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# ANTIMICROBIAL ACTIVITY OF *PIPER CUBEBA* LEAVES

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## ABSTRACT

The aim of this study was to evaluate the antimicrobial effects of Ethanol, Aqueous, Chloroform & Methanol solvent against human pathogenic bacteria like *Bacillus cereus, Staphylocous aureus, Escherichia coli, Pseudomonas aeruginosa* & was determined by in vitro agar diffusion method. The herbal plant *Piper Cubeba* Leaves is selected and was evaluated for its phytochemical constituents, physicochemical property & antimicrobial activity. The extract was prepared by maceration extraction process by using aqueous solvent, Chloroform solvent & Ethanol solvent & also by Continuous hot extraction-Soxhelation process by using Methanol solvent. The extracts was formulated in different concentration of 1%, 2%, 3% & 4% by using same solvent & compared with the standard antibiotic like Amoxicillin (10µg/ml). Agar well diffusion method was used and the extract showed extensive zones of inhibition beside the tested isolates. Better antimicrobial activities were obtained with extract which gave better minimum inhibitory concentration (MIC) for the selected .In this study it has been showed that the 4% Ethanol extract of *Piper Cubeba* Leaves (E.E.P.C.L.) which was prepared by hot extraction process (soxhelation) had wider range of antimicrobial activity on used organisms than the aqueous, chloroform & methanol extract by maceration extraction process. This indicates that the *Piper Cubeba* leaves which are extracted by ethanol solvent using soxhelation process may contain more active components. This study supports the traditional medicines (herbal extracts) to cure many diseases like Diabetes Mellitus, intestinal tract infection, diarrhea, Wound Healing, ear infections, fever and throat infections.

**KEYWORDS:** Antimicrobial activity, Agar well diffusion method, *Piper Cubeba Leaves*, Human pathogen.

## INTRODUCTION

Herbal medicine is making amazing response and increasing number of patients visiting position by medicine clinics. Due to side effects of synthetic drug it has seen very danger because of drug interactions.[1] Herbal plants constructed for antimicrobials stand a massive undamaged genetic mechanisms of action of resistance and to remain studies source for drugs and additional analysis of plant to progress new drugs either synthetic or natural. Antimicrobial of plant is dangerous objective to offer right and professional origin have huge remedial potential.[2] Now a day due to fewer side effects about 80% of the world's people in Asian, Latin American, African and Middle Eastern countries is using plants as traditional health remedies.[3,4] In tropical and subtropical countries of the world human antimicrobial infections mainly those involving microorganisms i.e. fungi, viruses, bacteria, etc. they cause unembellished infections.

In current years due to use of marketable antimicrobial herbal numerous drugs resistances in human pathogenic microorganisms has been developed against microorganisms so herbal plants are one of the management of such diseases.[5,6] Antibiotics are one of the superior important part valuable discoveries of the 20$^{th}$ century that had support against stern bacterial infections but, only one third of the infectious diseases known have been treated from these synthetic drugs. This is due to presence of different pathogens that is absent from endowment the significance of years of general casual use, nonstop and mistreatment of antibiotics.[7] The microorganism & bacteria have the hereditary capacity to pass on and acquire resistance to drugs which are applied as therapeutic agents.[8] It is quite dangerous to human being to obtain resistance in future so to overcome this reduces antibiotics & develop a new formulation from herbal plants. Until a natural formulation have been appropriate as new antibacterial drugs there is an imperative need to recognize novel substances active towards highly resistant pathogens.[9,10] In India from initial times parts of herbal plants have been used to treat particular disorders. Currently there is extensive variety of result in drugs resultant from plants. This is very significance for the assurance that herbal medicines are not dangerous and authentic compared with expensive synthetic drugs which have more adverse effects. Natural antimicrobials can be resulting from plants, animal tissues or microorganisms. The limitations of the drugs offered today, thrust the advance of new

