

## Antibacterial Efficacy And Minimum Inhibitory Concentrations of Medicinal Plants against Wound Pathogens

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### ABSTRACT

The medicinal plants are god's gift to cure a number of diseases among the living organisms. The aim of the present study was to evaluate the antibacterial property and Minimum Inhibitory Concentration (MIC) of *Azadirachta indica*, *Centella asiatica*, *Clitoria ternatea* against Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Klebsiella species*). The ethanolic extract of these plant species was prepared and demonstrated the antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella species*. It exhibits broad spectrum of inhibition against the pathogens. The Minimum inhibitory concentration was determined by two fold serial dilution methods. The Concentration of plant sample of about 551.0991g of *Azadirachta indica*, 681.1751g of *Centella asiatica*, 651.1751g of *Clitoria ternatea* showed good activity against *Staphylococcus aureus* whereas 557.4621g, 623.1831g, 6137.6921g against *Pseudomonas aeruginosa* and 213.3441g, 611.6391g, 480.2411g against *Klebsiella* sp respectively. These studies prove that the plant samples exhibit good activity against the pathogen even in low concentration and it is a good antibacterial agent against the bacteria which causes wound infection.

**Keywords:** Medicinal Plants, Minimum Inhibitory Concentration (MIC), antibacterial, *Klebsiella species*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Centella asiatica*, *Clitoria ternatea* and *Azadirachta indica*.

### INTRODUCTION

Wounds are the result of injuries or disruption of cellular and functional continuity of living tissue. Normal wound healing is a complex process of tissue repairing response which begins with injury. The expanding bacterial resistance to antibiotics has become a growing concern worldwide (Gardam, 2000)<sup>1</sup>. Various plant materials have been used for the treatment of wounds over the years. Ethano medicine has encouraged many developments in

drug discovery. Bio active compounds are normally accumulated as secondary metabolites in all plant cells but their concentration varies from parts to parts. Plants are rich in a wide variety of secondary metabolites which have been found *invitro* to have antimicrobial properties (Mahady, 2005)<sup>2</sup>. The use of plant extracts and photochemical both with known antimicrobial properties can be a great significance in therapeutic treatments. The medicinal plants are god's gift to cure a number of diseases among the living organisms<sup>3</sup>. Leaf is one of the highest



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accumulated plant parts of such compounds and it is preferred for therapeutic purposes. Some of the active compounds inhibit the growth of disease causing microorganisms. Authentication of the use of plants for medicinal purposes dates some 60,000 years back in both Western and Eastern culture<sup>4</sup>. The uses of plants and plant derivatives for treating various diseases have been a common practice since years. At present about 65% of Indians are dependent on the traditional system of medicine<sup>5</sup>. Herbal formulations are used due to their lesser side effect and low cost. For instance, the plants are generally readily available and their products are biodegradable. Antimicrobial drug resistance is a global problem today as the resistant microorganisms have emerged and spread throughout the world because of their genetic plasticity<sup>6,7</sup>.

Literature survey revealed that *Centella asiatica*, *Clitoria ternatea* and *Azadirachta indica* has the antimicrobial, anti oxidant activity and wound healing. In this study, by preparing the organic solvent extracts of *Clitoria ternatea*, *Centella asiatica* and *Azadirachta indica* to check potential activity against the *Staphylococcus aureus*, *Klebsiella species* and *Pseudomonas aeruginosa*. *Staphylococcus aureus* is a gram positive, non motile and does not form spores<sup>8</sup> which cause wound infection, food poisoning<sup>9</sup> pyogenic infections. *Klebsiella* species are gram negative, non motile, oxidase negative, rod shaped bacteria with a polysaccharide based capsule<sup>10</sup> which cause pneumonia, blood stream infection, urinary tract infection, diarrhea, wound or surgical site infection and meningitis<sup>11,12</sup>. *Pseudomonas aeruginosa* is a common bacterium which causes soft tissue infection, Urinary Tract infection and pyogenic infections. *Pseudomonas* growth with on the human body can be asymptomatic until the bacteria from a biofilm, which overwhelms the immune system. These biofilms are found in the lungs of cystic fibrosis and primary ciliary dyskinesia and can prove fatal<sup>13-18</sup>. The demonstration of the antibacterial activity against both Gram negative and Gram positive bacteria are an indication that the plants are potential source for production of drugs with broad spectrum of activity. The study supports the use of this herbal preparation by not only as dietary supplement but also as agents to prevent or control bacterial infections. The main aim of the present study is to evaluate the antibacterial

