Evaluation of Antibacterial Activity of *Calotropis gigantea* Latex Extract on Selected Pathogenic Bacteria.

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ABSTRACT

The search for novel antibiotics like compounds from medicinal plants continues to be of extreme importance in recent research programs around the world because of the increase of multidrug resistance and toxicity of some used antibiotics. One such medicinal plant used since ancient times is *Calotropis gigantea*. The plant produces large quantity of latex with various medicinal properties. The present study was aimed to evaluate the antibacterial effect of partially purified *C.gigantea* latex extract on some human pathogenic bacteria. The antibacterial activity was tested against three Gram positive and three Gram negative bacteria. The inhibitory effect was assessed by well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined by serial dilution method. The ethanolic extract was subjected to qualitative phytochemical screening for the presence of bioactive ingredients. The ethanolic extract shows the presence of many biologically active molecules such as flavonoids, alkaloids, triterpenoids, steroids, saponins and glycosides. The latex extract shows significant zone of inhibition in dose dependent manner. The MIC and MBC values for the latex extract against both Gram positive and Gram negative bacterial strains varies from 1mg to 5mg and the results are comparable with chloramphenicol. From this study, we conclude that the latex extract possesses potent bactericidal activity which may be due to the presence of biologically active ingredients with antimicrobial activity in the ethanolic extract of *C. gigantea*. The present study would also contribute to the acceptance of *C. gigantea* latex in traditional medicine and to the solution of growing problems of drug resistance by microorganisms.

Keywords: *Calotropis gigantea*, antibacterial activity, minimum inhibitory concentration, minimum bactericidal concentration, zone of inhibition

INTRODUCTION

*Calotropis gigantea* R.Br (Asclepiadaceae) is a xerophytic, erect shrub, growing widely throughout the tropical and subtropical regions of Asia and Africa. This plant is popularly known because it produces large quantity of latex. The plant has potential pharmacological properties.[1] Fractionation of the latex into its rubber and rubber-free fractions affords better insight into its potentials and limitations.[2]

Based on folklore claims, the present study was aimed to evaluate the antibacterial activities of *C. gigantea* latex extract against prominent human pathogenic bacteria by well diffusion method. Besides, the biological activity of the extract in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined. The latex extract has also been qualitatively analyzed for the presence of different phytochemicals.

MATERIALS AND METHODS

Plant material and latex collection

The fresh latex of *C. gigantea* was aseptically collected from the aerial parts of the healthy plants as described by Aworh *et al.*[3] in clean glass tubes containing distilled water to yield a dilution rate of 1:1 (v/v). The plant exsiccate was deposited at the herbarium of the Centre for Advance Studies in Botany, University of Madras, where the plant was identified by a plant taxonomist. The latex mixture was gently handled to maintain homogeneity during transport to the laboratory where it was kept overnight at 4°C.[1]

Preparation of *C. gigantea* latex extract

The supernatant was selectively decanted and centrifuged at 5000xg for 20 min at 25°C. The precipitated material showing rubber aspect (poly-isoprene) was pooled apart and the supernatant was decanted carefully and subjected to exhaustive dialysis using a membrane of 8000 MW cut off against distilled water at 25°C. Finally the samples were centrifuged as previously described and the clear soluble supernatant was collected and lyophilized. The lyophilized fraction was subjected to ethanolic extraction (70% (v/v)) in Soxhlet extractor at room temperature and the extraction process was performed repeating 4 cycles.[4]

The extract was filtered through Whatman No.1 filter paper and filtrate was concentrated with a rotary evaporator under reduced pressure at 60°C to afford crude ethanolic extract. The dry-crude extracts were irradiated with ultraviolet light for 24 h for sterilization; sterility was confirmed by plating the sample suspension on Muller-Hinton (MH) agar and stored in labeled sterile brown glass contain-
Phytochemical screening of *C. gigantea* latex extract

The phytochemical screening of the ethanolic extract of *C. gigantea* latex was performed qualitatively for the presence of alkaloids, glycosides, flavonoids, polyphenols, tannins, saponins, sterols, triterpenes according to the method of Harborne et al.\(^{[5]}\)

**Bacterial strains and growth medium**

Three Gram positive bacteria (*Staphylococcus aureus*, MTCC-740; *Bacillus subtilis*, MTCC-441 and *Staphylococcus epidermidis*, MTCC-3615) and three Gram negative bacteria (*Escherichia coli*, MTCC-4437; *Shigella dysenteriae*, ATCC-1457 and *Salmonella typhi*, MTCC-733) were used in the present study. The bacterial strains were all standard laboratory strains obtained from the stock cultures of the Division of Microbiology, CAS in Botany, University of Madras, Chennai and maintained on slopes of Muller Hinton Agar (MHA) and sub-cultured every 15\(^{th}\) day to prevent pleomorphic transformation. The bacterial cultures were appropriately diluted in sterile normal saline solution to obtain the cell suspension at 10\(^8\) to 10\(^9\) CFU/mL.