pharmacotherapeutic agents in medicinal plants. To find out the reliable and advancement of the use of herbal medicine, it is simple to increase the study of medicinal plants that determine place in legends.[11] Indian traditional medicinal system includes many medicinal plants associated to multiple effects.[12] It has been assessed that 14-28% of higher plant species are used medicinally and that 74% of pharmacologically vigorous plant derived components were discovered after follow up on ethno medicinal use of the plants.[13] Currently 30% or more of the pharmacological drugs are derived directly or indirectly from plants and their extracts directing in traditional medicine systems and a common element in Ayurveda, Homeopathic and Naturopathic etc.[14] Therefore traditional herbal drugs, fruits, spices, vegetables, and medicinal plants have expanded popularity over the past eras owing to their safety and efficacy. *Piper Cubeba* in unani medicine it is called as Kabab Chini which go to family Piperaceae. It is commonly distributed in tropical and subtropical regions and this medicinal herb is cited in traditional Unani books as an efficient therapy for Fever, Renal disorder, Gonorrhea, Leucorrhoea, Rheumatism, Diabetes etc.[15] *Piper Cubeba* plant has two types. 1. Long 2. Short. *Piper Cubeba* leaves are thin in shape the flower of *Piper Cubeba* is whitish yellow and grown into the stiff soil. The variety of Piper to the family Piperaceae & has more than 1000 variation spotted in hemispheres so the progress obviously in the form of stiff or shrubs, climbing herbs, or less frequently trees. Members of the genus Piper are used for many functions habitually in the spices and foods, fish bait, hallucinogens, fish poison, insecticides, oils, perfumes, ornaments and for lots of medicines for limited pain, diuretics, dysentery, fragrant refreshment, sedatives and carminatives.[16]

## MATERIALS AND METHODS

### Source of Plant Materials

The *Piper Cubeba Leaves* was collected from herbal garden of Sant Gajanan maharaj college of pharmacy, Takuka- Gadhinglaj, Dist.- Kolhapur (M.S.). The collected leaves are inspected for their pathogenic infections. Healthy leaves are selected after inspecting carefully. The leaves are washed in running tap water for removing the surface contaminants. The washed leaves are dried at room temperature for ten to fifteen days under shades. After drying the leaves are powdered using blender.[17]

### Chemicals and reagents

Methanol, Ethanol, Sulphuric Acid, Drangendroff's reagent, Molisch's reagent, Acetone etc.

**Equipment's**

Soxhlet apparatus, Incubator, Digital balance, Bunsen burner, PH meter, Glass wares, blender & Magnetic stirrer.

**Media**

Nutrient Agar and Muller Hinton Agar Media.

**Organism**

Human pathogens of two gram positive viz; *Bacillus cereus, Staphylocous aureus* & two gram negative *Escherichia coli, Pseudomonas aeruginosa* are used for antimicrobial activity.

**Preparation of Extracts**[18]

Extracts are prepared by using following procedure.

**Ethanol extract of *Piper Cubeba* Leaves**

This extraction process is carried out by using ethanol (95%) in a soxhelation apparatus (Continuous Hot Extraction Process) in expectation of extraction was finished. Then extraction is evaporate by heated on a water bath. Dark green colour extract was obtained. The extract was then stored in desiccators for further research investigation. The percentage yields of the above extracts were calculated & represented in Table No.1.

**Aqueous extract of *Piper Cubeba* Leaves**

Marc left after ethanol extract was collected & dried then it was macerated by using distilled water in a 2litre of round bottom flask not less than 72 hrs. Then added 10 ml of chloroform prevent it from fungal growth. After attainment of extraction the extraction was filtered & extract was removed by evaporation to dryness on a water bath. Green colored extracts were obtained which was sticky in nature & it was stored in a desiccators for removal of unnecessary moisture. Then the dried extracts were packed in air tight glass container for further research investigation. The percentage yields of the above extracts were calculated & represented in Table No.1.

**Methanol extract of *Piper Cubeba* Leaves**

This extraction process is carried out by using Methanol (95%) macerated it for not less than 72 hrs. After getting an extract the solvent was evaporate by heating on a water bath. Dark green colour extract was gated. The extract was preserved in desiccators for further research

investigation. The percentage yields of the above extracts were calculated & represented in Table No. 1.