efficacy and minimum inhibitory concentration of the plant extracts against the pathogens such as *Staphylococcus aureus*, *Klebsiella species* and *Pseudomonas aeruginosa* which cause wound infection.

## MATERIALS AND METHODS

### Collection of Plant Material

The three medicinal plants *Clitoria ternatea*, *Centella asiatica* and *Azadirachta indica* used in this study were collected from Trivandrum, India. These three medicinal plants were used as a traditional medicine for various microbial infections and disorders. The plant samples were authenticated by Botanical Survey of India, Tamilnadu Agriculture University, Coimbatore, India. The authentication number of *Clitoria ternatea*, *Centella asiatica* and *Azadirachta indica* were BSI/ SRC/ 5/ 23/ 2016/ Tech/ 112, BSI/ SRC/ 5/ 23/ 2016/ Tech/ 113, BSI/ SRC/ 5/ 23/ 2016/ Tech/ 115 respectively. All the collected plant leaves were washed with water to remove the soil and dust particles. Then they were shade dried and were grind. After grinding, the collected grind plant tissue was passed through a 0.125 mesh sieve to remove debris, and stored in a sterile air tight container.

### Organoleptic evaluation of Plant Material

The plant parts were organoleptically evaluated for various sensory parameters like the colour, appearance of the plant parts mainly size and shape, external texture, fracture (granular, splintery, smooth) and external marking (furrow, wrinkles, ridges, annular, out growth) of the plant part were examined. The fragrance, test for odour (aromatic, balsamic, camphoraceous, spicy, pleasant, irritating) and taste (sweet, bitter, sour, astringent, pungent, acidic, alkaline) were evaluated<sup>19, 20</sup>.

### Preparation of Extracts

The plant leaves were used for the preparation of extracts and they were washed thoroughly with distilled water to remove extraneous matter and dried under shade. The dried leaves were powdered using a mortar and pestle. 20 grams of powdered leaves were extracted successively with 100ml of ethanol. The extracts were evaporated drily and the resulting pasty form extracts were stored in a sterile plastic container.

### Screening for Phytochemical Constituents

The dried plant extracts were screened for the presence of phytoconstituents like alkaloids, saponins, terpenoids, glycosides, flavonoids, sterols and steroids, tannins and phenolic compounds, carbohydrates<sup>21</sup>.

### Antimicrobial assays

#### Determination of antibacterial activity

##### Selection of Bacterial Strains

The antibacterial activities of the medicinal plant extracts were determined against three predominant bacterial strains such as one from gram positive and other two from negative strains. These strains were screened through pus samples from the patients with wound infection.

### Isolation and identification of bacteria

Totally 150 pus samples were collected from wound infected patients. Among that three strains (Two gram negative and one gram positive pathogen) were isolated and identified based on Gram staining, biochemical characterization and

colony morphology. These isolates were confirmed on selective medium. Then the bacterial culture were maintained in nutrient agar slant and used for further studies.

### Preparation of inoculums

Nutrient broth medium was prepared by adding the nutrient broth in the distilled water and sterilized with the help of an autoclave at a pressure of 151Lbs and temperature of 120°C for 15 minutes. The bacterial strain was inoculated in the nutrient broth medium and the cultured was maintained at 28°C ± 2°C. The culture was inoculated over the Muller Hinton Agar (MHA) plates. The determination of antimicrobial susceptibility was confirmed through medicinal plant extracts.

