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# EVALUATION OF PHYTOCHEMICAL AND ANTIMICROBIAL PROPERTIES OF *Calotropis procera* LATEX

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***Abstract:*** ***C. procera*** **is one of the important member of traditional herbal medicine in every home of India. Phytochemicals are natural bioactive secondary metabolites found in plants that act as a defense system against disease. The latex is applied to painful joints and swelling. Fresh leaves are also used for the same purpose. Oil which the leaves have been boiled is applied to paralyzed part. Some of the medicinal properties may be facilitated by its microbial endophytes. The interactions between the host plant and their endophytic microorganisms are not yet fully understood. Hence in this study an attempt is made to evaluate the phytochemicals present in the latex. Further, antimicrobial activity of the latex was also evaluated. Endophytic microorganisms that resides in the leaf and latex was isolated and the plant growth promoting ability of the isolates were analyzed by a series of assay.**

***Keywords:*** **traditional herbal medicine, Endophytic microorganisms, natural bioactive.**

## 1. INTRODUCTION

Plants have been endowed with innate ability to synthesize aromatic substances. These secondary metabolites have therapeutic properties. Due to the potential health benefits with medicinal value, plant extracts and their exudates have been widely used as natural antimicrobials (Hsieh *et al*., 2001). Some of the metabolites of plant origin have been successfully used in the treatment and prevention of infectious diseases, cancer or in stimulating the immune system. The medicinal value attributed to plants is a function of the bioactive phytochemical constituents that produce definite physiological action on the human body (Akinmoladun *et al*., 2007). The most important of these plants bioactive chemical constituents are flavonoids, alkaloids, tannins and phenolic compounds (Edeoga *et al*., 2005). Phytochemicals are natural bioactive secondary metabolites found in plants that act as a defence system against disease. These phytochemicals are divided into two groups based on their functions in plant metabolism as primary and secondary constituents. Primary constituents comprise sugars, amino acids, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoids and phenolic compounds (Krishnaiah *et al*., 2009) and many more such as flavonoids, tannins, etc.

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further they protect plants from disease and damage and contribute to the plant's colour, aroma and flavour.

*Calotropis procera* is a species of flowering plant which belongs to the family Asclepaiadaceae. It is commonly known as Apple of Sodom (Irvine, 1961). The English name is milk weed. In Western part of Nigeria, it is called Bomubomu by the Yorubas, the Hausas call it tumfafia, in Sudan it is called Oshar. It is called calotrops in Italian, pomme de sodome in French and kisher in Arabic. *C. procera* is an evergreen shrub with a simple architecture, comprising of a single or few primary

stems emerging from the soil and giving rise to several secondary and tertiary stems. The plant is native to South Western Asia and Africa (Abbasi *et al*., 2003). In North-Eastern Brazil, especially in the dry forest called Caatinga, it is adapted to the sandy, acid and high aluminium content soils, and has become widespread, (Oliveira *et al*., 2009). *C. procera* is also found under high saline conditions along the coastal dune vegetation of North Eastern Brazil.

*C. procera* is a well- known medicinal plant in India. *C. procera* is one of the important member of traditional herbal medicine in every home of India. It strongly recommended in leprosy, hepatic and splenic enlargements, dropsy and worms. The latex is applied to painful joints and swelling. Fresh leaves are also used for the same purpose. Oil which the leaves have been boiled is applied to paralyzed part. The milky juice is used as purgative, while flowers are considered as digestive, stomachic, tonic and useful in cough, asthma and loss of appetite. The root bark is said to promote secretion and to be useful in treating skin disease, enlargement of abdominal viscera, intestinal worms, ascites and anasarca.

Traditionally the leaves are warmed and tied around any body organ in pain. It is practically useful in backache and in joint pains. Warm leaves also relieve from stomach ache if tied around. Inhalation of burnt leaf cures headache. The traditional folk healers use the milky latex for several ailments. Leaf latex if applied on fresh cut, stops bleeding immediately. Recent investigations have found that the alkaloids, calotropin, calotaxein and uskerin are stimulant to the heart. Flowers and roots are used in Ayurvedic medicine (Poovizhi and Krishnaveni, 2015).

Parotta (2001) reported that the secretions of *C. procera* are used traditionally in India for the treatment of skin diseases, enlargements of abdominal viscera and intestinal worms. In Nigeria, it has been used traditionally to treat diseases like fever, eczema, diarrhoea, leprosy, ringworm, cough, asthma and convulsion (Odugbemi, 2006). *C. procera* has many important medicinal properties with proven pharmacological potential. Some of the medicinal properties may be facilitated by its microbial endophytes. Biodiversity of endophytic microbes in the leaves of *C. procera* and their antimicrobial activity was studied by Nascimento *et al*., (2015).

Medicinally important parts of this plant are flower, terminal leaf pairs, root and latex. Out of that the most significant part which drew attention of researchers is latex because of its medicinal properties, economic use for rubber production. Latex contain majority of hydrocarbon contents (Erdman and Erdman, 1981). Kumar *et al*., (2001) have established this plant as a source of bio energy. The plant also shows good antibacterial activity (Parabia *et al*., 2008).

Endophytes reside asymptomatically in apoplastic spaces and/or within the living cells of plants for all or at least a significant part of their life cycles (Petrini, 1991). Evolving in this very specific ecological niche, where the endophyte and its host are subjected to constant and unique interactions, may have favoured the ability of endophytes to produce bioactive natural compounds, some of which are potentially useful in medicine, agriculture and industry (Strobel, 2003).

The dynamics of the interactions between the host plant and their endophytic microorganisms are not yet fully understood. In addition to environmental factors (temperature and humidity), variations in chemistry, anatomy and maturity of colonized host tissue affect the abundance and community composition of endophytes (Sanchez-Azofeifa *et al.*, 2012). The endophytic community may dramatically change in relation to plant age (Fernandes *et al*., 2011 and Hilarino *et al*., 2011) and even with the type of plant tissue (Tejesvi *et al*., 2005; Gond *et al*., 2007; Sanchez-Azofeifa *et al*., 2012). These inconspicuous microorganisms are found in virtually all plants (Arnold, 2007). Strobel (2003) suggested that the chance of finding new and potential active substances produced by the endophytes are higher in plants in unique environments that present new strategies for survival under stress conditions, and those with a history of ethno botanical interest. To ensure the success of investigations with endophytes with pharmacological potential, scientists have suggested to isolate endophytes preferably from plants with medicinal value.