**Chloroform extract of *Piper Cubeba* Leaves**

Marc left after methanol extraction was dried & then extraction is carried with chloroform up to extraction was completed. After getting an extraction the solvent was evaporate by heating it on a water bath. Greenish brown colored extract was obtained. The extract was then stored in desiccators for further research investigation. The percentage yields of the above extracts were calculated & represented in Table No.1.

**Preparation of Formulation**

After collection of each dried extract i.e. aqueous, chloroform, methanol (By maceration) & ethanol (By Soxhelation) the formulation was prepared in different concentration of 1%, 2%, 3% & 4% for each extract by using same solvent .The formulations are prepare as for Ethanolic extract (F1 to F4) , Aqueous Extract (F5 to F8), Chloroform extract (F9 to F12) & Methanolic extract (F13 to F16).

**Preparation of the tested organisms[19]**

**Preparation of standard bacterial suspensions**

The average number of viable *Bacillus cereus, Staphylocous aureus, Escherichia coli, Pseudomonas aeruginosa* organisms per ml of the stock suspensions was determined by means of the surface viable counting technique. About (108-109) colony forming units per ml was used. Each time a fresh stock suspension was prepared the experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

**Evaluations**

**Phytochemical analysis[20]**

The each extract obtained after maceration & soxhelation was subjected to various phytochemical screening as per the standard procedure to reveals the presence of various active phytoconstituent phytochemical evaluation of each plant extract is given in Table No. 2.

**Physicochemical parameters[21]**

Preliminary evaluation of *Piper Cubeba* Leaves was carried out as per given below & expressed in Table No. 3.

**Color and Odour**

Color and odour was examined by visual examination.

**PH**

The pH of various extracts was determined by using Digital pH meter. Each extract was dissolved in 100 ml of same solvent of extract and stored for two hours. The measurement of pH of each concentration was done in triplicate and average values were depicted in Table No.3.

**Stability Studies**

The stability studies were carried out for each extract at different temperature conditions ($4^0$C, $25^o$ C and $37^o$ C) for 3 months. The average values were given in Table No.3.

**Preparation of Sample**

The extracts were weighed & prepared different formulation (F1 to F16) of different concentrated by using same solvent & 0.05 ml was used for activity studies.

**Preparation of Control**

The same solvents which are used for extraction process is used as control for the antimicrobial studies.0.05 ml solvents are used for activity study.

**Preparation of Standard**

Amoxicillin serve as a standard control for antimicrobial activity.

(Amoxicillin10µg/ml)

**Preparation of Medium and Nutrient broth[22]**

Weigh accurately 0.4gm of nutrient soup and dissolved in 30ml of distilled water. Then the soup was hanged in each of test tube. The Muller Hinton agar medium was prepared which contain 9.7gm of MHA was hanged in 250ml of water. Then broth the medium and soup were for sterilization. After sterilization the nutrient soup was allowed to cool and then the organisms were inoculated for 4 hrs. The MHA medium was poured in the petridish before cooling and allowed to solidify for about 3-4 hrs.

**Methodology**

Antimicrobial activity was determined according to the method described by Okeke (2001) with in triplicate. For each extract 1 to 4 mg. extract was used for antimicrobial activity and

formulations was prepared from F1 to F16 for each extract. *Bacillus cereus* culture was swabbed on the surface of sterile nutrient agar plate in triplicate. In agar plate with five wells were prepared with the help of sterilized cork borer of 6mm diameter namely 1,2,3,4 & 5. The (5) center well is served as standard, (C) served as control, (1) served as 1% extract, (2) served as 2% extract (3) served as 3% extract & (4) served as 4% extract as show in figure No 2 . Each well was filled aseptically with 0.05ml Standard, Control & Extracts by using micropipette. Similarly the antimicrobial activity of *Staphylocous aureus, Escherichia coli & Pseudomonas aeruginosa* was carried out. These plates were incubated at 37$^{o}$C overnight for observation. The occurrence of inhibition was noted and compared with the standard. The susceptibility of the test organism to the tested plant extract was resolute by observing the zone of inhibition around each well.