**Preparation of inoculum**

The suspension for inoculation was prepared from the broth culture. Few colonies of similar morphology of the respective bacteria were transferred with the aid of a sterile inoculating loop to a Muller-Hinton broth and were incubated until adequate growth of turbidity equivalent to McFarland 0.5 turbidity standard (10\(^8\) CFU/mL) were obtained.

**Preparation of the McFarland standard**

Add 0.5 ml of 0.048M BaCl\(_2\) to 99.5 ml of 0.18M H\(_2\)SO\(_4\) with constant stirring. Distribute the standard in to screw cap tubes of the same size and with the same volume as those used in growing the broth culture. Seal the tubes tightly to prevent loss by evaporation. Store protected from light at room temperature. Vigorously agitate the turbidity standard on a vortex mixture before use. Standards may be stored for up to 6 months, after which time they should be discarded.

Antibacterial activity of the ethanolic extract of *C. gigantea* latex was evaluated by agar well diffusion method.\(^{[6]}\) The inocula with respective tested bacteria were homogenously seeded onto the 90mm Petri dishes containing 20 ml of cooled molten MH agar medium using a sterile swab in such a way as to ensure thorough coverage of the plates and a uniform thick lawn of growth following incubation.\(^{[7]}\) Wells were dug in the medium with the help of a sterile cork borer. Stock solution of the latex extract (2.5 mg/ml) was prepared in sterile distilled water. Dilutions of the stock solution containing 50, 100, 150, 200 and 250 µg were also prepared in sterile distilled water. 100 µl of each dilution was added to their respective wells with a sterile pipette. Control wells received only 100 µl of sterile distilled water. The plates were kept for 1 h at room temperature for the diffusion of the extract into the agar. Subsequently, all the plates were incubated at 37°C for 18-24 h. Following incubation the plates were examined for signs of microbial growth. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the wells. Chloramphenicol (30 µg/ml) was used as positive control. Each experiment was carried out in triplicates.

**Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) assays**

A serial of 2-fold macro-broth dilution method was performed to determine the MICs and MBCs of *C. gigantea* latex extract for the respective tested bacterial suspensions (concentration) as recommended by the Clinical and Laboratory Standards Institute (CLSI).\(^{[8]}\) The stock solutions of *C. gigantea* latex extract was diluted suitably as required from stock solution. The ranges should be prepared one step higher than the final dilution range required that if a final dilution range of 0.5, 1, 2, 4, 8, and 16 mg/ml is required then a range of 1, 2, 4, 8, 16 and 32 mg/ml should be prepared to compensate for the addition of an equal volume of inoculums. Two rows of 12 capped test tubes were arranged in the rack. In a sterile 30 ml (universal) screw capped bottle, 8 ml of MH broth containing the required concentration of *C. gigantea* latex extract for the first tube in each row was prepared from the appropriate stock solution already made. The contents of the universal bottle were mixed using a sterile pipette and transferred 2 ml to the first tube in each row. Using a fresh sterile pipette, 4 ml of broth was added to the remaining 4 ml in the universal bottle, mixed well and transferred 2 ml to the second tube in each row. Dilutions were continued in this way to as many as 10 tubes. 2 ml of broth free from *C. gigantea* latex extract was added to the last tube in each row. The density of the bacterial suspension was adjusted (10\(^6\) CFU/ml) to equal that of the 0.5 McFarland standard by adding sterile distilled water as detailed above. The bacterial suspension was suitably diluted (10\(^5\) CFU/ml) and added to the tubes containing MH broth. Chloramphenicol (30 µg) was used as positive control. After incubation at 37°C for 24 h, turbidity of the tubes was assessed visually by comparison to uninoculated control. The MIC is expressed as the lowest concentration of the latex extract where bacterial growth was not detected by lack of turbidity in the tube. All the assays were tested in triplicate.