The structure and composition of the endophytic microbial community of *C. procera* is under explored (Gherbawy and Gashgari, 2013; Aharwal *et al*., 2014). This invasive plant has a great pharmacological potential, as indicated by its widespread use in African, Indian, and Middle Eastern cultures (Iqbal *et al*., 2005). Various studies have shown some of its pharmacological activities. These biological properties include its anti-inflammatory (Kumar and Basu, 1994), analgesic (active ingredients similar to aspirin) (Dewan *et al.*, 2000), antidiarrhoeal (Kumar *et al.*, 2001), insecticidal (Ahmed *et al*., 2006), antifungal and antibacterial (Kareem *et al*., 2008; Yesmin *et al*., 2008) activities. Therefore, the further knowledge of the endophytic community in *C. procera* represents an important step towards a better understanding of its pharmacological potential. The goals of this study is to evaluate the physicochemical constituents in the latex exudates of *C. procera*, occurrence of endophytic bacteria in the latex and in the leaves of *C. procera* and the influence of leaf age on the occurrence of endophytic bacteria is analysed. Further, the endophytic bacteria is characterized, their probiotic potential

and biofilm activity is elucidated. Finally the antibacterial activity and anti-inflammatory activity of the plant exudates is analysed.

**Objectives**

- Phytochemical analyses of *C. procera* latex.
- Elucidation of antimicrobial activity of *C. procera* latex.
- Isolation of endophytic bacteria and fungi from the leaves and latex of *C. procera*.
- Screening of plant growth promotion potential of the bacterial isolates.
- *In-vitro* anti-inflammatory activity of the latex.
- Susceptibility of the bacterial isolates to different commercially available antibiotics.

## 2. MATERIALS AND METHODS

***Collection of plant samples***

Fresh leaves and latex of *Calotropis procera* was collected from the campus of Aditanar College of Arts and Science, Virapandiapatnam. The plant was characterized and authenticated at the Department of Botany, Aditanar College of Arts and Science, Virapandiapatnam. The leaves were hand plucked aseptically and cleaned for debris using tap water and then rinsed in sterile distilled water and surface sterilized. The leaves were airdried in the shade at room temperature. The dried leaves were blended using a domestic electric blender, powdered samples were stored in air tight glass containers protected from sunlight for subsequent extraction and further bioassay.

***Collection and preparation of latex extracts***

The method of crude latex collection described by Nenaah and Ahmed (2011) was employed. Latex from the young leaves close to the tip of branches was collected as the leaves were plucked from the branches. The latex was collected into a sterile bottle. The bottle was shaken slightly during collection to avoid coagulation. This was air-dried under shade. Petroleum ether was then added and filtered, so as to remove chlorophyll pigments and rubber materials that could be present. The obtained latex extract was dried and stored at 4º C for further analyses.

***Test organisms***

The aqueous latex extract was tested for antibacterial and antifungal activities. The antimicrobial activity was tested against four bacterial species (*Streptococcus* sps., *Bacillus* sps., *Micrococcus* sps., and *Pseudomonas* sps.) and two species of fungi (*Fusarium* sps., *and Penicillium* sps.).

***Antimicrobial activity***

*In vitro* antimicrobial test was performed by agar well diffusion method on Mueller Hinton agar and Sabouraud Dextrose agar for bacteria and fungi respectively. Mueller Hinton Agar with a pH of 7.2 ± 0.2 medium was poured into the plates to a uniform depth of 7mm and refrigerated for solidification. Prior to use, the plates were transferred to the incubator at

37º C for 30 minutes to clear the moisture content that develops on the agar surface. The plates were inoculated with selected bacterial strains separately by means of cotton swab to ensure the confluent growth of the organism. Wells of 7 mm in diameter were cut into these agar plates and 50 µL of latex was added in each well. The plates were incubated for 24 hours at 37º C and were subsequently examined for the clear zones surrounding each well. Zone of inhibition was measured by the diameter of the zone in millimetres (mm) and the results were recorded.

***Antifungal activity***

Antifungal activity of the test extracts were evaluated by the agar well diffusion method against *Fusarium* and *Penicillium* was described by Afolayan and Ashafa (2009) with slight modification. Briefly, to a prepared potato dextrose agar, about 500 µl of the actively growing *Fusarium and Penicillium* were inoculated. Wells of 7 mm bored on the plates 0.05 ml of the exudates was then introduced in the well. The plates were then incubated for 48 h at 37º C, after which the inhibition zone obtained around the well was measured. Control were plates containing microorganisms without the extracts. The experimenters were carried out in triplicates under aseptic conditions.

***Physicochemical analyses***

The methods described by Krishnaiah *et al*., (2009) and Mikail (2010) were adopted for the determination of phytochemicals in the latex of *Calotropis procera.*

***Test for alkaloids***

500 µL of latex was mixed with 2.5 mL of 2% hydrochloric acid. The solution was divided into 2 aliquots; to the first test tube which acted as a reference, 1 mL of distilled water was added. To the second test tube, 2 drops of Mayer's reagent (Potassium mercuric iodide solution) was added. Appearance of turbid white precipitate in the second test tube illustrates the presence of alkaloids (Raffauf, 1962).

***Test for Tannins***

0.5 mL of latex was boiled with 10 mL of distilled water in a boiling tube and filtered through a filter paper. Few drops of 0.1 % of $FeCl_3$ was added to this filtrate. Appearance of blackish blue colour indicated the presence of Gallic tannins and green blackish colour indicated catechol tannins.

***Test for Flavonoids***

500 µL of latex was added with one mL of 1 % Ammonia solution. Appearance of yellow color illustrates the presence of flavonoids in the sample.