**Screening of Microbial activity**

Antimicrobial activities of *Piper Cubeba* Leaves extracts was evaluated by using agar well diffusion method.

**Agar well diffusion method[23]**

Agar well diffusion method was used to find out antimicrobial activity. Nutrient agar (NA) and Potato Dextrose Agar (PDA) plates were swabbed (sterile cotton swabs) with 8 hour old broth culture of respective bacteria. Five wells (6mm diameter and about 2 cm a part) were made in each of these plates using sterile cork tool. *Piper Cubeba* Leaves extract of different concentration 1, 2, 3 & 4 mg/ml was used. About 0.05 ml of extract is subjected into well by using micropipette and permitted to diffuse at room temperature for 2hrs. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 hrs. for bacterial pathogens. The diameter of the inhibition zone (mm) was measured and considered. The readings were taken in three different fixed directions and the average values were recorded.

**Determination of relative percentage inhibition[24]**

The relative percentage inhibition of the *Piper Cubeba* Leaves extract with respect to standard, control was calculated by using the following formula

$$\text{Relative percentage inhibition of the test extract} = \frac{100 \text{ X } (x-y)}{(z-y)}$$

Where,

**x:** total area of inhibition of the test extract

**y:** total area of inhibition of the solvent

**z:** total area of inhibition of the standard drug

The total area of the inhibition was calculated by using area = πr2

Where, r = radius of zone of inhibition

**Measurement of antimicrobial activity using Agar well diffusion Method**

The antimicrobial activity of *Piper Cubeba* Leaves was assessed according to their zone of inhibition against various pathogens and the results (zone of inhibition) were compared with the activity of the standards viz. Amoxicillin antibiotic. The results discovered that the extracts are potent antimicrobials against all the microorganisms studied. Among of these different concentrations the 4% Ethanolic Extract of Piper Cubeba formula (E.E.P.C.L.F4) showed high degree of inhibition.

The antimicrobial activity of different extracts of *Piper Cubeba* Leaves is listed from Table No. 4 to table No 7.

**Evaluation of Percentage yield of various extract of *Piper Cubeba* Leaves**

**Table 1: Percentage yield of various extract of *Piper Cubeba* Leaves.**

| Sr. No. | Solvent Used | Raw material used (gm.) | Yield (gm.) | % Yield |
|---|---|---|---|---|
| 1. | Aqueous extract | 500 | 69.7 | 13.9 |
| 2. | Chloroform extract | 500 | 62.0 | 12.4 |
| 3. | Methanol extract | 500 | 71.1 | 14.2 |
| 4. | Ethanol extract (Soxhelation) | 500 | 74.1 | 15.0 |

**Evaluation of phytoconstituent of various extract *Piper Cubeba* Leaves**

**Table 2: Phytochemical screening of the extract of *Piper Cubeba* Leaves.**

| Test | *Piper Cubeba* Leaves Extracts | | | |
|---|---|---|---|---|
| | Aqueous Extract | Methanol Extract | Ethanol Extract | Chloroform Extract |
| Alkaloid | + | + | + | + |
| Flavanoids | + | - | + | - |
| Glycosides | + | + | + | - |
| Reducing Sugar | - | - | - | - |
| Tannins | - | + | + | + |
| Saponins | - | - | - | - |
| Terpenoids | - | + | - | - |
| Polysaccharides | + | - | + | - |
| Phytosterols | - | - | + | - |
| Phenols | + | + | + | - |