The MBC was derived by sub-culturing 100 µl from each tube from the MIC assay onto substance free MH agar plates. The plates were incubated at 37°C for 24 h and the MBC was defined as the lowest concentration of substance that allows no visible growth on the agar plate.

**RESULTS**

In the present study, the ethanolic extract of *C. gigantea* latex yield was 15.5%. The qualitative phytochemical screening of *C. gigantea* latex showed the presence of alkaloids, flavonoids, tannins, steroids, triterpenoids, saponins and glycosides.

Table 1 shows the antibacterial activity of ethanolic extract of *C. gigantea* latex against three different Gram positive and Gram negative bacterial strains. The antibacterial potency of *C. gigantea* latex extract was evaluated by the presence or absence of inhibition zones and zone diameters (mm). From the results, it is evident that the
Table 1. Antibacterial activity of Calotropis gigantea latex extract - Zone of inhibition in diameter (mm)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Bacterial species</th>
<th>Control</th>
<th>50 µg</th>
<th>100 µg</th>
<th>150 µg</th>
<th>200 µg</th>
<th>250 µg</th>
<th>Chloramphenicol (30 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Staphylococcus aureus</td>
<td>-</td>
<td>2.5</td>
<td>9.0</td>
<td>14.0</td>
<td>21.5</td>
<td>22.0</td>
<td>23</td>
</tr>
<tr>
<td>2.</td>
<td>Bacillus subtilis</td>
<td>-</td>
<td>-</td>
<td>8.5</td>
<td>16.0</td>
<td>25.0</td>
<td>26.0</td>
<td>25</td>
</tr>
<tr>
<td>3.</td>
<td>Staphylococcus epidermidis</td>
<td>-</td>
<td>3.0</td>
<td>10.0</td>
<td>17.5</td>
<td>20.0</td>
<td>21.5</td>
<td>24</td>
</tr>
<tr>
<td>4.</td>
<td>Escherichia coli</td>
<td>-</td>
<td>4.0</td>
<td>10.3</td>
<td>18.0</td>
<td>23.0</td>
<td>23.0</td>
<td>20</td>
</tr>
<tr>
<td>5.</td>
<td>Salmonella typhi</td>
<td>-</td>
<td>1.2</td>
<td>9.5</td>
<td>19.0</td>
<td>22.0</td>
<td>23.5</td>
<td>27</td>
</tr>
<tr>
<td>6.</td>
<td>Shigella dysenteriae</td>
<td>-</td>
<td>-</td>
<td>4.0</td>
<td>9.0</td>
<td>15.0</td>
<td>17.0</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 2. MICs and MBCs of Calotropis gigantea latex extract on Gram positive and Gram negative bacteria

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Minimum Inhibitory Concentration (MIC)</th>
<th>Minimum Bactericidal Concentration (MBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. gigantea latex extract (mg/ml)</td>
<td>Chloramphenicol (µg/ml)</td>
</tr>
<tr>
<td>Gram positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Gram negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

ethanolic extract of C. gigantea latex showed a maximum inhibitory zone in a dose dependant manner. However, there was no significant difference between the levels of zone of inhibition at the concentration of 200 µg and 250 µg. The antibacterial potency of C. gigantea latex extract on the Gram positive bacteria, B. subtilis showed a larger diameter of clearance than that of other Gram positive bacteria used in this study. Similarly, C. gigantea latex extract showed a maximum zone of clearance in the Gram negative bacteria, S. typhi than that of other Gram negative bacteria. Moreover, the zone of clearance achieved by C. gigantea latex extract is comparable to that of standard antibiotic, chloramphenicol.

The minimum inhibitory concentration and minimum bactericidal concentration of C. gigantea latex extract and the standard antibiotic, chloramphenicol was depicted in Table 2. The MIC value of C. gigantea latex extract against both Gram positive and Gram negative bacterial strains varies from 1 mg to 5 mg and the results are comparable with the standard antibiotic, chloramphenicol.

**DISCUSSION**

Drug resistance to human pathogenic bacteria has been reported all over the world and the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. Plants are important source of potentially useful structures for the development of novel chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial assay. Samy and Ignacimuthu have studied the antibacterial activity of some folkloric medicinal plants used by tribes in Western Ghats of India. Only the alcoholic extract was used in the present study, as ethanol was reported to be a better solvent for extracting the antimicrobial active substances from Calotropis compared to other solvents. The bacterial strains used in the present study were chosen on the basis of their clinical importance. For example, *S. aureus* is the most common species found in purulent wound and is often associated with high microbial load in chronic wounds. It acquires resistance due to the presence of penicillin-binding protein of high molecular weight and has a very low affinity for lactamic antibiotics.

