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# Phytochemical Analysis of Leaves of *Coleus aromaticus* Benth. and it's Antibacterial Activity against *Staphylococcus aureus*

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**Keywords:** *Staphylococcus aureus*, Lamiaceae, Antibacterial activity, Phytochemical, Coleus aromaticus, Saponin, Flavonoid

**ABSTRACT**

The expanding bacterial resistance to antibiotics has become a growing concern worldwide. Increasing bacterial resistance limits therapeutic options and hence attention has turned towards plants as alternative therapy against resistant strains. The search for antibiotic resistance modulators in plants represents a new dimension to addressing the problem of antibiotic resistance. Medicinal plants are promising and offer considerable potential for the development of new agents effective against infections currently difficult to treat. Medicinal plants, since time immemorial, have been used as a source of medicine in most parts of the world. Leaves of plant *Coleus aromaticus* Benth. were taken for herbal drug potential studies belonging to family Lamiaceae. In India, the plant is commonly known as Patharchur. In the present study phytochemical analysis of leaves was done in acetone, aqueous, ethanol and methanol solvents. Results showed that leaves have flavonoids, saponin, phenolic compound, diterpenes and carbohydrates. The antibacterial activity of *Coleus aromaticus* was evaluated against human pathogenic bacteria *Staphylococcus aureus* by well diffusion method. *A staphylococcus aureus bacterium was* isolated from the sputum samples of T.B. negative patients. It has been observed that all solvent extract exhibit antibacterial activity. The alcoholic extract showed maximum activity followed by aqueous extract while acetonic extract exhibit minimum antibacterial activity against *Staphylococcus*. From this study, it is concluded that leaves of *Coleus* are effective in the inhibition of *Staphylococcus aureus* growth *in vitro* conditions.

## INTRODUCTION

Disease causing bacteria have always been considered a major cause of morbidity and mortality in humans. Antibiotic resistance has become a challenge in research industry. Increasing bacterial resistance limits therapeutic options and hence attention has turned towards plants as alternative therapy against resistant strains (Alviano and Alviano, 2009). Research and scientific data supports the antimicrobial activity of extracts and biologically active compounds isolated from medicinal plants. (Laj *et al*., 2007). A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds (Mahady, 2005). Medicinal plants are promising and offer considerable potential for the development of new agents effective against infections currently difficult to treat (Iwu *et al*., 1999). In recent years, secondary plant metabolites (Phytochemicals) of plants have been extensively investigated as source of medicinal agents. Since olden days, various medicinal plants have been used to treat human diseases. It has been estimated that between 60-90% of the populations of developing countries use traditional and herbal medicines almost exclusively and consider them to be a normal part of primary healthcare (WHO, 2002). Plants are rich in a great diversity of phytochemicals such as phenolic acids, flavonoids, tannins, lignin, and other small compounds. (Cowan, 1999). *Coleus aromaticus* Benth. is a commonly available medicinal herb in India. The plant *Coleus aromaticus* Benth., belonging to the family Lamiaceae. It is a large succulent aromatic perennial herb, much branched, fleshy highly aromatic pubscent herb with distinctive smelling leaves. The plant is distributed throughout India and is cultivated in the gardens.

### Objective of the study

- Isolation and identification of bacteria causing respiratory tract infection from samples collected from Chirayu hospital, Bhopal.
- Qualitative analysis of phytoconstituents of leaf extract of *Coleus aromaticus* plant.
- *In vitro* investigation of antibacterial activity of plant extract against isolated pathogens.

## MATERIAL AND METHODS

### Collection, Isolation and Identification of Respiratory Tract Pathogens

The patients suffering from different respiratory tract infections of Chirayu Hospital, Bhopal (M. P.) were the sources for the clinical samples. Throat swabs and sputum samples were aseptically collected from suspected patients referred by physicians to diagnostic laboratory, Chirayu Hospital, Bhopal (M. P.).