**Present = (+) & absent = (-)**

**Evaluation of Physiochemical properties of various extract *Piper Cubeba* Leaves**

**Table 3: Physiochemical properties of extracts of *Piper Cubeba* Leaves.**

| Sr. No. | Solvent | Color | Odour | PH | Consistency |
|---|---|---|---|---|---|
| 1. | Aqueous extract | Dark Green | Characteristics | 6.2 | Sticky |
| 2. | Chloroform extract | Greenish Brown | Characteristics | 5.2 | Sticky |
| 3. | Methanol extract | Dark Green | Characteristics | 6.4 | Sticky |
| 4. | Ethanol extract (Soxhelation) | Dark Green | Characteristics | 7.1 | Sticky |
| 5. | Stability | $4^0$c | Stable | Stable | Stable |
| | | $25^0$c | Stable | Stable | Stable |
| | | $37^0$c | Stable | Stable | Stable |

**Evaluation of antimicrobial activity of *Piper Cubeba* Leaves**

**a) Evaluation of antimicrobial activity of Ethanolic Extract of *Piper Cubeba* Leaves(E.E.P.C.L)**

**Table 4: Antimicrobial activities of different concentration of Ethanolic Extract of Piper Cubeba Leaves (E.E.P.C.L.) extract formulation.**

| Sr. No. | Formulations | Concentrations | Zone of Inhibited Diameter in mm | | | |
|---|---|---|---|---|---|---|
| | | | *Bacillus cereus* | *Staphylocous aureus* | *Escherichia coli* | *Pseudomonas aeruginosa* |
| 1 | E.E.P.C.L.F1 | 1Mg. | 5 | - | 4 | 3 |
| 2 | E.E.P.C.L.F2 | 2Mg. | 7 | 4 | 8 | 7 |
| 3 | E.E.P.C.L.F3 | 3Mg. | 15 | 12 | 16 | 16 |
| 4 | E.E.P.C.L.F4 | 4Mg. | 19 | 16 | 20 | 19 |
| | | Standard | 18 | 17 | 18 | 19 |
| | | Control | - | - | - | - |

**b) Evaluation of antimicrobial activity of Aqueous Extract of *Piper Cubeba* Leaves(A.E.P.C.L)**

**Table 5: Antimicrobial activities of different concentration of Aqueous Extract of Piper Cubeba Leaves (A.E.P.C.L.) extract formulation.**

| Sr. No. | Formulations | Concentrations | Zone of Inhibited Diameter in mm | | | |
|---|---|---|---|---|---|---|
| | | | Bacillus cereus | Staphylocous aureus | Escherichia coli | Pseudomonas aeruginosa |
| 1 | A.E.P.C.L.F5 | 1Mg. | - | - | - | - |
| 2 | A.E.P.C.L.F6 | 2Mg. | - | - | - | - |
| 3 | A.E.P.C.L.F7 | 3Mg. | 6 | - | - | 5 |
| 4 | A.E.P.C.L.F8 | 4Mg. | 8 | 4 | - | 8 |
| | | Standard | 18 | 17 | 18 | 19 |
| | | Control | - | - | - | - |

**C) Evaluation of antimicrobial activity of Chloroform Extract of *Piper Cubeba* Leaves(C.E.P.C.L)**

**Table 6: Antimicrobial activities of different concentration of Chloroform Extract of Piper Cubeba Leaves (C.E.P.C.L.) extract formulation.**

| Sr. No. | Formulations | Concentrations | Zone of Inhibited Diameter in mm | | | |
|---|---|---|---|---|---|---|
| | | | *Bacillus cereus* | *Staphylococ us aureus* | *Escherichia coli* | *Pseudomonas aeruginosa* |
| 1 | C.E.P.C.L.F9 | 1Mg. | - | - | - | - |
| 2 | C.E.P.C.L.F10 | 2Mg. | - | - | - | - |
| 3 | C.E.P.C.L.F11 | 3Mg. | - | - | 3 | - |
| 4 | C.E.P.C.L.F12 | 4Mg. | 4 | - | 5 | 5 |
| | | Standard | 18 | 17 | 18 | 19 |
| | | Control | - | - | - | - |