### Antibacterial activity by Disc diffusion method

Antimicrobial susceptibility was carried out by disc and well diffusion technique. First the Muller Hinton Agar (MHA) plate was prepared and after that the solidification of the culture was swabbed on the plates, and then commercially available antibiotic

**Table 1: Organoleptic Evaluation of Medicinal Plants**

Parameteres	<i>Azadirachta indica</i>	<i>Clitorea terennatea</i>	<i>Centella asiatica</i>
Colour	Green	Green	Green
Odour	Pungent	Raw	Raw
Appearance	Dark green when powdered	Dark green when powdered	Dark green when powdered
Taste	Bitter	Pleasant	Pleasant

**Table 2: Phytochemical screening of Ethanolic extract of *Azadirachta indica*, *Centella asiatica* and *Clitorea terennatea***

Phytochemical constituents	Ethanolic extract of Medicinal plants		
	<i>Azadirachta indica</i>	<i>Centella asiatica</i>	<i>Clitorea terennatea</i>
Alkaloids	+	+	+
Saponins	+	+	-
Terpenoids	+	+	+
Glycosides	+	+	+
Flavanoids	+	-	+
Steroids and sterols	+	+	+
Tannins and phenols	+	+	-

discs were introduced on the upper layer of the seeded agar plate. The sterile discs impregnated with methanol were used as control test discs they were aseptically applied to the surface of the agar medium.

Then plates were incubated for 24 hours 37° C after the incubation period the zone of inhibition was measured. Gentamicin (30mcg), Netilmicin (30mcg), Cefazolin (30mcg), Cephalexin (30mcg), Cefuroxime

**Table 3: Biochemical Characterization of isolated pathogens**

Biochemical Reaction	<i>S. aureus</i>	<i>Klebsiella</i>	<i>P. aeruginosa</i>	<i>Proteus</i>	<i>E.coli</i>	<i>S. pyogenes</i>
Indole	-	-	-	-	+	-
Methyl Red	+	-	+	+	+	-
Voges Proskauer	+	+	+	-	-	-
Citrate Utilization	-	+	-	-	-	-
Urease	+	+	-	+	-	-
Nitrate reduction	+	-	+	+	-	-
Hydrogen sulfide Production	-	-	-	+	-	-
Oxidase	-	-	+	-	-	-
Catalase	+	+	+	+	+	-
Coagulase	+	-	-	-	-	-
Motility	-	-	+	+	+	-

**Table 4: Triple Sugar Iron and Carbohydrate Fermentation Test of Isolated Pathogens**

Test bacteria	TSI reaction		Carbohydrate fermentation				
	Slant	Butt	Gas	H <sub>2</sub> S	Glucose	Sucrose	Lactose
<i>S. aureus</i>	Acid	Acid	+	-	+	+	+
<i>P. aeruginosa</i>	Alkaline	Alkaline	-	-	+	-	-
<i>Klebsiella Sp</i>	Acid	Acid	+	-	-	±	+
<i>Proteus</i>	Alkaline	Acid	+	+	+	+	+
<i>E.coli</i>	Acid	Acid	+	-	+	±	+
<i>Streptococcus</i>	Alkaline	Acid	-	-	+	+	+

**Table 5: Organisms isolated from Wound and Pus samples**

S. NO	Isolated Organism	Percentage of occurrence
1	<i>Staphylococcus aureus</i>	58.77%
2	<i>Klebsiella species</i>	19.08%
3	<i>Pseudomonas aeruginosa</i>	11.45%
4	<i>Proteus species</i>	3.81%
5	<i>Escherichia coli</i>	3.81%
6	<i>Streptococcus pyogenes</i>	3.53%

(30mcg), Cefexime (5mcg), Cefotaxime (30mcg), Vancomycin (5mcg), Cloxacillin (5mcg), Penicillin- G (1 unit), Cotrimoxazole (25mcg), Ampicillin (10mcg), Ticarcillin (75mcg) and Piperacillin (100mcg) were the commercially available antibiotics were used for this study.

