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# BACTERICIDAL PROPERTY OF *CLERODENDRUM PANICULATUM* AND *SARACA ASOKA* AGAINST MULTIDRUG RESISTANT BACTERIA, RESTORING THE FAITH IN HERBAL MEDICINE

Govindaraju Shruthi [1], Monisha P. Joyappa [1], S. Chandrashekhar [2], Shiva Prasad Kollur [3], M. N. Nagendra Prasad [4], Ashwini Prasad [1] and Chandan Shivamallu[*1]

Division of Bioinformatics and Biotechnology [1], Faculty of Life Sciences, Jagadguru Sri Shivarathreeshwara University, Mysore - 570015, Karnataka, India.
Department of Biotechnology [2], Yuvaraja's College, University of Mysore, Manasagangotri, Mysore - 570005, Karnataka, India.
Laboratory of Synthetic and Materials Chemistry [3], Manipal Centre for Natural Sciences, Manipal University, Manipal, Udupi - 576104, Karnataka, India.
Department of Biotechnology [4], JSS Science and Technology University, Campus Roads, Mysuru - 570006, Karnataka, India.

**Keywords:**

*Clerodendrum paniculatum*, *Saraca asoka*, Antimicrobial assay, Biochemical and molecular characterization, PCR analysis

**Correspondence to Author:**
**Dr. Chandan Shivamallu**

Division of Bioinformatics and Biotechnology, Faculty of Life Sciences, Jagadguru Sri Shivarathreeshwara University, S. S. Nagar, Mysore - 570015, Karnataka, India.

**E-mail:** chandans@jssuni.edu.in

**ABSTRACT:** Bacterial pathogens are known to pester Mankind from times immemorial; even though significant efforts in getting rid of these harmful microbes have been made the results are very faint with only few organisms that have been eradicated, such as Small pox virus. But the efforts to eradicate bacterial diseases have shown no passable results as in case of viruses. This is due to the exceptional adaptation and transformation abilities of bacteria to varying environmental Conditions. Though a large number of antibiotics are being used from decades now, there are no affirmative solutions available due to resistance developed towards antibiotics by these bacteria. This resistance developed by the bacteria calls for dire necessity to discover new drugs which can at least reduce the hazards posed by these microbes if not eradication. Hence in this study we have focused on bio prospecting of *Clerodendrum paniculatum* and *Saraca asoka* against the highly virulent and extremely adaptable organisms *E. coli* and *K. pneumoniae* which currently pose a severe threat to humans due to their acquired resistance to large number of antibiotics.

**INTRODUCTION:** All living organisms are prone to disease, the severity of which may vary from mild to lethal conditions depending on the pathogenic microorganism. Humans are highly susceptible to microbial infections from microbes present in nature. These microbes can be either beneficial or harmful to the organism in which they colonize, Some of the beneficial microbes include intestinal commensals and the skin micro flora, which are highly essential, the beneficial aspects of these organisms are indispensible but at the same time there are instances when these microbes become lethal when they enter the blood stream, along with these commensals there exist microbes which exist in

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nature, that are detrimental to the organisms which they infect. The vast numbers of microbes that are infectious are regarded as pathogens.

There are umpteen numbers of pathogenic microbes in nature which infect humans, leading to increased death rate. Some of the well known diseases that are commonly seen in animals include pneumonia, typhoid, cholera, diarrhoea, hepatitis, nephritis, *etc.* Even though there are plenty of treatment options available for a range of well known diseases, the effectiveness of the existing treatment options are found to be reducing over time due to multidrug resistant variants resulting owing to abuse of antibiotics in growing population [1]. In present study pneumonia, diarrhoea, nephritis which is caused by *Klebsiella pneumonia* and *Escherichia coli* respectively are focused in this study.

*E. coli* and *K. pneumoniae* are persistently present in the nature currently, even with enormous efforts and numerous ways employed to curb their spread and virulence abilities in humans there is no significant result hence in this study *Clerodendrum paniculatum* and *Saraca asoka* were screened for antimicrobial activity against these dreaded microorganisms [4]. *Saraca asoka* (the Asoka tree; lit., "sorrow-less") is a plant belonging to the fabaceae family and Caesalpinioideae subfamily. It is an important tree in the cultural traditions of the Indian subcontinent and adjacent areas. It is sometimes incorrectly known as *Saraca indica* [5].