According to Kareem et al., agar well diffusion method allows better diffusion of the extracts into the medium, thus enhancing contact with the organisms. Paper discs may act as a barrier between the extract and the organisms, thus preventing total diffusion of active components absorbed by the discs into the medium and may reflect in antibacterial activity. Hence, agar well diffusion method was performed in the present study to investigate the antibacterial activity of C. gigantea latex extract. The latex extract evidenced different inhibitory properties against Gram positive and Gram negative bacterial strains. The highest activity (diameter of zone of inhibition 27 mm) was demonstrated by the ethanolic extract of C. gigantea against *S. subtilis*, while the lowest activity was observed against *S. dysenteriae*. The greater resistance of Gram negative bacteria to latex extract may be due to the differences in the cell wall structure between Gram positive and Gram negative bacteria; the Gram negative bacteria outer membrane acting as a barrier to many substances, including antibiotics. However, the results of the *in vitro* antibacterial assay revealed that the growth of both Gram positive and Gram negative bacteria strains were affected by the latex extract by forming clear inhibition zones. The observed low bioactivity of the crude latex extract would result either from dilution of its active constituents or from the antagonism in the bioactivity among extract constituents.

Minimum inhibitory concentrations are considered the “gold standard” for determining the susceptibility of microorganisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definite answer when a borderline result is obtained by other methods of testing, or
when disc diffusion methods are not appropriate. Results of MICs and MBCs showed that S.typhi had the highest MIC (5 mg/ml) and MBC (6 mg/ml) while the lowest MIC of 1 mg/ml was demonstrated by E.coli and S.epidermidis. The observed susceptibility may be due to the difference in the cell wall composition of Gram positive and Gram negative bacteria.[17] It has been reported that antibiotics at their sub MIC have numerous effects on bacteria, including morphological changes, modifications of cell wall structure, altered growth kinetics, inhibition of enzyme or toxin production and loss of adhesive properties.[18]

Studies on the antibacterial activity of C.gigantea latex are scanty in the literature. However, few reports are available on the antimicrobial activity of C.procera. Recently, Parabia et al. have reported that the crude fractions of apical twigs and latex of C.procera possess significant inhibitory effect on twelve tested bacterial strains.[19] However, they found that the apical twigs of the plant produced wider inhibition zones than the fractions of latex. Similarly Kareem et al. reported that the ethanolic extract of C. procera latex and leaves have demonstrated strong inhibitory effect on the test microorganisms and the inhibitory effect was more pronounced in the latex extract.[13]

The aqueous and methanolic extracts of leaves of C.procera were found to exhibit both antibacterial and antioxidant properties. However, the aqueous extract was reported to have mild antioxidant activity.[20] The presence of pharmacologically active phytochemicals in the ethanolic extract of C. gigantea may provide a justification for the observed antibacterial activity. Phytochemical constituents such as flavonoids, alkaloids, tannins and other aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and herbivores. Hassan et al. have reported the presence of alkaloids, flavonoids, tannins, steroids, triterpenoids, saponins and saponin glycosides in the leaves and root fractions.[21] However, the stem part extract was reported to contain only flavonoids, triterpenoids and saponins. Results so far have already been reported by Mossa et al.[22] Akhtar et al. isolated and characterized a novel free cardenolide named proceragenin from latex extract of C. gigantea with potent antibacterial activity from C. procera.[23] The demonstration of antibacterial activity of C.gigantea latex extract against both Gram positive and Gram negative bacteria may be an indicative of the presence of broad spectrum antibiotic compounds.[24]

To our knowledge, the present study is the first one which systematically reports the antibacterial activities of C. gigantea latex extract. The results of our preliminary screening assays justify the use of C. gigantea latex extract in the Indian ethnomedicine. However, it is important to note that the crude extract of C. gigantea latex need to be further purified through antibacterial activity guided fractionation to isolate and identify the compounds responsible for antibacterial activity.

In conclusion, the remarkable bactericidial effects of C. gigantea latex extract suggest that the latex may be a useful source for the development of novel antibiotics against pathogenic bacteria.

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