In aseptic condition, the swabs and sputum were taken out from the container and streaked (Collins and Lyne *et al*., 1995) on the nutrient agar plates for primary isolation of organisms. The samples were also plated on to Blood agar and Mannitol salt agar media. The streaked plates were kept in the incubator at $37^0$C for 24 to 48 hrs for further observations. Plates were observed after overnight incubation for colony characteristics from nutrient agar media. Characteristic colonies from the plates were isolated and then subcultured to obtain a pure culture. The isolated organisms were identified based on colony morphology, microscopic observation and various biochemical tests according to standard laboratory methods (Ananthanarayan and Paniker, 2009; Bhattacharya, 2006).

### Biochemical Identification of bacterial pathogens

All the selected test isolates based on colony characteristics and microscopic features were subjected to the further biochemical tests.

Basic biochemical test like catalase, coagulase, DNAse and IMViC were conducted as per the procedure prescribed by Collins and Lyne *et al*. (1995).

### Molecular Characterization of bacterial pathogens

Genomic DNA was isolated from the bacterial isolates and the 16S rRNA region of the DNA was amplified using universal 16SrRNA primers in thermal cycler. The PCR reaction conditions were; initial denaturation for 5 min at 94°C, denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C, extension at 72°C for 2 min and final extension at 72°C for 15 min. The PCR amplified products were then run on agarose gel, eluted, purified and sequenced using Sanger's method.

**Collection and Identification of Indigenous Medicinal plant**

Twigs of *Coleus aromaticus* were collected from Bhopal (M. P.). The plants were identified from Govt. M.V.M. College with the help of the project report of Dr. Madhuri Modak, who has confirmed the identification from Botanical Survey of India, Allahabad. The fresh leaves were separated from plant. The separated leaves were washed repeatedly with tap water and finally with distilled water, shade dried and then finely ground to a powder with the help of mechanical grinder, sieved and stored separately at room temperature for further studies. The voucher specimen is preserved in the Department of Botany, Sri Sathya Sai College for Women, Bhopal (M.P.) (Satish *et al.*, 2007).

**Scientific Classification**

Kingdom: Plantae

Order: Lamiales

Family: Lamiaceae

Genus: *Coleus*

Species: *aromaticus*

**Figure No. 1: Habit of *Coleus aromaticus***

**Preparation of plant extracts**

50 gm of dried powdered sample was extracted with 300 ml of solvents such as water, methanol, ethanol and acetone in a Soxhlet apparatus. The soxhlet extraction method is the most commonly used semi-continuous method applied for extraction of active principles. The powdered plant material was filled in the thimble with appropriate amount of solvent in flask for extraction process. Temperature was maintained at the boiling point of respective solvent.

| | |
|---|---|
| Ethanol | 78.3°C |
| Methanol | 65°C |
| Petroleum ether | 60-80°C |
| Water | 100°C |
| Acetone | 56.3°C |

Soxhlet exhaustion was continued till the solvent become colourless in siphon tube. The extracts were collected and then concentrated by using a hot air oven at temperature between 40°-50°C. Dried extract was collected and stored in refrigerator for further analysis. (Abeysinghe, 2010).

**Determination of plant yield**

The percentage yield of the extract obtained was calculated by using this formula:

$$\frac{W_2 - W_1}{W_0} \times 100$$

Where $W_2$ is the weight of the extract and the container, $W_1$ the weight of the container alone and $W_0$ the weight of the initial dried sample. (Anokwuru, 2011)

**Preliminary phytochemical screening**

Phytochemical screening of plant extracts were carried out qualitatively for the presence of carbohydrates, terpenoids, tannins, flavonoids, phenolic compounds, saponins, quinones and alkaloids using standard procedures (Evans, 1996).

**1. Detection of alkaloids**

Extracts were dissolved individually in dilute Hydrochloric acid and filtered. The filtrates were used to test for the presence of alkaloids.

**a) Mayer's Test**: 2-3 ml of filtrates were treated with Mayer's reagent (Potassium Mercuric iodide). Formation of a yellow cream precipitate indicates the presence of Alkaloids.

**b) Wagner's Test**: Filtrates were treated with Wagner's reagent (Iodine in potassium iodide) Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

**c) Hager's Test**: Filtrates were treated with Hager's reagent (saturated picric acid solution). Formation of a yellow colored precipitate indicates the presence of alkaloids.