The principal constituents of this tree are a steroid component and a calcium salt. The bark of *Saraca asoka* contains an estrogenic compound called ergosterol. The earliest chronicled mention is in the Ayurvedic treatise, the Charka Samhita (100 A.D.), in which Asoka is recommended in formulations for the management of pain with relation to uterus (Gynecological) as Anodynes. The Bhavprakasha Nighantu, commonly known as the Indian *Materia medica* (1500 A.D.), cites the plant as a uterine tonic that is effective in regularizing the menstrual disorders. The bark has a stimulating effect on the endometrium and ovarian tissue and is useful in menorrhagia during uterine fibroids. It also has great benefits for its uterine activity [5]. Flowers of this tree are used to treat cervical adenitis, biliousness, syphilis, hyperdipsia, burning sensation, hemorrhagic dysentery, piles, scabies in children and inflammation. Scant literature is available on the antimicrobial effect of their extracts [5, 6].

*Saraca asoka* contains tannin, catechol, sterol and organic calcium compounds. Its methanol fraction contains haematoxylene, tannin, and water-soluble glycoside. The latter has glucose, galactose and mannose as sugars [7]. The *Clerodendrum paniculatum* (pagoda flower), so called because of its tall, pyramidal inflorescences, is one of the most spectacular *Clerodendrum* species. The pagoda flower is found throughout tropical and subtropical Asia, from Bangladesh to the Moluccas. It is widely cultivated and often establishes as a garden escapee in these regions, so that its original distribution is not entirely clear [8].

The most distinctive features of *Clerodendrum paniculatum* are the large terminal inflorescences (thyrses, though often erroneously called panicles) up to 45 cm long, bearing numerous red-orange flowers. Each slender, tubular flower is 1.2-2 cm long with five small lobes, these usually being slightly paler than the tube. Butterflies are the main pollinators. They extend their long, thin proboscides into the flower tubes during which process pollen adheres to their bodies from the long-exerted stamens. The large, glossy, lobed leaves and fairly robust stems with an almost square cross-sectional form are also prominent characteristics of *C. paniculatum*. Their ability to produce root suckers allows pagoda flowers to spread vegetatively and they can form apparently colonel stands of several plants together [9].

The pagoda flower has a number of medicinal uses in Asia. In Malaysia an infusion is drunk as a purgative and is applied externally to distended stomachs. Various magical attributes have been recorded; indeed the Malay vernacular name pangil-pangil refers directly to the ‘summoning’ of spirits. *Clerodendrum paniculatum* is also supposed to confer protection from harm and is used as an elephant-medicine. Substances produced by several *Clerodendrum* species are undergoing more rigorous scientific trials in order to evaluate their medicinal potential. To date, results are promising, and antipyretic and anti-inflammatory properties have been verified, as well as antiviral activity[10].

**Enterohemorrhagic *E. coli*:** *E. coli* is a gram negative proteobacteria which is present ubiquitously in nature and is found to cause a range of different infections in humans leading to numerous differences in symptoms and pathogenesis. This is due to presence of numerous different species and mutants of *E. coli* which have evolved to evade antibiotic action simultaneously with discovery and evolution of antibiotics. One of the threats with conventional antibiotic therapy to treat *E. coli* is that upon lysis of outer membrane of *E. coli* the toxic verocytoxin which is also known as shiga toxin is released into the host cell environment.

Shiga toxin which is a two subunit protein is found to inhibit protein synthesis in the host cell leading to apoptosis of the host cell. The Shiga toxins have high affinity towards GB3 receptors and bind to cells expressing these receptors. Some of the cells which are found to express these GB3 cells are Epithelial cells, renal glomerular epithelium *etc*. When the toxin bind to these receptors the cells internalize the toxin thereby exposing themselves to toxicity of shiga toxin resulting in cell death leading to conditions like nephritis, thrombo-cytopenia and microangiopathic haemolytic anaemia [11 - 25].

Currently there is no treatment available for Enterohemorrhagic *E. coli* infection as the conventional antibiotics aggravate the shiga toxin-mediated cytotoxicity. It is also found that shiga toxin production is enhanced with antibiotic treatment as stx gene expression which results in lysis of *E. coli* cell envelope, resulting in discharge of shiga toxin into the host environment [11, 23, 26 - 35].