#### Assay of Antibacterial activity by agar well diffusion method

The agar well diffusion method expresses the results as the width of the inhibition zone produced by the plant extract<sup>22</sup>. The plant extracts were tested against bacterial strains. For agar well

diffusion method, 5mm size well was prepared in the in cultural strain swabbed plates with the help of a well cutter. Then, 5µl of plant extract (ethanolic plant extract) was added in the well by using micro pipette. Methanol added well considered as a control. Then plates were incubated at 37° C 24 hours. After the incubation period the zone of inhibition was

measured. A well was prepared in the plates with the help of a cork-borer (5mm)<sup>23</sup>.

#### Minimal inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) is the lowest concentration of a chemical

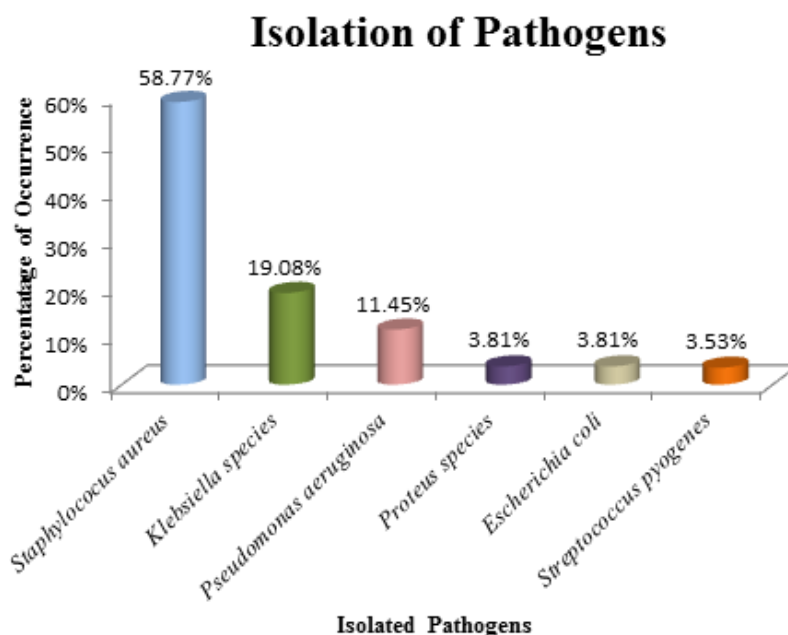


Fig. 1: Organisms isolated from Wound and Pus samples

Table 6: Antibiotic Susceptibility Pattern of Pyogenic Bacterial Isolates

Antibiotics	<i>Staphylococcus</i>		<i>Klebsiella</i>		<i>Pseudomonas</i>	
	%of S	%of R	%of S	%of R	%of S	%of R
Gentamicin	56.32	43.6	37.93	62.06	6.66	93.33
Netilmicin	50.57	49.42	41.37	58.62	33.33	66.66
Cefazolin	66.66	33.33	27.58	72.41	13.33	86.66
Cephalexin	52.87	47.12	10.34	89.65	6.66	93.33
Cefuroxime	62.06	37.93	34.49	65.51	20.00	80.00
Cefexime	ND	ND	27.58	72.41	13.33	86.66
Cefotaxime	43.67	56.32	24.13	75.86	26.66	73.33
Vancomycin	94.25	5.74	ND	ND	ND	ND
Cloxacillin	70.11	29.88	ND	ND	ND	ND
Penicillin- G	2.29	97.70	3.44	96.55	0	100
Cotrimoxazole	36.78	63.21	17.24	82.75	13.33	86.66
Ampicillin	1.14	98.85	3.44	96.55	0	100
Ticarcillin	ND	ND	ND	ND	60.00	40.00
Piperacillin	ND	ND	ND	ND	53.33	46.66