**2. Detection of glycosides**

Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

**a) Modified Borntrager's Test**: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

**3. Detection of Cardiac glycosides**

**a) Keller Killani test:** Crude extract was mixed with 2 ml of glacial acetic acid containing 1 – 2 drops of 5 % solution of $FeCl_3$. Then mixture was treated with 2 ml conc. $H_2So_4$. A brown ring (green – blue) at the interphase indicated the presence of cardiac glycosides.

**4. Detection of phytosterols**

**a) Salkowski's Test:** Extracts were treated with chloroform and filtered. The filtrates were treated with a few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

**b) Liebermann Burchard's test**: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of brown ring at the junction indicates the presence of phytosterols.

**5. Detection of saponins**

**a) Froth Test**: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

**b) Foam test:** Small amount of extract was shaken with little quantity of water. If foam produced persists for ten minutes it indicates the presence of saponins.

**6. Detection of tannins**

**a) Gelatin Test:** To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

**7. Detection of flavonoids**

**a) Alkaline Reagent Test**: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

**b) Lead acetate Test:** Extracts were treated with few drops of lead acetate solution. Formation of a yellow colour precipitate indicates the presence of flavonoids.

**8. Detection of phenols**

**a) Ferric Chloride Test**: Extracts were treated with few drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

**9. Detection of Phlobatannins**

Aqueous extract of plant was boiled with 1% aqueous HCl. Formation of red PPT indicates the presence of Phlobatannins.

**10. Detection of Anthraquinones**

0.5 gm of extract was boiled with 10% HCl and filtered. Few drops 10% ammonia were added to the mixture and heated. Formation of rose pink colour indicates the presence of Anthraquinones.

**11. Detection of resins**

**a) Acetone-water Test**: Extracts were treated with acetone. Small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

**12. Detection of fixed oils & fats**

**a) Stain Test:** Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oil.

**13. Detection of carbohydrates**

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

**a) Molisch's Test:** Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml of conc. sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of Carbohydrates.

**b) Benedict's test**: Filtrates were treated with Benedict's reagent and heated on a water bath. Formation of an orange red precipitate indicates the presence of reducing sugars.

**c) Fehling's Test:** Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehlings A and B solutions. Formation of a red precipitate indicates the presence of reducing sugars.

**d) Iodine test:** Crude extract was mixed with 2 ml of Iodine solution. A dark blue or purple colour indicates the presence of carbohydrates.

**14. Detection of proteins and amino acids**

**a) Millon's Test:** Crude extract when mixed with 2 ml of Millon's reagent white ppt appeared which turned red upon gentle heating that confirms the presence of protein.

**b) Xanthoproteic Test:** The extracts were treated with few drops of concentrated Nitric acid solution. Formation of yellow colour indicates the presence of proteins.

**c) Ninhydrin test**: To the extract, 0.25% ninhydrin reagent was added and boiled for few a minutes. Formation of blue colour indicates the presence of amino acid.

**d) Biuret Test:** The extracts were treated with 1 ml of 10% sodium hydroxide solution and heated. To this, a drop of 0.7% copper sulphate solution was added. Formation of purplish violet colour indicates the presence of proteins.

**Preparation of inoculums (Test Sample):** Suspension of bacteria was prepared by inoculating a loopful culture of target bacteria (24 hour old culture) in 25 ml nutrient broth and incubated at 37°C for 18-24 hours till a moderate turbidity was developed.

**Procedure for Performing Antibacterial Assay:** The antibacterial activity of different plant species was evaluated by agar well diffusion method. The required amount of nutrient agar plates was prepared. 100 µl of standardized cell suspension was spreaded on nutrient agar plate using a spreader in order to obtain uniform microbial growth. A sterile cork borer was used to bore wells with a diameter of 6 mm to the plate of the agar. Wells were seeded with 50 µl plant extract and permitted to stand at room temperature for 1 hr. Three concentrations 25, 50 and 100 mg/ml were used for each extract. The plates were incubated at 37 °C for 24hrs. Ciprofloxacin was used as positive control.