***Klebsiella pneumonia*:** Pneumonia has been one of the most dreaded diseases from centuries and has continued to haunt human lives with high virulence, pathogenesis and increased rate of deaths each year. The severity and intensity of the infection depends on the mode of acquiring the disease and also on the organism responsible for the disease. Based on the mode of transmission pneumonia is broadly classified into 5 types such as Community acquired, Aspiration, Healthcare associated, Hospital acquired, Ventilator associated pneumonia [1-3, 10, 29, 36 - 56].

## MATERIALS AND METHODS:

**Isolation of Test Organism:** The standard human bacterial culture *E. coli* and *K. pneumoniae* were obtained from JSS Hospital, Mysuru, Karnataka, India and maintained in nutrient agar slants. Nutrient broth of about 50ml was prepared, sterilized and kept for pre-incubation at 37 °C for 24 hr and in the next day the fresh cultures of respective bacterial strains were inoculated into respective inoculation tubes and these bacteria were used as test organism for further experiments.

**Characterization of Bacterial Pathogens:** Isolated bacteria were sub-cultured onto the nutrient agar medium (NA; Peptone 5.0g/l; Beef extract 3.0g/l; Agar 20g/l) and 24-36 hr old-cultures were used for biochemical characterization. The biochemical tests were carried out with appropriate controls which are as follows;

**Biochemical Characterization:**

**Gram's Reaction:** A loop full of bacterial suspension was smeared onto a glass slide, air-dried and heat fixed by passing the slide rapidly two to three times using a Bunsen burner. The smear was flooded with crystal violet solution for 1 min. The slide was washed with a gentle stream of distilled water and flooded with Lugol's iodine for 1 min. Again the slide was washed with distilled water and blot dried, and decolorized by washing with a gentle stream of 95% ethyl alcohol for 30 s to remove excess stain, then blot dried and counter stained by flooding with safranin for 20 s. The slide was again washed with distilled water and blot dried. The preparation was observed under a compound microscope at different magnifications for pink-red or blue-violet stained bacteria representing gram negative or gram-positive nature respectively.

**Non-Gram Staining or Potassium Hydroxide (KOH) Solubility Test:** A loopful of bacteria from a well-grown colony was mixed with a toothpick on a clean glass slide with a drop of 3% aqueous KOH solution. The toothpick was raised a few centimeters above the slide to observe the formation of a mucoid thread. The formation of viscous strands represents the bacteria as gram-negative. Gram-positive bacteria do not produce any strands even on repeated strokes of the toothpick.

**Starch Hydrolysis:** Plates with starch medium (Soluble starch 2.0g/l; Peptone 5.0g/l; Beef extract 3.0g/l; Agar 20.0g/l. Dissolve the nutrient agar powder in the water by heating. Dissolve the starch in 10ml distilled water and add to molten agar) were streaked with bacterial isolates and were incubated for 2-4 days at 28 ± 2 °C. Plates were flooded with Lugol's iodine solution and observed for the zone of hydrolysis. Appearance of clear zone of hydrolysis around and under the bacterial growth indicates starch has been hydrolyzed. If not, the medium turns blue indicating starch has not been hydrolyzed (Lelliott and Stead, 1987).

**Kovac's Oxidase Test:** A loopful of bacteria grown in King's B medium (Protease peptone 20.0 g/l; Glycerol 15.0ml/l; $K_2HPO_4$ 1.5 g/l; $MgSO_4.7H_2O$ 1.5g/l; Agar 20.0g/l; Adjust pH to 7.2 before autoclaving the medium) was applied onto a disc of Whattman filter paper No.1, impregnated with 3-4 drops of a 1% aqueous solution of Tetramethyl-paraphenylenediaminedihydrochloride at the center as per the procedure given by Kovac's (1956). The change in the color of the reagent to purple within 10 is regarded as the positive test result, if there is no change in the color of the reagent, the test result is negative.