R: Resistant, S: Sensitive, %: Percentage, ND: Not Done

which prevents visible growth of a bacterium. This is in difference to the minimum bactericidal concentration (MBC) which is the concentration resulting in microbial death as defined by the inability to re-culture bacteria. The closer the MIC is to the MBC, the more bactericidal the compound<sup>24</sup>. Minimal inhibitory concentration (MIC) was determined by two fold serial dilution methods<sup>25</sup>. *Klebsiella*, *Staphylococcus* and *Pseudomonas* were used as

the indicator organisms. Samples were dissolved in DMSO (Dimethyl sulfoxide), which was a widely used commercial solvent derived from trees as a byproduct from the production of paper<sup>26</sup>, to a final concentration of 10mg/ml and added in increasing concentration such as 31.25, 62.5, 125, 250, 500, 1000 µg respectively and incubated overnight at 37°C. Growth was observed by visual inspection and by measuring the optical density (OD) at 630 nm

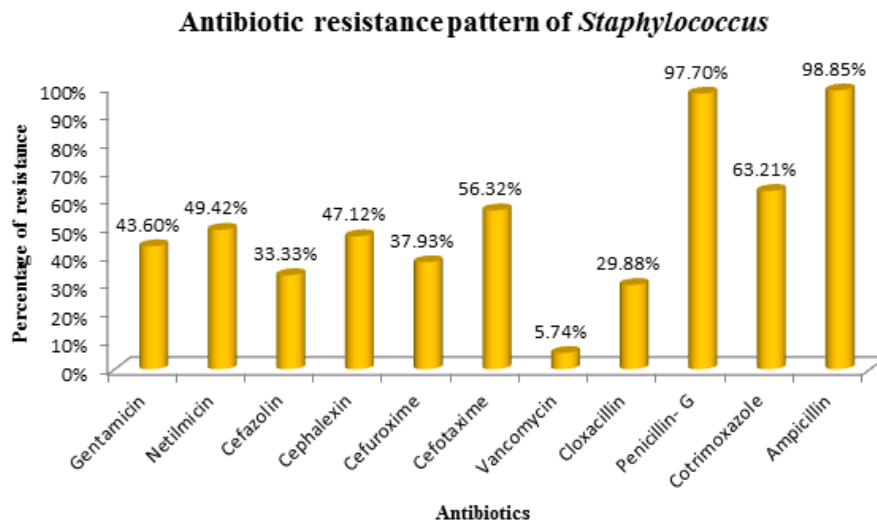


Fig. 2: Antibiotic resistance pattern of *Staphylococcus aureus*

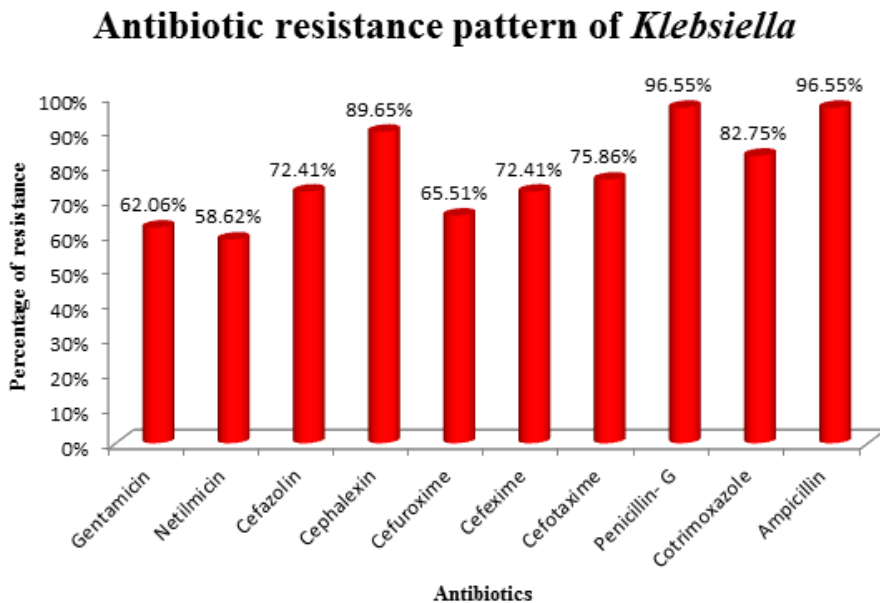


Fig. 3: Antibiotic resistance pattern of *Klebsiella Sp*

### Antibiotic resistance pattern of *Pseudomonas*

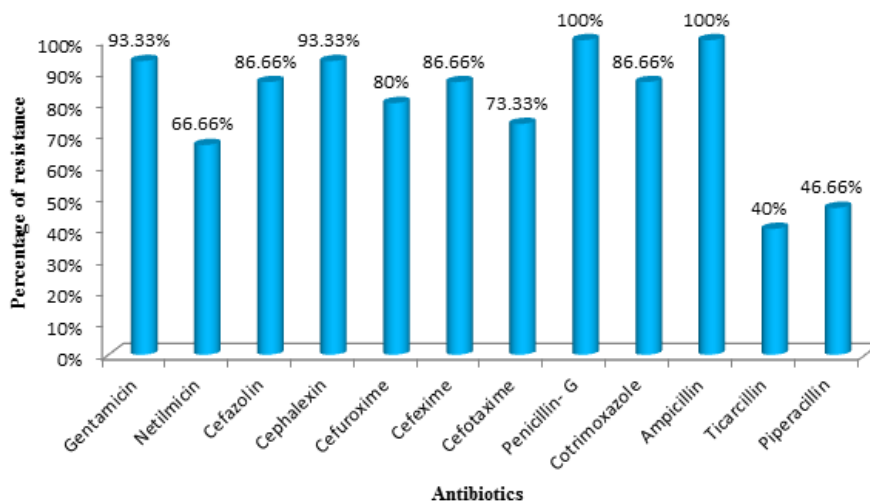


Fig. 4: Antibiotic resistance pattern of *Pseudomonas aeruginosa*