***Test for Steroids***

500 µL of latex was dissolved in 0.25 mL of acetic acid followed by the addition of 0.25 mL chloroform. The solution was pipetted out into a dry test tube followed by the addition of 500 µL con. $H_2SO_4$. A brown-red ring at the interface between the two liquids and a green supernatant indicated the presence of steroids.

***Test for Terpenoids***

2.5 mL of latex was mixed with 1 mL of chloroform followed by the addition of con. $H_2SO_4$ on the sides of the tube. Appearance of reddish brown color illustrates the presence of terpenoids in the sample.

***In vitro anti-inflammatory analysis***

Anti-inflammatory activity of latex was carried out under *in vitro* conditions.

Membrane stabilization test described by Angajala *et al.* (2014) was followed for anti-inflammatory analyses.

***Membrane Stabilization test***

Human blood (10mL) was collected from a healthy volunteer, transferred to heparinized centrifuge tubes and centrifuged at 3000 rpm for 10 min. After centrifugation, the pellet was washed and reconstituted to 10 % with normal saline. Two milliliter of latex at different concentrations (50, 100, 150, 200, 250 µL/mL) were mixed with equal volume of 10% v/v of RBC suspension and incubated in a water bath at 50° C for 30 min. Reaction mixture was centrifuged at 2500 rpm for 5 min and absorbance of the supernatant was measured at 560 nm. A control was maintained without the addition of Latex.

***Screening of bacterial isolates***

Bacteria were isolated from the latex by serial dilution as per the procedure described by Cappuccino and Sherman, (1999). One milliliter of latex was mixed with 9 mL of sterile distilled water, followed by serial dilution in the range $10^{-1}$ to $10^{-10}$. 200 µL of the diluted samples were spread over the nutrient agar plate and incubated for 24 hours. Similarly the endophytic bacteria were isolated after surface sterilizing the leaves of *Calotropis procera* by serial dilution technique. Bacterial colonies were differentiated according to the colony morphology and individual colonies were streaked on fresh nutrient agar plate to attain pure culture. All pure bacterial colonies were maintained on nutrient agar slant and stored at 4° C.

***Screening for plant growth promotion attributes Phosphate solubilization***

Phosphate solubilizing ability of the isolates was determined qualitatively and quantitatively using Pikovskaya agar medium (Pikovskaya, 1948) with 0.5 % tricalcium phosphate [$Ca_3$ $(PO_4)_2$] as inorganic phosphate source. The endophytic isolates were streaked on Pikovskaya agar medium and incubated at 30˚C for 48 h. Formation of clear halo around the isolates on the agar plate after 48 h of incubation indicates phosphate solubilization. For quantitative estimation, 1 mL of bacterial

suspension ($3x10^7$ cells) was inoculated into 100 mL of Pikovskaya broth medium and incubated for 72 h at 37º C without shaking. The phosphate content in the culture supernatant (10,000 rpm for 10 min) was quantified spectrophotometrically (600 nm) by stannous chloride method (Gaur 1990) at different time intervals. The change in pH of the medium following tricalcium phosphate solubilization was also recorded using a pH meter.

***Indole acetic acid production***

Indole acetic acid (IAA) production by the bacterial isolates was determined following the method described by Patten and Glick (2002) with slight modifications. One millilitre of bacterial suspension ($3x10^7$ cells) was inoculated in 100 mL of nutrient broth amended with L- tryptophan (100 µg $mL^{-1}$) and incubated for 72 h at 30º C with shaking at 125 rpm. The concentration of IAA in cell free supernatant (10,000 rpm for 10 min) was quantified at different time intervals. The culture supernatant (5 mL) was mixed with two drops of ortho phosphoric acid and 4 mL of the Salkowski reagent (50 mL of 35 % of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution) and allowed to stand at room temperature for 20 min. The concentration of IAA in the supernatant was determined against a standard using a spectrophotometer (540 nm).

***Detection of hydrogen cyanide and ammonia production***

The isolates were screened for hydrogen cyanide production following the method of Bakker and Shipper (1987). The bacterial isolates were streaked over the nutrient agar plates supplemented with glycine (0.4g/100 mL). Sterilized filter paper impregnated with 2 % sodium carbonate in 0.5 % picric acid solution was placed at the top of the plate, sealed and incubated for 48 h at 30° C. Change in the colour of the filter paper from yellow to orangebrown indicates cyanide production. Ammonia producing ability of the isolates were determined by adding Nessler's reagent (0.5 mL) to the bacterial culture in peptone water (10 mL) at mid log phase. Development of brown colour indicates ammonia production.

***Media for isolation***

Sabouraud agar medium was used for culturing fungi. An antibiotic (Streptomycin) was added with the medium after sterilization at a concentration of 150 µg per ml, to inhibit bacterial growth. 0.1 ml, of $10^{-1}$ and $10^{-2}$ dilutions were spread over the solid agar surface. The plates were then incubated at room temperature for 4 days to ensure complete development of fungal colonies. Nutrient agar containing petriplates were used for culturing bacterial colonies 0.1 ml of $10^{-3}$, $10^{-4}$ and $10^{-5}$ dilutions were used for isolation of colonies using spread plate technique. A replica was maintained for each concentration. The petriplates were incubated at 37° C for 48 hours. Pure culture of bacterial colonies was obtained by streak plate method, which established a concentration gradient of bacteria across the surface of solid agar medium in petriplates.

***Selective media for Escherichia coli***

Pure culture of *E. coli* colonies was obtained using Eosin Methylene Blue agar media.

***Selective media for Staphylococci***

Mannitol – Salt agar was used for the isolation of *Staphylococcus sp*.

***Identification of Fungi***

The fungi isolated were identified by observing the colony morphology of the isolates and by observing the microscopical appearance of the spores. Microslide culture method developed by Riddell (1950) was adopted for studying the microscopic morphology.

***Identification of Bacteria***

The isolated endophytic bacteria was then assessed for colony characteristics followed by morphological tests *viz*., cell shape, Gram reaction, and motility. The strain were identified by an array of biochemical tests following the procedure described by Cappucino and Sherman (1999). Finally, they were confirmed by their growth on selective media.

***Cell shape***

The purified cultures, at log phase were observed microscopically for the cell morphological characteristics.