The plates were observed for bacterial growth inhibition indicated by the clear zone around the well. The zone of inhibition was measured in millimetre. No inhibition zone was interpreted as the absence of activity. All experiments were carried out in triplicates under aseptic conditions and the zone of inhibition around each well was measured for susceptibility and resistance.

## RESULTS

### Identification of *Staphylococcus aureus*

Sample streaked on NAM was observed for colony characteristics. A yellow colour colony on plates was observed. For identification, the cultures were Gram's stained and studied at 100x (oil immersion) objective of microscope. Gram positive purple coloured cocci were seen. The identified bacterial colonies were pure cultured on NAM. For further confirmation of *S. aureus*, the cultures were streaked on MSA and Blood Agar media. The growth of small colonies fermenting mannitol with changing the colour from pink to yellow on MSA confirmed the culture to be *S. aureus*. Further β- haemolysis was (complete haemolysis of blood) observed on Blood agar media confirmed the results. Biochemical testing was done for confirmation of identified *S. aureus* cultures. It was observed that culture was catalase, coagulase, methyl red and DNAase positive. In carbohydrates fermentation test, organism fermented mannitol, sucrose, lactose, glucose and dextrose. The 16S rRNA sequencing was carried out for *Staphylococcus aureus* using universal primer. Sequence alignment of the 16S rRNA using BLAST analysis was carried out to confirm the identification of bacteria. The

analysis revealed 97% similarity to *Staphylococcus aureus* sequences available in GenBank database. The 16S rRNA sequence was then submitted to GenBank database and accession number was obtained (MN014310).

**Table No. 1: Colony morphology of *S. aureus***

| Sr. No. | Culture media | Morphology |
|---|---|---|
| **1.** | Nutrient agar media | Small, Smooth, entire edge, low convex, glistening, opaque, golden in color |
| **2.** | Mannitol salt agar | Small colonies fermenting mannitol with changing the color from pink to yellow |
| **3.** | Blood agar | β -haemolytic colony |

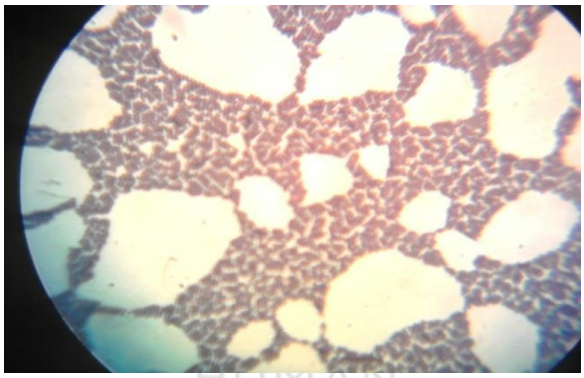

**Figure No. 2: Gram positive cocci of *S. aureus***

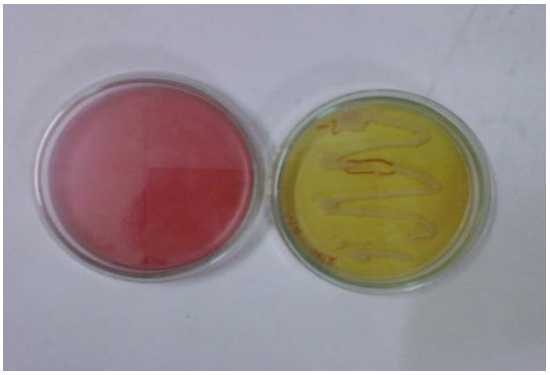

**Figure No. 3: *S. aureus* on Mannitol salt agar**


**Figure No. 4*: S. aureus* on Nutrient agar**

**Figure No. 5: *S. aureus* on Blood agar**

**Table No. 2: Biochemical and Carbohydrate Fermentation Test**

| Biochemical Test | | Carbohydrate test | |
|---|---|---|---|
| **Indole** | -ve | **Mannitol** | Fermented |
| **Methyl red** | +ve | **Sucrose** | Fermented |
| **Voges proskauer** | -ve | **Glucose** | Fermented |
| **Citrate utilization** | -ve | **Maltose** | Nonfermented |
| **Catalase** | +ve | **Lactose** | Fermented |
| **Cogulase** | +ve | **Dextrose** | Fermented |
| **DNAse** | +ve | | |