**Lipase Activity:** Sterilized Tween 80 agar plates (Peptone 10.0 g/l; NaCl 5.0 g/l; $CaCl_2$, $H_2O$ 0.1 g/l; Agar 15.0 g/l; Dissolve by heating and adjust pH to 7.2-7.4. Sterilize the base and Tween 80 separately by autoclaving at 121 °C for 15 min. Add Tween 80 to the molten base to give a final concentration of 0.1 % mix well and pour plates) were inoculated with the bacterial isolates and incubated up to 7 days. Development of a milky-white precipitate (opaque zone) of the calcium soap, due to crystals formation around the colonies demonstrates the ability to hydrolyze the lipid Tween 80 and indicates the positive result to lipase activity (Lelliott and Stead, 1987).

**Gelatin Hydrolysis:** Test tubes containing gelatin medium (Yeast extract 3.0 g/l; Peptone 5.0 g/l; Gelatin 120.0 g/l; allow the solids to stand in the water for 15 min and dissolve by heating. Adjust pH to approximately 7.0 if necessary. Dispense into test tubes to a depth of approximately 5cm. Sterilize by autoclaving at 121 °C for 15 min) were stab inoculated with the bacterial isolates, along with positive control and uninoculated sets, experiment was conducted in duplicates. Tubes were incubated for 7-14 days at 22 ±2 °C, for every 2-3 days amount of liquefaction was recorded. On the final day, tubes were cooled at 5 °C for 30 min before reading the results. If the test culture fails to solidify, indicates that gelatin hydrolysis is positive. If the test culture solidifies, incubate the tubes in a tilted position at room temperature or at 30 °C in an incubator for 30 min. If the culture liquefies before the control / uninoculated tube, it is a weak positive reaction; if the culture takes as long as the control for liquefaction, then it is a negative reaction (Lelliott and Stead, 1987).

**Extraction of Phytochemicals from Plants:** Both *Clerodendrum paniculatum* and *Saracaasoka* plants were washed thoroughly using soap water after which they were rinsed thoroughly with distilled water to get rid of dust, mites and other contaminants. After washing the plants the plants were dried in shade for 10 days. The dried plants were powdered using domestic blender the powdered plants were then subject to extraction using soxhlet apparatus. Around 40g of plant powder was taken in thimble and extracted using methanol solvent system in Soxhlet apparatus, followed by drying the extracts to get rid of residual methanol solvent, the extracts which were in paste form were stored in Eppendorf tubes.

**Antimicrobial Property of Plant Extracts:** The antibacterial property of *Saraca asoka* and *Clerodendrum paniculatum* were analyzed against both *E. coli* and *K. pneumoniae* at concentration of 25µg/ml and were compared along with standard antibiotic ampicillin with concentration same as that of plant extracts.

**Molecular Characterization:**

**Primer Design and *Insilico* PCR:** The 16s rRNA sequence of *Klebsiella pneumonia* and *Escherichia coli* were obtained from Genbank. The primers for the sequences obtained were designed *in silico* using primer designing tool of Fast PCR (ver.6.0). Two sets of specific primers were synthesized for *Klebsiella pneumonia* and *Escherichia coli*. The designed primers were further analyzed for their amplification ability and specificity using *in silico* PCR tool of Fast PCR (ver.6.0).

***In vitro* PCR Analysis:** The bacterial DNA was isolated for PCR from the pathogen harvested from 24h-old-cultures grown on NB and suspended in 1M NaCl by vigorous vortexing as described by Chan and Goodwin (1995). The tubes were centrifuged at 12,000 rpm for 10 min, supernatant was discarded and the process was repeated to reduce and separate the cells from the polysaccharide xanthum gum. The cells were finally washed twice in sterilized, distilled and deionized water to reduce the salt concentration; the pellet was suspended in 500µl of Tris EDTA and Sodium dodesylsulphate (SDS) extraction buffer containing 50mg/ml proteinase K. The samples were vortexed thoroughly, mixed and placed in a water bath at 65 °C for 30 min. One-half volume (250 µl) of 7.5M ammonium acetate mix was added and the samples were incubated on ice or at -5 °C in refrigerator for 10 min.

