media and allowed to dry for 5 min. About 5 µl of extract was loaded to each disc. The loaded discs were placed on the surface of the medium. Negative control was prepared using the respective solvents. The plates were incubated for 24 h at 37 °C for bacteria and for 48 h at 28 °C for fungi. Zones of inhibition were recorded in millimeters.

**Antibacterial and antifungal activity**

All extracts of C. guianensis have shown antibacterial and antifungal activity. Leaf extract of C. guianensis exhibited promising activity against bacteria and fungi using disc diffusion method. The activity of all extracts against bacteria and fungi are given in table 2. Among all extracts of leaf chloroform extract showed maximum inhibition zone on S. aureus (Fig.2 A). Leaf extract of C. guianensis was more effective on Gram positive bacteria than Gram negative bacteria. Similar observation have been made by many researchers that Gram positive bacteria are more susceptible to plant’s extracts as compared to Gram negative bacteria [29-30]. Chloroform extract showed higher antibacterial activity, it could be due to nature of extraction solvent.
Antimicrobial activity of leaf extract was also observed by [21] and reported significant activity on different bacteria. Antibacterial activity of chloroform extract of C. guianensis fruits was also reported [31]. The leaf extract of C. guianensis showed antimicrobial activity against S. aureus, S. Typhimurium and S. faecalis. These bacteria are known pathogen.

Table 1: Phytochemical screening from leaf extract of Couroupita guianensis

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Tests</th>
<th>Aqueous extract of leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Mayer’s reagent</td>
<td>Strongly present</td>
</tr>
<tr>
<td></td>
<td>Wagner’s reagent</td>
<td>Strongly present</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Lead acetate solution</td>
<td>Moderately Present</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Liebermann’s test</td>
<td>Present</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>Hydrochloric acid</td>
<td>Present</td>
</tr>
<tr>
<td>Saponins</td>
<td>Foam test</td>
<td>Absent</td>
</tr>
<tr>
<td>Steroids and Terpenoids</td>
<td>Chloroform and sulphur acid</td>
<td>Present</td>
</tr>
<tr>
<td>Tannins</td>
<td>Ferric chloride</td>
<td>Present</td>
</tr>
</tbody>
</table>

Fig. 2: Antibacterial activity of leaf extract of C. guianensis: 10, 11, 12 represent methanolic, ethanolic and chloroform extract respectively. A) Inhibition zone in S. aureus B) S. Typhimurium

Table 2: Antibacterial and antifungal activity of different extract of C. guianensis leaf (5 µl of 100 mg/ml extract)

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Inhibition zone (mm)</th>
<th>Methanolic extract</th>
<th>Ethanolic extract</th>
<th>Chloroform extract</th>
<th>Standard drugs (5 µl/disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram positive</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>09.00±0.57</td>
<td>10.00±0.06</td>
<td>16.00±0.57</td>
<td>22.60±0.88 (Ampicillin)</td>
<td></td>
</tr>
<tr>
<td>E. aerogens</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>25.00±0.58(Ampicillin)</td>
<td></td>
</tr>
<tr>
<td>S. faecalis</td>
<td>07.00±0.46</td>
<td>07.00±0.26</td>
<td>07.00±0.40</td>
<td>19.50±0.29 (Ampicillin)</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>07.00±0.52</td>
<td>07.00±0.34</td>
<td>08.00±0.61</td>
<td>21.23±0.67 (Ampicillin)</td>
<td></td>
</tr>
<tr>
<td>Gram negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>09.00±0.17</td>
<td>11.00±0.57</td>
<td>10.00±0.50</td>
<td>26.93±0.58 (Ciprofloxacin)</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>26.33±0.33 (Ciprofloxacin)</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>24.66±0.13 (Ciprofloxacin)</td>
<td></td>
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<tr>
<td>Fungus</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C. albicans</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>24.63±0.41(Fluconazole)</td>
<td></td>
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<tr>
<td>C. tropicalis</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>00.00±0.00</td>
<td>21.00±0.53(Fluconazole)</td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>08.00±0.43</td>
<td>07.00±0.25</td>
<td>08.00±0.60</td>
<td>25.80±0.41 (Fluconazole)</td>
<td></td>
</tr>
</tbody>
</table>

All data represented mean±SE of three independent experiments. 00.00±0.00 showed that no inhibition zone for respective microorganisms.

Leaf extract of C. guianensis also showed antifungal activity against Candida parapsilosis. Methanolic extract was slightly more effective in comparison to the other extract (table 2). C. parapsilosis shows significant drug resistance against azole family drugs such as fluconazole and voriconazole. The plant extract of this plant can be used against C. parapsilosis. However, leaf extract was ineffective on Candida albicans and Candida tropicalis.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was found different for different leaf extract for S. aureus. Ethanolic extract showed higher activity than methanolic and chloroform extract. MIC was 25 mg/ml for ethanolic, 50 mg/ml for methanolic and 100 mg/ml for chloroform extract.

ACKNOWLEDGEMENT

Prof. Uma Jaiswal, Department of Botany, Mahila Mahavidyalaya, Banaras Hindu University, Varanasi, India, is acknowledged for the identification of this plant. University Grant Commission is acknowledged for financial support.

CONFLICT OF INTERESTS

The authors have no conflict of interest for publication of this research article

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