***Gram staining***

Gram staining was carried out following the procedure described in Cappucino and Sherman (1999). The procedure divides most of the bacteriological assortment of rods, cocci and spirals in to two large additional groupings. After adding Gram stain, cells that looked identical become separable as purple gram-positive organisms and pink gram-negative ones. The gram stain technique is a differential staining procedure that distinguish various microorganisms, according to their ability to retain certain dyes.

In Gram staining a primary stain, crystal violet was applied to a dried, beat fixed smear of bacteria. Then a mordant, (a substance that intensifies the reaction between cells and stain) Gram's Iodine was poured on to the smear. The mordant reacts with crystal violet and the bacteria. After a quick water rinse, a decolouriser usually 95% Ethyl alcohol was briefly applied (a decolouriser removes colour from cells). Gram – negative cells decolourise faster with alcohol than do gram-positives. After another water rinse, counter stain Safranin was added to differentiate gram-negative cells. The slides were viewed with the light microscope under oil-immersion. Gram-positive bacteria appear violet and gram-negative bacteria appear pinkish red.

***Spore staining***

Spore staining was done as follows. The bacterial smear on a glass slide was air dried and fixed with minimal flaming. The slide was placed over a beaker of boiling water, resting on the rim with the bacterial film uppermost. After a few seconds, when large water droplets were condensed on the underside of the slide, the smear was flooded with 5% aqueous solution of malachite green and allowed to react for a minute. The same procedure was repeated twice. Then the slide was washed in cold water. Later, the slide was treated with 0.5% Safranin and washed with tap water. It was allowed to air dry and examined under the oil immersion objectives (Cruickshank R. 1975).

***Biochemical tests***

The following biochemical tests were performed to identify the species of bacteria as described by Cappuccino and Sherman (1999).

***Oxidase test***

Two drops of the bacterial isolates were added in the sterile oxidase discs and the slides were observed for the colour change from pink to maroon and finally to purple within 30 sec indicated a positive reaction.

***Nitrate reduction test***

The endophytic isolates were inoculated into 10 mL of nitrate broth taken in test tubes and the tubes were incubated at 30° C. After 14 days, 2 mL of the broth was tested by adding equal amounts of sulfanilic acid and alpha naphthylamine. These chemicals react with nitrate and produce red colour. Development of red colour indicated that nitrate had been reduced to nitrite. To confirm the presence of nitrate in the negative solution, a bit of zinc added reduces nitrate to nitrite and develops red colour which confirms the negative reactions.

***Hydrogen sulphide production***

Sulfide indole motility (SIM) agar stabs were inoculated with the isolates and incubated at 30° C for 48 hr. Formation of insoluble black colour precipitate along the line of stab inoculation indicated $H_2S$ production.

***Catalase activity***

A loopful of 24h old culture of bacterial isolates maintained on nutrient broth were transferred to a glass slide and mixed thoroughly with few drops of 3% hydrogen peroxide solution and observed for the presence of the effervescence. Development of gas bubbles indicates the positive result.

***Indole production***

The isolates were inoculated into sterilized glucose tryptone broth taken in test tubes and the tubes were incubated at 37° C. After 48 h of incubation, 0.3 mL of Kovacs reagent was added and mixed well. The reddening of the alcohol layer within few minutes indicated indole production.

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***Methyl red and Voges- Proskauer test***

The Methyl red and Voges-Proskauer (MR-VP) broth prepared in two sets was inoculated with the isolates and incubated for 48 h at 37° C. To the first set of tubes, few drops of an alcoholic solution of methyl red were added. The development of distinct red colour was indicative of positive reaction for MR test. Naphthol solution (5 per cent solution in 70 per cent ethyl alcohol) was added to the second set of tubes and shaken gently for 15 min. The positive reaction of acetyl methyl carbinol production was indicated by development of red colour. This indicates positive result for the VP test.

***Citrate Utilization test***

The isolates were inoculated into test tubes having Simmons citrate agar medium and incubated for 48 h at 37° C. Simmons citrate agar contained citrate as its only carbon and energy source. The presence of growth and change of colour from green to blue due to pH change indicated positive reaction.

***Antibiotic susceptibility test***

The susceptibility of bacterial isolates to different antibiotics was determined by placing standard antibiotic discs on the surface of Muller Hinton agar medium spreaded with isolates as a lawn. The plates were observed for zone of inhibition after 24 hrs incubation at 37$^{o}$ C.

## 3. RESULTS

**Table 1. Phytochemical Constituents in the leaf and latex of Calotropis procera**

| **Phytochemicals** | **Name of the test** | **Response** | |
|---|---|---|---|
| | | **Leaf extract** | **Latex** |
| Tannins | $FeCl_3$ test, Lead acetate test | - | - |
| Steroids | Salkowski test | + | + |
| Flavanoids | Ammonia test | + | - |
| Saponins | Frothing test | + | + |
| Protein and aminoacids | Ninhydrin test | + | + |
| Alkaloids | Wagners test | + | + |
| Carbohydrates | Molischs test | - | - |
| Cardiac glycosides | Keller Killiani test | _ | - |
| Terpenoids | Salkowski test (Modified) | + | + |

Table 1 reveals the qualitative analysis of the phytochemical constituents in the latex and aqueous leaf extract of *Calotropis procera*. Steroids terpenoids, alkaloids, saponins were found to be present in the latex and leaf extract. Steroids and saponins were analysed by Salkowski test and Frothing test respectively. Tannins, cardiac glycosides and carbohydrates (reducing sugar) were found to be absent. The notable feature is the absence of flavonoids in the latex which was found to be present in the aqueous leaf extract.