The tubes were centrifuged for 15 min at 20800 g and the supernatant was transferred to a new tube and equal volume (500 µl) of ice-cold isopropanol was added and incubated at -20 °C for 1-2hr. Tubes were centrifuged for 10 min at 13,000 rpm to pellet the DNA, the supernatant was decanted and DNA pellets were washed with 800µl of cold 70% ethanol and air dried. DNA was eluted from the pellet with twice repeated extractions with 250µl of 1x TE buffer (10mM Tris-HCL (pH 8), 1mM EDTA) each time centrifuge to avoid collecting pelleted polysaccharides. Solution containing DNA was transferred to a 1.5ml micro centrifuge tube and 5µl of RNaseA (20 mg/ml) was added and incubated at 37 °C for 60 min and the DNA was recovered and air-dried.

The 16S rRNA gene was amplified by PCR for both the isolates using the primers 16S forward primer (5'-TGGTAGTCCACGCCCTAAAC-3') and 16S reverse primer (5'- CTGGAAAGTTCC-GTGGATGT-3') for *Klebsiella pneumonia* and 16S forward primer (5'-AGTCCACCAGGGCCACTT-3') and 16S reverse primer (5'-CTGCAAAGGC-GCGTTCTC-3') for *Escherichia coli* designed using Fast PCR software (Ver. 6.0). Amplification reactions (25µl) contained 1x PCR buffer (2.5µl), 3U/ml Taqpolymerase (0.5µl), 1mM dNTPs (2.0 µl), 10 pmol/µl of primer (2.0µl) and 100ng of genomic DNA/µl (1.0µl) of reaction mixture. PCR was performed in a thermocycler (Eppendorf, Mastercycler gradient, Japan). Initial denaturation at 94 °C for 5 min was followed by 30 cycles (1 cycle consists of denaturation for 30 s at 94 °C, annealing for 30s at 60 °C and extension for 45 s at 72 °C). A final extension of 72 °C for 2 min was incorporated into the programme, followed by cooling to 4 °C until the recovery of the samples. Amplicons were electrophoresed using 1.5 % agarose gel in 1xTBE buffer along with ethidium bromide, DNA detecting stain along with the 100 bp ladder that were photographed under UV light.

**DNA Sequencing:** PCR products generated by PCR amplification with all the test bacteria using 16S rRNA primers were sent for sequencing at Chromous Biotech, Bangalore, India.

**RESULTS:** The bacterial isolates were characterized based on the morphological and biochemical properties. The results of the biochemical tests are as follows.

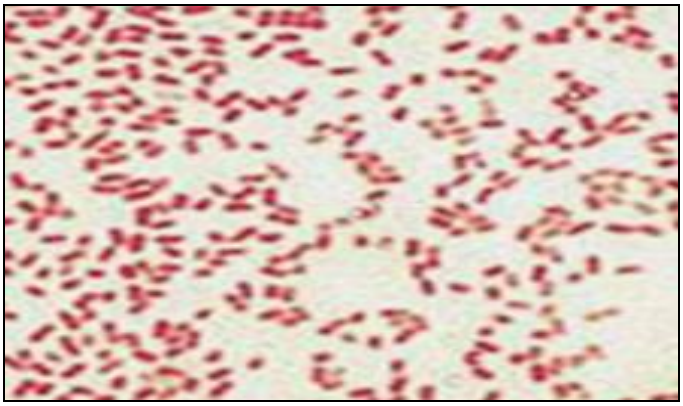

**FIG. 1: GRAM'S STAINING; PINKISH-RED COLOURED BACTERIAL CELLS SHOWING GRAM NEGATIVE NATURE**

**TABLE 1: BIOCHEMICAL CHARACTERIZATION OF *K. PNEUMONIAE* AND *E. COLI* PATHOGENS**

| Biochemical tests | Results | |
|---|---|---|
| | ***Klebsiella*** | ***E. coli*** |
| Gram's staining | - | - |
| KOH solubility | + | + |
| Starch hydrolysis | - | - |
| Kovac's oxidase test | - | + |
| Gelatin hydrolysis | + | + |
| Lipase test | - | + |

*All the tests were conducted in four replicates and were repeated twice. + indicates positive reaction, - indicates negative reaction

**Gram's Staining:** The tested bacterial isolates were stained pink-red indicating that bacteria are gram negative in nature (**Fig. 1** and **Table 1**).