**Figure No. 6: Catalse test**

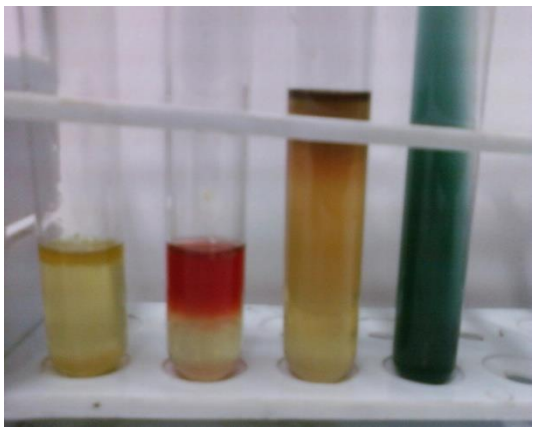

**Figure No. 7: IMViC for *S. aureus***

**Figure No. 8: Coagulase test**

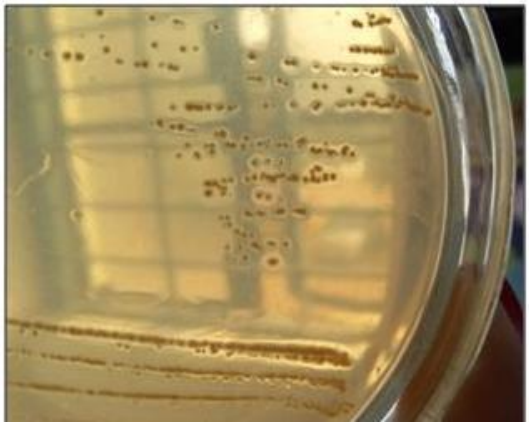

**Figure No. 9: DNAse test**

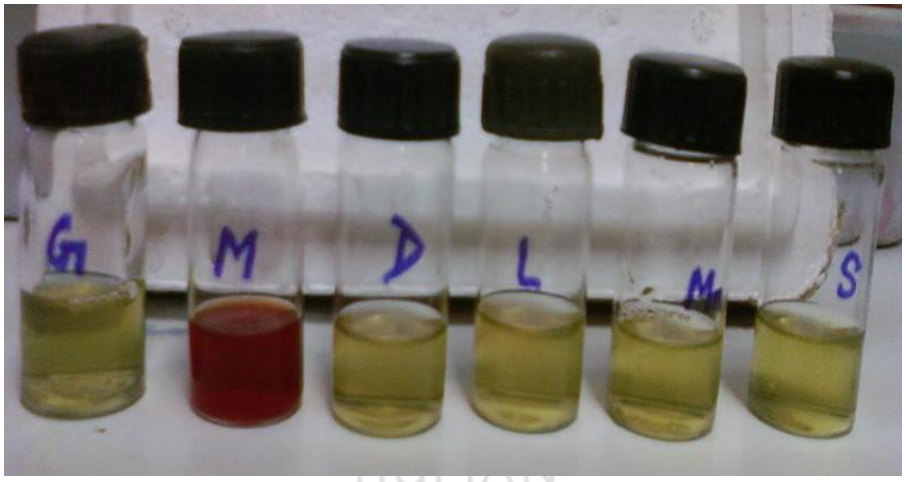


**Figure No. 10: Sugar fermentation test for *S. aureus***

**Phytochemical Screening of extracts of *Coleus aromaticus***

Phytochemical analysis of leaf extracts of *Coleus aromaticus* in different solvents tested are summarized in Table no. 3.