**Table 2. Prevalence and Colony forming unit of endophytic bacteria and fungi in leaves and latex of C. procera**

| **Source** | **Bacteria** | | **F** | **ungi** |
|---|---|---|---|---|
| Leaves age | CFU | No | CFU | No |
| 1 | 1.2 $X10^2$ | 2 | 1.0 $X10^2$ | 3 |
| 3 | 1.0 $X10^2$ | 3 | 1.0 $X10^2$ | 3 |
| 5 | 0.8 $X10^2$ | 4 | 0.6 $X10^2$ | 3 |
| 7 | 0.6 $X10^2$ | 2 | 0.6 $X10^2$ | 3 |
| Latex | 0.6 $X10^2$ | 2 | 1.0 $X10^2$ | 5 |

No- Number of different bacteria isolated; CFU – Colony Forming Unit

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**Figure 1. Different leaf ages of C. procera**

Endophytic bacteria and fungi in the latex and leaves of *C. procera* categorized into different leaf ages (Fig. 1) namely 1 to 7 (Distal leaf, youngest =1; last leaf at the stem bassenescent =7) were enumerated and categorised. The results are presented in Table 2. Compared to the latex leaves of different ages found to harbour more number of microbes. Of the two different microbes analysed fungal population outnumbered bacteria. Isolated endophytic bacteria and fungi were distinguished morphologically. Based on the morphological evaluation all the bacteria isolated from leaves were differentiated into two strains. The two strains isolated from latex were different from that isolated from the leaves.

So, totally four different endophytic bacteria were isolated from the leaves and latex of *C. procera*. Endophytic fungal occurrence was found to be greater in number in latex than in fungi. A total of five different fungal strains were identified in the latex of the five only three where found to be present in different leaves of *C. procera.*

**Table 3. Morphological and biochemical characteristics of the bacterial isolates**

| **Biochemical tests** | **BCLA1** | **BCLA2** | **BCLE1** | **BCLE2** |
|---|---|---|---|---|
| Colour | Pale Orange | White | Pale orange | White |
| Gram Staining | Negative | Positive | Positive | Positive |
| Shape | Rod | Rod | Cocci | Cocci |
| Indole Production test | Positive | Negative | Negative | Negative |
| Catalase | Positive | Positive | Positive | Positive |
| Methyl Red | Positive | Negative | Positive | Negative |
| $H_2S$ Production | Negative | Negative | Positive | Negative |
| Voges-Proskauer test | Negative | Positive | Positive | Positive |
| Urease | Negative | Negative | Positive | Positive |
| Citrate Utilisation test | Negative | Positive | Positive | Positive |
| Nitrate Reduction Test | Positive | Positive | Positive | Positive |
| Oxidase Test | Negative | Negative | Negative | Negative |
| Species | *Eschericia coli* | *Bacillus* species | *Staphylococcus* species | *Micrococcus* species |

The four endophytic bacteria isolated were identified based on Gram staining reaction and biochemical properties. The morphological and biochemical characteristics of the isolates are presented in the Table 3. The isolates were named initially with the first letter of the plant followed by first two letter from where they have isolated, for example in BCLE1, B for bacteria C for *Calotropis procera*, LE for leaf and subsequent numerical represent the series number (Table 3). Similarly FCLE1 F for fungi C for *Calotropis procera*, LE for leaf and subsequent numerical represent the series number.

A total of 5 different endophytic fungi were isolated from the latex of *C. procera*. Of the five three were found to be present in the different age groups of *C. procera* leaves. The fungi present in the leaves and latex were identified as *A. flavus*, *A. niger*, *A. fumigatus* based on the macro and micro morphological features. In present study, aqueous extracts of leaf and latex of *Calotropis procera* was tested for their antibacterial and antifungal activity against selected strains of bacterial and fungal pathogens collected from microbiology lab. The results obtained are present in table 4.

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**Table 4. Anti-bacterial and anti-fungal activity of Calotropis procera latex against test organisms**

| S.No | Bacterial pathogens | Zone of inhibition (mm) | |
|---|---|---|---|
| | | Aqueous Leaf Extract | Latex (100ul) |
| 1. | *Streptococcus* sps. | 15.00 (±0.52) | 12.00 (±0.57) |
| 2. | *Bacillus* sps. | 10.00 (±0.08) | 6.33 (±0.33) |
| 3. | *Micrococcus* sps. | 10.00 (±0.09) | 8.00 (±0.25) |
| 4. | *Pseudomonas* sps. | 13.00 (±0.15) | 10.28 (±0.32) |
| | **Fungal pathogens** | | |
| 5 | *Fusarium* sps. | 6.00 (±0.23) | 7.00 (±0.17) |
| 6 | *Penicillium* sps | 4.87 (±0.57) | 5.00 (±0.18) |

It is evident from the table that the bacteria were more susceptible than the fungi to the latex of *C. procera*. Of the four bacteria analysed *Streptococcus* was found to be more susceptible followed by *Pseudomonas*, *Micrococcus* and *Bacillus* species (Fig. 2 & 3).