**KOH Solubility Test:** The isolates showed thin viscid mucoid strand when loopful of test bacteria were mixed with 3% KOH solution and lifted slightly, indicating the test isolates are positive for KOH test, which indicated gram negative nature of the bacteria (**Fig. 2** and **Table 1**).

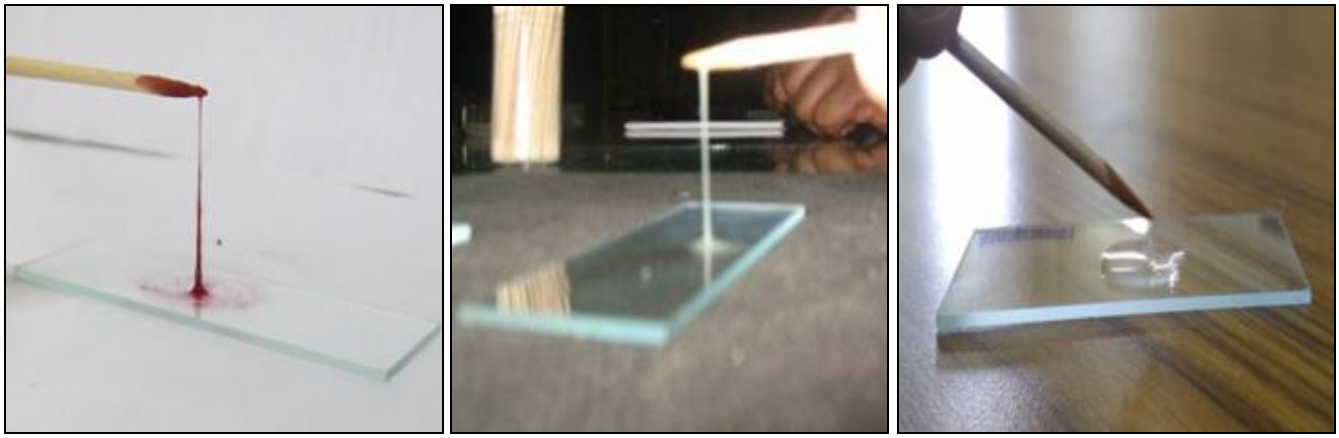

**FIG. 2: KOH SOLUBILITY TEST; MUCOID THREAD FORMED SUGGESTING A GRAM-NEGATIVE NATURE OF THE BACTERIUM**

**Starch Hydrolysis:** A clear zone of hydrolysis was failed to form around the bacterial colonies of the test isolates when the culture plates were flooded with Lugol's iodine. Hence the test bacteria were negative for the starch hydrolysis (**Fig. 3** and **Table 1**).

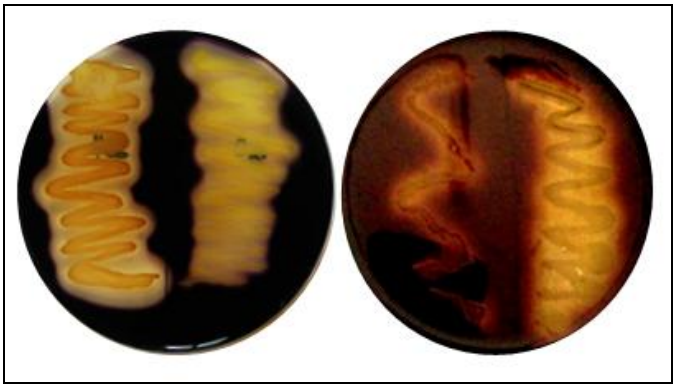

**FIG. 3: STARCH HYDROLYSIS: NO CLEAR ZONE AROUND *KLEBSIELLA* INDICATING NEGATIVE RESULT. POSITIVE RESULTS SEEN IN *E. COLI***

**Kovac's Oxidase Test:** The color of the test isolates failed to turn into violet colour after few minutes of rubbing onto the Whatman No. 1 filter paper impregnated with Kovac's oxidase reagent, which showed the test isolate was positive for Kovac's oxidase test (**Fig. 4** and **Table 1**).

**Lipase Test:** The formation of white precipitate around the colonies of the test isolate indicated that the test isolates were positive for lipase activity (**Fig. 5** and **Table 1**).