**c) Evaluation of antimicrobial activity of Methanolic Extract of *Piper Cubeba* Leaves(M.E.P.C.L)**

**Table 7: Antimicrobial activities of different concentration of Methanolic Extract of Piper Cubeba Leaves (M.E.P.C.L.) extract formulation.**

| Sr. No. | Formulations | Concentrations | Zone of Inhibited Diameter in mm | | | |
|---|---|---|---|---|---|---|
| | | | *Bacillus cereus* | *Staphylococ usaureus* | *Escherichia coli* | *Pseudomonas aeruginosa* |
| 1 | M.E.P.C.L.F13 | 1Mg. | - | - | - | - |
| 2 | M.E.P.C.L.F14 | 2Mg. | 5 | - | - | 3 |
| 3 | M.E.P.C.L.F15 | 3Mg. | 7 | 5 | - | 5 |
| 4 | M.E.P.C.L.F16 | 4Mg. | 10 | 6 | 4 | 6 |
| | | Standard | 18 | 17 | 18 | 19 |
| | | Control | - | - | - | - |

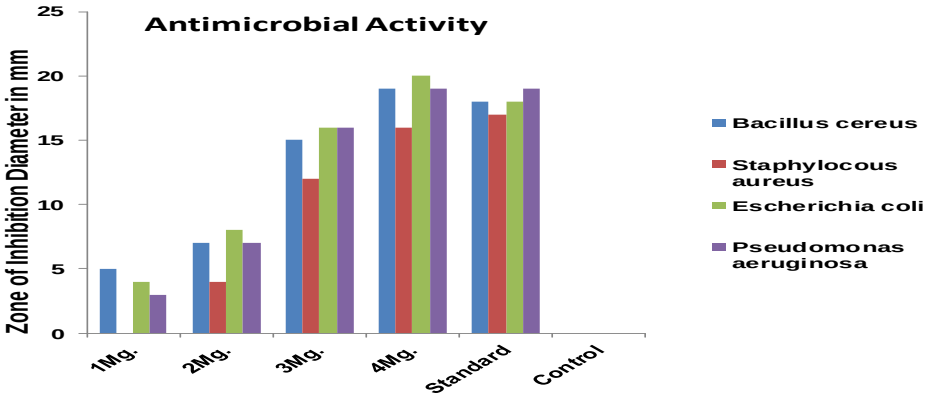


**Figure 1: Graphical Presentation of Antimicrobial activity of different concentrated Piper Cubeba Leaves (E.E.P.C.L.) extract formulation.**

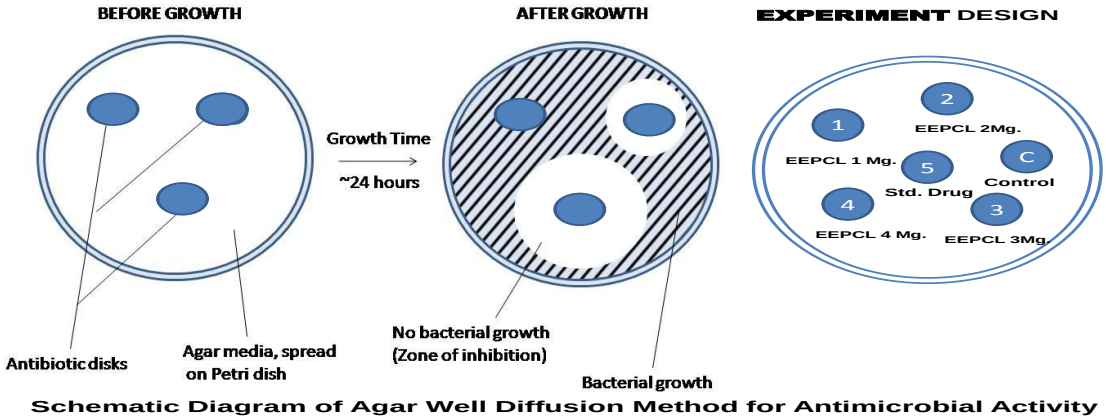


**Figure 2: Schematic diagrams were showing use of methodology for antimicrobial activity of different concentrated formulation.**