**Table No. 3: Phytochemical Screening of extracts of *Coleus aromaticus***

| S. No. | Constituents | Acetone extract | Methanol extract | Ethanol extract | Aqueous extract |
|---|---|---|---|---|---|
| 1. | Alkaloids | - | - | - | - |
| 2. | Glycosides | - | - | - | - |
| 3. | Flavonoids | + | + | + | + |
| 4. | Saponins | - | + | + | + |
| 5. | Phenolics | - | + | + | + |
| 6. | Amino Acids | - | - | - | - |
| 7. | Carbohydrate | - | + | + | + |
| 8. | Proteins | - | - | - | - |
| 9. | Diterpenes | + | + | - | - |

From table no. 3 it is clear that acetone, Methanol Ethanol and Aqueous extracts of *Coleus aromaticus* were taken for phytochemical analysis. Extracts were prepared by soxhlation using respective solvent. The analysis revealed the presence of flavonoids, saponins, phenols, carbohydrate and diterpenes in different extracts. In acetone leaves extract flavonoids and diterpenes were present while methanol, ethanol and aqueous leaves extract showed the presence of flavonoids, saponins, phenols and carbohydrate. Diterpenes was only present in acetone and methanol extracts. Maximum phytoconstituents were present in methanol extract.

**Antimicrobial Activity of *Coleus aromaticus* Extracts**

The present investigation in this research work, the antimicrobial activity of an extract obtained from the *Coleus aromaticus* was evaluated against microbial pathogen *Staphylococcus aureus* used under present study. Extracts obtained from the plant used to suitably dilute up to the concentrations of 100, 50 and 25 mg per ml and applied onto the test organism using the well diffusion method. The results of the experiment are being presented in Table 4 and 5 and fig 11 which clearly shows the Antimicrobial activity of extracts of *coleus aromaticus*. In the present *in vitro* study, antimicrobial activities of *coleus aromaticus* extracts have been assessed for Gram-positive *Staphylococcus aureus* using agar well diffusion method. Ciprofloxacin antibiotic was used as standard (Table 4). Among all the

three concentrations tested, the maximum result of antimicrobial activity was observed in 100 mg/ml concentration of all the four extracts of *coleus aromaticus.* For *Staphylococcus aureus* maximum zone of inhibition was recorded in ethanol extract (22±0.816 mm) > methanol (21±0.816 mm) > aqueous extract (20±0.942 mm)> acetone extract of *Coleus aromaticus* (10±0.942 mm). The alcoholic extracts of leaves were more effective than other extracts.

**Table No. 4: Antimicrobial activity of *standard drug* on selected microbes**

| S.N | Name of drug | Microbes | Zone of inhibition | | |
|---|---|---|---|---|---|
| | | | 10 µg/ml | 20 µg/ml | 30 µg/ml |
| 1 | Ciprofloxacin | *Staphylococcus aureus* | 17±1.69 | 18±2.62 | 22±2.16 |

**Table No. 5: Antimicrobial activity of *Coleus aromaticus* on selected microbes**

| S. No. | Name of microbes | Zone of inhibition | | |
|---|---|---|---|---|
| | | Acetone extract | | |
| | | 25mg/ml | 50 mg/ml | 100mg/ml |
| 1. | *Staphylococcus aureus* | 7±2.62 | 8±1.88 | 10±0.942 |
| | | Methanolic extract | | |
| 2. | *Staphylococcus aureus* | 19±0.471 | 20±0.942 | 21±0.816 |
| | | Ethanolic extract | | |
| 3. | *Staphylococcus aureus* | 17±2.16 | 20±0.942 | 22±0.816 |
| | | Aqueous extract | | |
| 4. | *Staphylococcus aureus* | 14±471 | 15±4.71 | 20±0.942 |