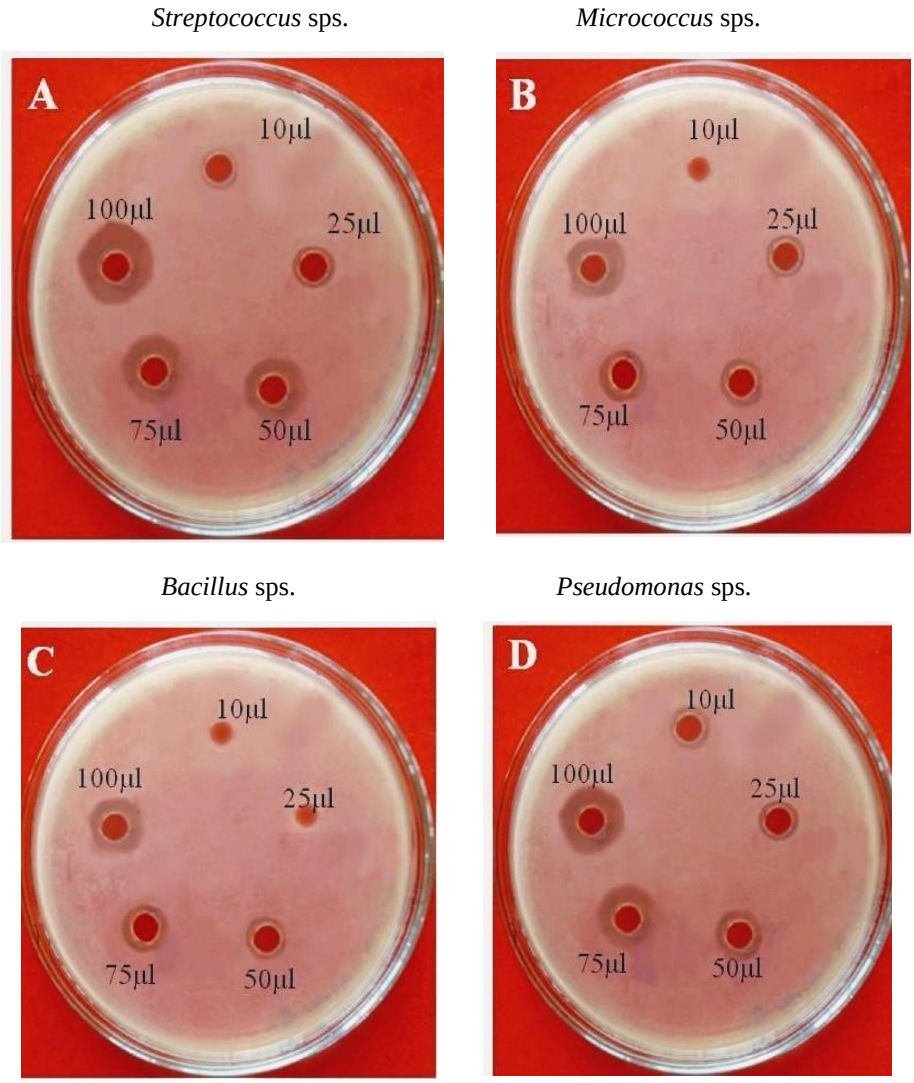


**Figure 2. Anti-bacterial activity of C. procera latex**

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*Fusarium* sps *Penicillium* sps.

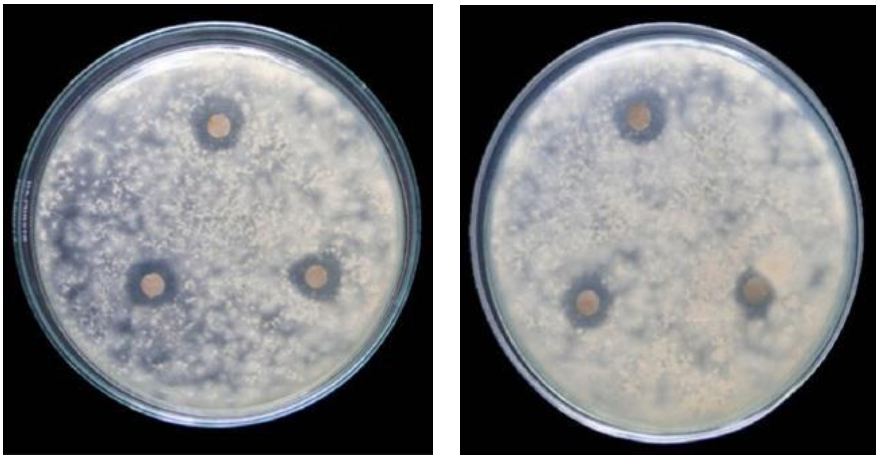

**Figure 3. Anti-fungal activity of C. procera latex**

Anti-inflammatory efficiency of the *C. procera* latex was determined by membrane stabilization inhibitory activity. The latex was found to possess high anti-inflammatory efficacy. Membrane stabilization was found to be maximum at 200 µl/mL of latex and it was found to be 0.58%. However, Aceclofenac®, a well known anti-inflammatory agent exhibited maximum membrane stabilization inhibition at 250 µl/mL and it was found to be 31.70 %. The anti-inflammatory activity of the *C. procera* latex extract was comparatively lesser than the standard drug. (Table 5 and Fig. 4).

**Table 5. Proteinase inhibitory and membrane stabilization activity of the latex of Calotropis procera**

| Concentration (µg/ml) | Percentage Inhibition | |
|---|---|---|
| | Standard | Latex |
| 50 | 42.38(±0.87) | 34.24 (±0.34) |
| 100 | 45.24 (±0.34) | 37.12 (±0.46) |
| 150 | 50.17 (±0.23) | 40.44 (±0.13) |
| 200 | 61.69 (±0.30) | 45.67 (±0.64) |
| 250 | 73.70 (±0.11) | 50.22 (±0.21) |

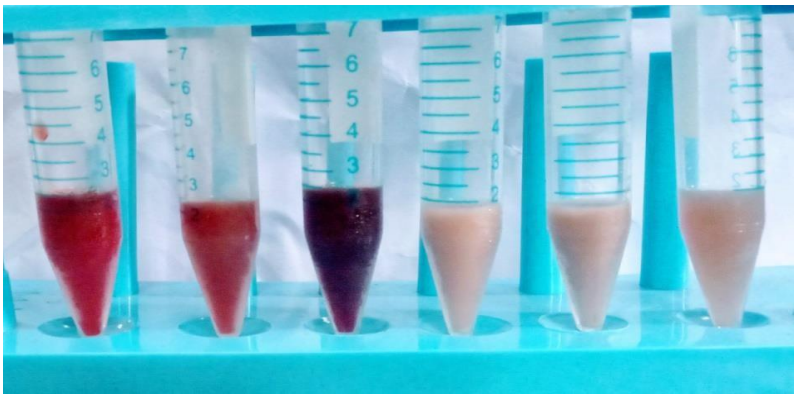

**Figure 4. Membrane stabilization assay of C. procera latex**

***Plant growth promotion assays***

Phosphate solubilizing ability of the isolates was determined qualitatively using pikovskaya medium. Development of clear halo on pikovskaya agar medium on the addition of cell free supernatant reflects P-solubilizing efficiency (Fig. 5). Phosphate solubilization was found to be maximum at 48 h of incubation in all the bacteria isolated. The level of phosphate solubilization by the isolates in pikovskaya agar medium was in the order of *Bacillus*, *Staphylococcus*, *Micrococcus* and *E. coli*. The zone diameter after 48 h of incubation was found to be 1.69 mm (*Bacillus*), 1.57 mm (*Staphylococcus*), 1.26 mm (*Micrococcus*) and 0.95 mm (*E. coli*).