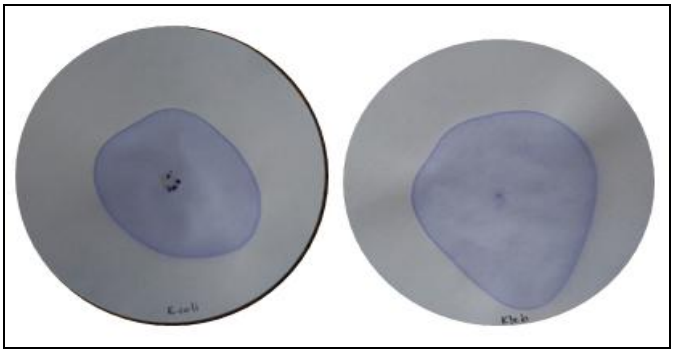

**FIG. 4: KOVAC'S OXIDASE TEST: CHANGE IN COLOR TO PURPLE, THUS *KLEBSIELLA* AND *E. COLI* WERE SEEN POSITIVE FOR THE TEST**

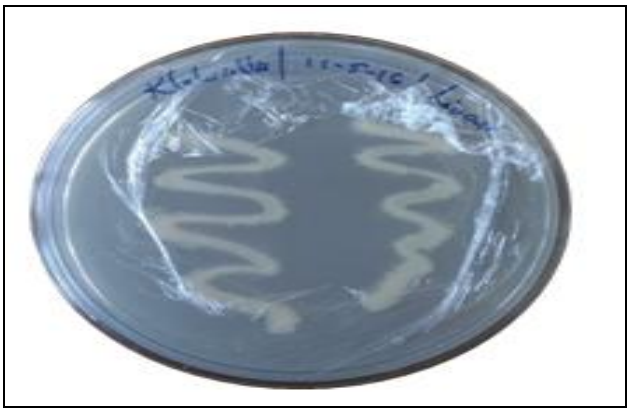

**FIG. 5: LIPASE ACTIVITY - THE FORMATION OF WHITE PRECIPITATE AROUND THE COLONIES OF POSITIVE RESULTS WERE SEEN *K. PNEUMONIAE***

**Gelatin Hydrolysis:** The isolates showed positive result for the test, by liquefaction of the gelatin medium after 2 days of incubation. (**Fig. 6** and **Table 1**).

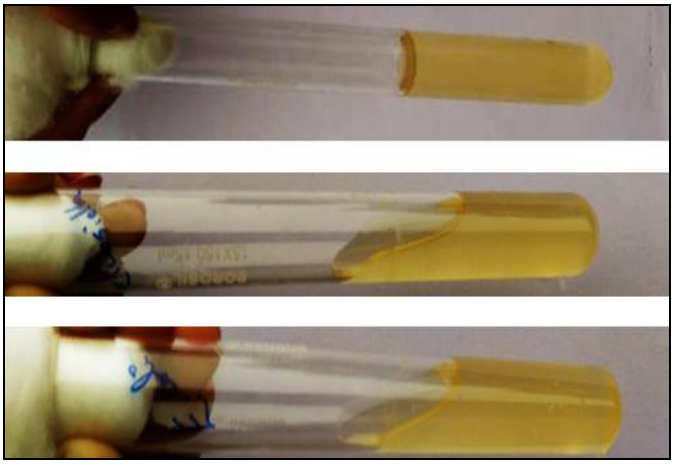

**FIG. 6: GELATIN HYDROLYSIS TEST: 1. BLANK, 2. *K. PNEUMONIAE*, 3. *E. COLI***

**Antimicrobial Property of Plant Extracts:** Both *Saraca asoka* and *Clerodendrum paniculatum* extracts were found to have significant antibacterial activity against *E. coli and K. pneumoniae* which were resistant to streptomycin and cephalosporin. *Saraca asoka* showed higher antibacterial activity against both the bacterial strains when compared to standard antibiotic Ampicillin whereas *Clerodendrum paniculatum* was found to have higher antibacterial activity against *E. coli* in comparison with standard antibiotic ampicillin and almost similar antibacterial activity against *K. pneumoniae* as that of Standard antibiotic ampicillin (**Table 2**).