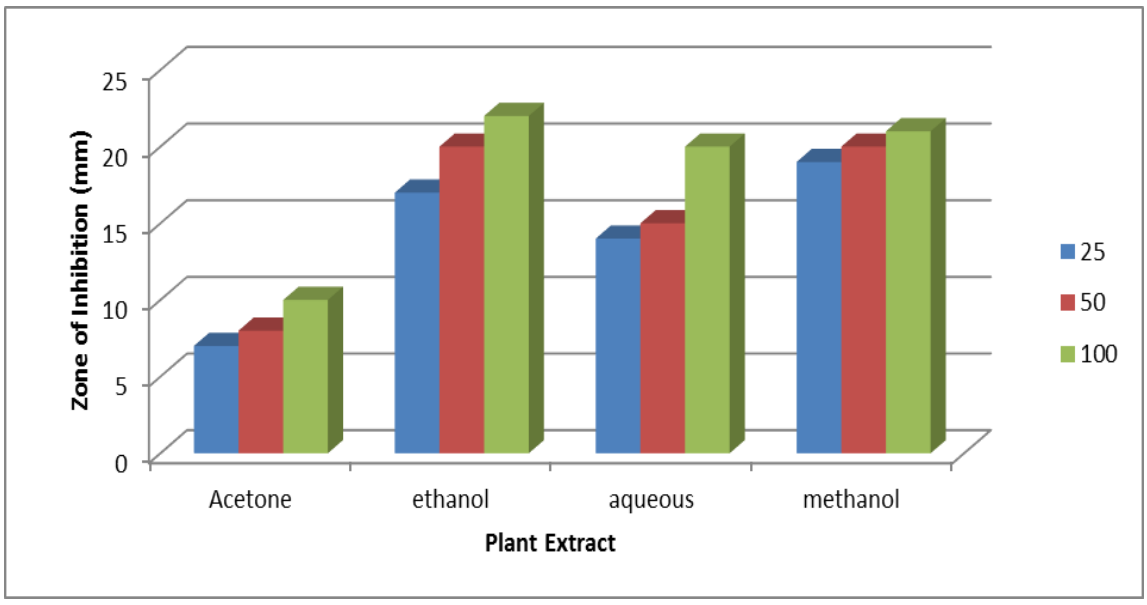


**Figure No. 11: Antimicrobial activity of ethanolic, acetone, aqueous and methanol extract of *Coleus aromaticus* against *Staphylococcus aureus***

## DISCUSSION

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents, Tona, *et al.,* (1998). The secondary metabolites which have been observed in our experimental plants include flavonoids, saponins, phenols, carbohydrates and diterpenes. Similar results were obtained by Krithiga and Jayachitra, (2012) and Bole and Jayashree, (2014). In contrast to our results Ramya *et al.,* (2012) reported tannin, alkaloids and glycosides in the phytochemical screening of *Coleus aromaticus.*

In our study for antibacterial activity, different solvent extracts showed different results. The methanolic and ethanolic leaf extracts showed strongest antibacterial activity. Similar findings and conclusions were drawn by Jayachitra and Chitra (2015), Ramya *et al.,* (2012) and Girish, (2016). Studies have demonstrated absence of toxicity of plant extracts *in vitro* and *in vivo* Parra *et.al* (2001), which makes the use of this species very safe in the treatment of multiresistant infections. According to Gurgel, *et.al* (2009) the plant extract damage cell membrane and bacterial cells. Antibacterial activity of *Coleus aromaticus* was also reported by Pritima and Pandian (2007). Similar findings was also reported by Chandrappa *et al.,* (2010) that *C. aromaticus* showed wide spectrum of antibacterial activity against human pathogenic bacteria. According to them alcohol extracts is rich in polyphenol and other bioactive compounds which exhibit antibacterial activity. Ramalakshmi *et al.,* (2014) also reported that ethanolic extract of *Coleus* leaves was evaluated against *Staphylococcus aureus* and *Salmonella typhi.* The highest antibacterial activity was observed against *S. typhi* and lowest against *S. aureus* bacteria.

## CONCLUSION

*Coleus aromaticus* is a rich source of secondary metabolites with various biological activities. It is easily available in India and leaves of the plant possess a wide range of antimicrobial properties and thus can be a source of low cost medicine. A systematic research and development work should be undertaken for the better economic and therapeutic utilization of *C. aromaticus.* Further studies are needed for confirmation of antibacterial action by isolating pure chemical constituents and also identify the compound responsible for antibacterial action of *C. aromaticus* leaves.

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