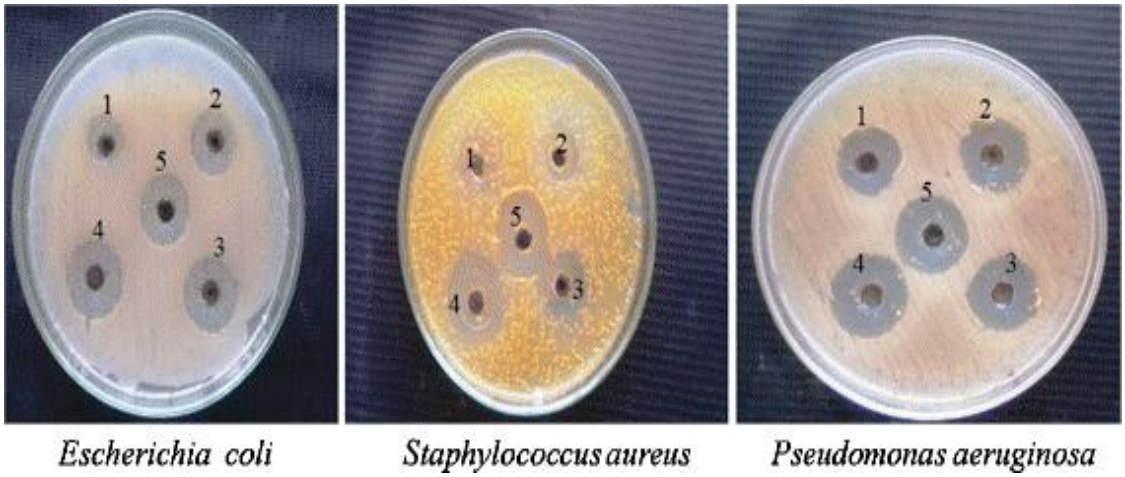


**Figure 3: Photographs of E.EP.C.L. Antimicrobial Activity.**

**Figure 4: Photograph of *Piper Cubeba* Leaves.**

## RESULTS AND DISCUSSION

This present study shown that the herbal plant *Piper Cubeba* Leaves was made to create different concentrations in different extracts and to evaluate for its physical parameter and to compare its antimicrobial activity with a standard antibiotic like Amoxicillin. *Piper Cubeba*

Leaves was extracted by maceration process using aqueous, chloroform & methanol solvents & again continuous hot extraction (Soxhelation) process by using methanol solvent. The preliminary Phytochemical reading show that crude extract of *Piper Cubeba* Leaves contain alkaloids, glycosides, Carbohydrates, Terpenoids, Flavonoids etc. The percentage yield of herbal plant extract (Aqueous, Chloroform, Methanol and Ethanol) of *Piper Cubeba* Leaves was shown in Table No. 1. From the discovered data in the percentage yield it was found that the Methanol & Ethanol extract by soxhelation extraction has maximum amount of the yield and the chloroform extract has minimum amount of the yield.

In the present work the *Piper Cubeba* Leaves extract was studied for its physical parameter, phytochemical constituents & different concentrated in vitro antimicrobial activity & was compared with standard antibiotic like Amoxicillin. The physical parameters were within the suitable range. The stability studies was carried out and concluded that the all concentrations shows no signs of instability. The antimicrobial activity of prepared formulations were compared with antibiotic like Amoxicillin using selected species of gram positive bacteria such as *Bacillus cereus, Staphylocous aureus* & gram negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa* and it was showed that 4% Ethanolic Extract of *Piper Cubeba* Leaves (E.E.P.C.L.F4) showed greater antimicrobial activity against given micro-organism as compared to antibiotic like Amoxicillin. It was also seen that the by increasing in a concentration of extract it show an increase in antimicrobial activity. The antimicrobial activity of Ethanolic Extract of *Piper Cubeba* Leaves is due to the presence of flavanoids and tannins. Hence the study concludes that the Ethanolic Extract of *Piper Cubeba* Leaves extracted by continuous hot extraction (soxhelation) process is an antimicrobial activity, it is also efficient antiseptic purpose with antimicrobial activities can be formulated from the ethanolic plant extracts of *Piper Cubeba* Leaves which can also be used for wound healing and various skin infections.

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