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*Bacillus* sps. *E. coli*

*Micrococcus* sps. *Staphylooccus* sps.

**Figure 5. Phosphate solubilizing ability of the isolates**

Indole acetic acid production by the microorganisms is considered as an important feature for plant growth promotion. IAA promotes plant growth by increasing the radical surface of the root. In the present study, IAA production by the isolates after 48 hrs of incubation was determined by the development of pink colour on the addition of salkowski reagent in growth medium containing tryptophan as a substrate. Of the four bacteria isolated three isolates were respond positively for indole acetic acid production (Table 6). Comparison of IAA production with phosphate solubilization capability, one of the four isolates that solubilise phosphate fails to produce (*Staphylococcus*).

Hydrogen cyanide (HCN) and ammonia production contributes to the antagonistic potential of the isolates against phyto pathogens. Change in the colour of the filter paper from yellow to orange brown while placed over the culture confirms cyanide production. All the four bacteria fails to produce HCN

In addition to antagonistic activity, ammonia produced by the isolates contributes in growth promotion of the plant. Ammonia production is confirmed by the development of yellow colour in the peptone water amended with Nessler's reagent. In the present study all the four isolates found to produce ammonia (Fig. 6).

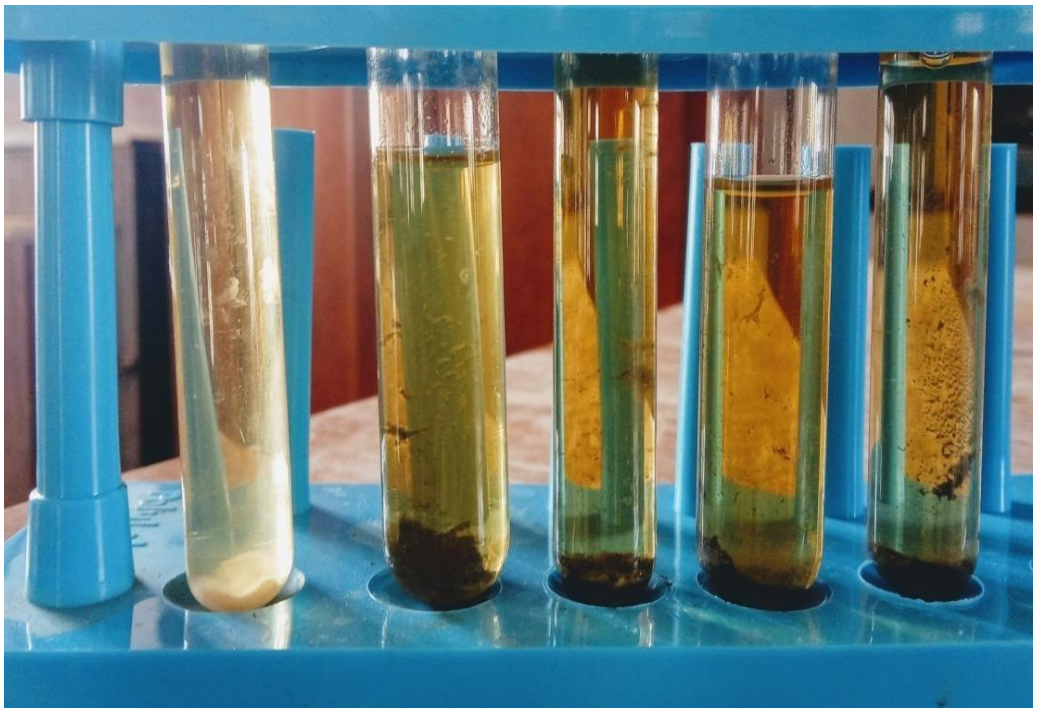

**Figure 6. Production of ammonia by the isolates (Nessler's reagent method)**

**Table 6. Illustration of plant growth promotion potential of the isolates**

| Endophytic Tests | *Bacillus* | *Staphylococcus* | *Micrococcus* | *E. coli* |
|---|---|---|---|---|
| Phosphate solubilizing | + | + | + | + |
| Indole Acetic Acid | + | - | + | + |
| Hydrogen Cyanide | - | - | - | - |
| Ammonia | + | + | + | + |

## 4. DISCUSSION

*Calotropis procera* has many important medicinal properties with proven pharmacological potential. The latex of *C. procera* is well known for its medicinal and toxic properties. The result of the qualitative phytochemical study of latex and the leaves of *C. procera* revealed the presence of alkaloids, saponins, terpenoids and steroids. Tannins were found to be absent in leaves and latex (Table 1). Besides the afore mentioned chemicals, others such as anthocyanins, proteolytic enzymes, cardenolides, and triterpenoids were reported by Kumar and Basu (1994).The medicinal value attributed to plants is a function of the bioactive phytochemical constituents that produce definite physiological action on the human body. The chloroform extract of *C. procera* root exhibits protective activity against carbon tetrachloride induced liver damage (Basu *et al*., 1992).

Importance of the phytochemicals in the different parts of *C. procera* have been reported by Mungole *et al*. (2010). Sodipo *et al*. (1991) reported that tannins prevent the development of microorganisms by making useful proteins unavailable to the organism and facilitate the precipitation of microbial protein. Plants rich in tannins have been found to be astringent in nature and are used for treating intestinal disorder (Mungole *et al*., 2010). Saponins in *C. procera* was reported to have expectorant activity by Ogbulie *et al*. (2007). Banso (2009) illustrated that phytochemicals exhibit antimicrobial activity against a number of microorganisms. Plants containing phytochemicals have been found to have anticancer properties (Nanasombat and Teckchuen, 2009). Latex and plant leaf extracts have been reported to be active against both gram positive and gram negative bacteria (Ashafa and

Afolayan, 2009). The broad pharmacological profile of *C. procera* could be interesting for the pharmaceutical industry to develop new drugs.

This study analysed the community of endophytic microbes both bacteria and fungi in the latex and in the leafs of the plant *C. procera.* A total of 4 different bacterial and 5 different fungal isolates were found to be present in the latex as well as in the leaves of *C. procera* at different stages of maturation. Of the five fungi three were common in both the tissues analysed. The rate of endophyte colonization was found to be increased with the leaf age/development. Endophytes reside asymptomatically in apoplastic spaces and/or within the living cells of plants for all or at least a significant part of their life cycles (Petrini, 1991).