**TABLE 2: ANTIBACTERIAL PROPERTY OF *SARACA ASOKA* AND *CLERODENDRUM PANICULATUM* AGAINST OF *K. PNEUMONIAE* AND *E. COLI* PATHOGENS**

| Tested Bacteria | Diameter of inhibition zone for *Saraca asoka* Extracts (mm) | Diameter of inhibition zone for *Clerodendrum paniculatum* Extracts (mm) | Diameter of inhibition zone for Ampicillin standard (mm) | Diameter of inhibition zone for streptomycin (mm) | Diameter of inhibition zone for Cephalosporin (mm) |
|---|---|---|---|---|---|
| *K. pneumoniae* | 19 | 14 | 15 | - | - |
| *E. coli* | 22 | 15 | 12 | - | - |

**Molecular Characterization:** The DNA obtained by using DNA extraction of bacterial strains was used for PCR analysis. The bacterial DNA from all the isolates used for molecular detection using 16S rRNA primers revealed that the *Klebsiella pneumoniae* and *Escherichia coli* recorded amplifications of 780 bp and 800 bp DNA fragments respectively thus confirming the pathogens (**Fig. 7**).

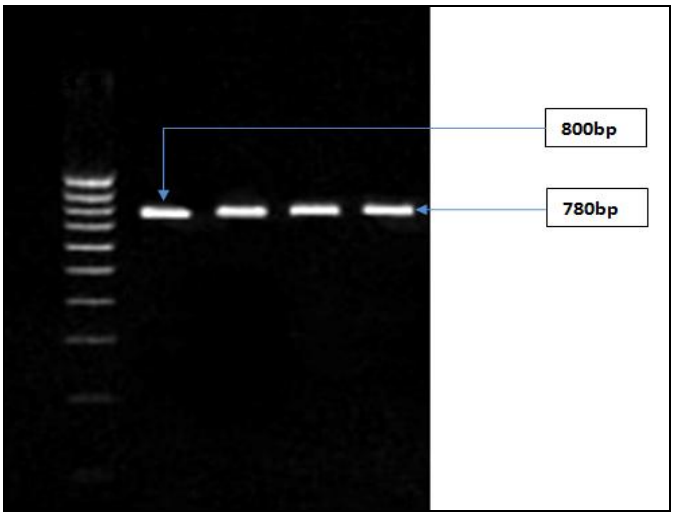


**FIG. 7: VISUALIZATION OF PCR AMPLICONS ON 1.2% AGAROSE GEL. DNA WAS EXTRACTED FROM THE BACTERIA, 16S rRNA PRIMERS USED FOR PCR. LANE M, 1000-BP MARKER, LANE 1 TO 2, 3 TO 4, ISOLATES OF *E. COLI* AND *K. PNEUMONIAE* RESPECTIVELY. LANE 40 NON DNA TEMPLATE**

**DISCUSSION:** Medicinal plants are being used to treat in numerous diseases from times immemorial and are found to be safest method of treatment with least number of side effects or cross reactions. In addition to their efficiency to cure diseases they are the most nontoxic forms of medications currently available and they are the most nontoxic forms of medications currently available. And they do not pose the threat of resistance development in microbes even with prolonged usage. Plants have been found to possess incredible biological properties with greater ability to work as protective agents to humans by providing innumerous benefits such as antioxidant property, Bactericidal property, bacteriostatic property, fungicidal property, and fungi static property.

Conventional synthetic antibiotics even though are potent have the threat of developing antibiotic resistance and also toxic side effects in such instances plant extracts and plant products come to rescue in such instance without causing toxic side effects or anaphylactic reactions in humans, it is also an excellent option in case of antibiotic resistance as there are no instances of resistance seen in case of plant extract or plant product usage. Plants contain numerous biologically active compounds such as flavanoids, terpenes,

pyrrolizidines and many other phyto-chemicals and have not been evaded by these microbes with change in time. Even though there are a few highly efficient drugs, they cannot be afforded by all classes of the economy. Hence this lack of highly efficient drugs at affordable price calls for discovery of alternative drugs which are both effective and affordable at the same time. Hence plants make an ideal choice to screen for antimicrobial components and their treatment [6].

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**CONFLICT OF INTEREST:** There is no conflict of interest whatsoever among the authors. All the authors concur with the submission.

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