Table 7: Antimicrobial activity of the Ethanolic extract of *A. indica*, *C. asiatica* and *C. ternateia* against the pyogenic organisms

Ethanol Extract	Concentration (mg/mL)	Zone of inhibition (mm)		
		<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella spp</i>
<i>Azadirachta indica</i>	10	26	19	22
	5	16	18	17
	2.5	11	14	15
	1.25	8	10	11
<i>Centella asiatica</i>	10	22	18	20
	5	16	17	21
	2.5	14	14	19
	1.25	10	8	9
<i>Clitoria ternateia</i>	10	24	17	21
	5	18	16	19
	2.5	12	12	16
	1.25	6	10	8

Table 8: Minimum Inhibitory Concentration (MIC) of *Azadirachta indica*, *Centella asiatica* and *Clitoria ternateia* against the pyogenic organisms

Plants	Minimum Inhibitory Concentration		
	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella spp</i>
<i>Azadirachta indica</i>	551.099µg	557.462µg	213.344µg
<i>Centella asiatica</i>	681.175µg	623.183µg	611.639µg
<i>Clitoria ternateia</i>	651.175µg	637.692µg	480.241µg



using a spectrophotometer. The OD was measured immediately after the visual reading. The growth inhibition for the test wells at each plant extract dilution was determined by the formula:

$$\text{Percentage of inhibition} = \frac{(\text{OD of control} - \text{OD of test})}{(\text{OD of control})} \times 100\%$$

The minimum and maximum values were 0% and 100%, respectively.