Evolving in this very specific ecological niche, may have favoured the ability of endophytic microbes to produce bioactive natural compounds, some of which are potentially useful in medicine, agriculture and industry (Strobel and Daisy, 2003).

The dynamics of the interactions between the host plant and their endophytic microorganisms are not yet fully understood. In addition to environmental factors (primarily temperature and humidity), variations in chemistry, anatomy and maturity of colonized host tissue affect the abundance and community composition of endophytic fungi (SanchezAzofeifa *et al*., 2012).

In addition to environmental factors and soil characteristics diversity of endophytic microbial flora in the tissues of the host plant reported to influence plant growth (Wang *et al*., 2009). Therefore, diversity and occurrence of endophytic microflora (bacteria & fungi) was analysed in this study.

The isolation procedure of endophytic bacteria is of key importance as it should recover the complete internal population alone. Incomplete surface disinfestations process, adsorption of bacterial cells to plant cell structures, or penetration of the sterilant into plant tissues hinders the isolation of endophytic microbes. Though, several techniques have been illustrated, for the isolation of endophytes in this study trituration technique was adopted as it is advantageous over other methods. Sterility check was performed in this study in order to avoid surface contaminants. Sterility check also helps to monitor the efficiency of the disinfestation procedure. Only after ensuring the absence of surface contaminants in the sterility check

process, the tissue samples were subjected to isolation of endophytic bacteria. Hallmann *et al*. (1997) stated that the sterility check process is inevitable to ensure the endophytic status of the isolate.

Endophytic bacteria were reported to colonize leaf tissues (Fisher and Petrini 1992), intercellular spaces, parenchyma and xylem vessels (Gyaneshwar *et al*., 2001) of the host plant. In the present study, latex of the plant found to harbour more number of endophytes (both bacteria and fungi) than the leaf tissues.

Morphological, biochemical and physiological characteristics of the four isolates distinguish them taxonomically as *Staphylococcus* sps., *Bacillus* sps., *Micrococcus* sps. *and Escherichia coli* (Table 3). All the four isolates possessed distinct biochemical and morphological characteristics. Three different fungi *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus* were isolated from the leaves and latex of *C.procera*. All the three isolates were found to be present in both the tissues analysed. The result of the antimicrobial activities of *Calotropis procera* latex was presented in Table 4.

Antimicrobial activity of the aqueous and ethanolic extract of *Calotropis procera* leaf and latex were investigated by Shobowale *et al*. (2013). Akimoladun *et al*. (2007) analysed the antagonistic activity of leaf extract of *C. procera* against pathogenic organisms using the Agar well diffusion method. Due to the potential health benefit of plants of medicinal value (Gomez – Flores *et al*., 2008), medicinal plant extracts in recent times, have been developed and proposed for use in food as natural antimicrobials (Hsieh *et al*., 2001).

Leaves and areal parts of the plant *C. procera* are used in the treatment of external swellings Latex is reported to contain purgative properties, procoagulant activity and wound healing activity. Different parts of the plant have been used in Indian traditional system of medicine for the treatment of leprosy, ulcers, tumors, piles and diseases of spleen, liver and abdomen (Kritikar and Basu, 1999). Sangraula *et al*. (2002) reported that the milky white latex obtained from the plant exhibits potent anti-inflammatory activity in various animal models that is comparable to standard anti-inflammatory drugs. The latex of the medicinal plant *C. procera* has anti-inflammatory activity.

In the present study, all the four different endophytic bacteria isolated were found to possess phosphate solubilizing activity (Fig. 5). It is a well-established that solubilization of available insoluble phosphorous in the rhizosphere enhance the overall plant growth and root development (Jones and Darrah, 1994). El-Azeem *et al.* (2007) reported a concomitant decrease in pH of the culture broth with phosphate solubilization activity of the endophytic bacteria under *in vitro* conditions.

Nimnoi and Pongsilp (2009) demonstrated that IAA producing endophytic bacteria enhanced root and shoot development of the host plant. Variation in IAA production as a function of incubation time was reported by Gray *et al*. (2001).All the four bacterial isolates were fail to produce HCN. However, all of them were found to produce ammonia. Results of this study fall in line with the findings of Cattelan *et al*., 1999.

## 5. SUMMARY

*Calotropis procera* is a well-known Indian medicinal plant. The latex and the leaf of *C. procera* is rich in organic compounds (alkaloids, cardiac glycosides, tannins, flavonoids, sterols and terpenes. The notable feature is that the organic constituents are not uniformly present in the latex and in the leaves of the plant. Absence of flavonoids in the latex which was found to be present in the aqueous leaf extract.

Screening of endophytic isolates in the latex and leaf of *C. procera* illustrate occurrence of fungi exceeds the bacterial presence. Compared to the latex, leaves of different ages found to harbour more number of microbes. Morphological evaluation and biochemical characterization of the bacteria revealed the occurrence of two different bacterial strains in the leaves and latex. The two strains isolated from the latex were different from that of the leaf isolates. A total of four different bacterial isolates and five different fungi were isolated from the two plant tissues. Of the five different fungi, three were found to be present in both the tissues. They were identified based on macro and micro morphological features.

The four different endophytic bacteria isolated were identified as *Bacillus* sps., *Staphylococcus* sps., *Micrococcus* sps., and *Escherichia coli*. The three different fungi isolated were identified as *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus*.

Antimicrobial potential of the *C. procera* latex revealed that bacteria were more susceptible than the fungi tested. Of the four bacteria analysed, *Streptoccus* was found to be more susceptible to the latex and of the two fungi tested *Fusarium* was found to be more susceptible than *Penicillium*.

Anti-inflammatory efficiency of *C. procera* latex was determined by membrane stabilization assay and compared to that of the standard drug Aceclofenac®. Plant growth promotion potential of the four endophytic bacteria were determined by a battery of assays.

All the four isolates found to possess plant growth promotion potential.

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