## RESULTS AND DISCUSSION

The collected medicinal plants were subjected to the organoleptic evaluation which was tabulated in Table 1. The phytochemical constituents of the plant materials play a major role in antibacterial activity. It was identified by various phytochemical reactions and screen the presence of phytoconstituents like alkaloids, saponins, terpenoids, glycosides, flavonoids, sterols and steroids, tannins and phenolic compounds, carbohydrates (Table 2).

Out of 150 samples, 131 positive cultures were isolated and identified from wound and pus samples. Among 131 positive cultures, the predominant pathogen were gram positive organisms (62.3%) followed by gram negative organism (38.15%). The isolates were subjected to biochemical characterization and carbohydrate fermentation test for the identification of the pathogens (Table 3, 4). The predominant pathogen isolated from the clinical samples was identified as *Staphylococcus aureus* (58.77%), *Pseudomonas aeruginosa* (11.45%), *Klebsiella* (19.08%) followed by *Proteus* (3.81%), *E.coli* (3.81%) and *Streptococcus pyogenes* (3.53%) (Table 5, Fig.1). From these pathogens the most frequent pathogens such as *Staphylococcus aureus* (58.77%), *Pseudomonas aeruginosa* (11.45%), *Klebsiella* (19.08%) were selected for the further studies.

The Antibiotic susceptibility pattern of screened pathogens was determined using commercial antibiotics by disc diffusion method. *S. aureus*, the predominant pathogen was sensitive to Gentamicin, Netilmicin, Cefazolin, Cephalexin, Cefuroxime, Vancomycin and Cloxacillin. In other hand gram negative organisms, the *Klebsiella* sp

show resistance to the antibiotics such as Gentamicin, Netilmicin, Cefazolin, Cephalexin, Cefuroxime, Cefexime, Cefotaxime, Penicillin-G, Cotrimoxazole and Ampicillin. In case of *Pseudomonas aeruginosa*, it shows sensitivity to Ticarcillin, Piperacillin and resistance towards all other antibiotics (Table 6). The antimicrobial activity of the selected plant was determined by agar well diffusion method and all the three medicinal plant showed good activity against the pathogens. *Clitoria ternatea*, *Centella asiatica* and *Azadirachta indica* showed good activity against the gram positive organism *Staphylococcus aureus* followed by gram negative organisms *Klebsiella* sp and *Pseudomonas aeruginosa* (Table 7, Fig.2, Fig.3, Fig.4). The minimum inhibitory concentration is the lowest concentration of an antimicrobial agents like plant extracts or bacteriostatic or bactericidal or an antifungal agent that inhibit the growth of microbial pathogen after overnight incubation. The MIC of the medicinal plants *Clitoria ternatea*, *Centella asiatica* and *Azadirachta indica* inhibits the growth of bacterial pathogens in the lowest concentration of about 213.344µg to 681.175µg (Table 8).

Antibiotic resistance has become a global concern. There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. Such increase has been attributed to indiscriminate the use of broad spectrum antibiotics, immune suppressive agents and ongoing epidemics of Human Immuno deficiency virus infections. Antimicrobial resistance leads to billions of death every year<sup>27</sup>. This has forced scientists to search for new antimicrobial substances from various sources like the medicinal plants<sup>28</sup>. The selection of plant material for this study was based on ethno botanical data on the traditional use of the plants in treatment of bacterial diseases. Table 1 shows the antibacterial activity of medicinal plants against Gram-positive and Gram-negative bacteria.

## CONCLUSION

In the present study, plants like *Centella asiatica*, *Azadirachta indica*, *Clitoria ternatea* were selected that could accelerate the wound healing. The results showed that ethanolic extracts inhibited



the growth of *Pseudomonas*, *Klebsiella* to a great extent followed by *Staphylococcus aureus*. The selection of plant material for this study was based on ethno botanical data on the traditional use of the plants in treatment of bacterial diseases. The antibacterial effect may be attributed to the

phytoconstituents present in the herbal extracts and further studies will be focused on formulations against the microbes using these extracts. This may lead to find the effective role of medicinal plant as alternative medicine.

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