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# *In vitro* antimicrobial evaluation of *Anthocephalus cadamba, Butea monosperma, Diospyrous melanoxylon* and *Ficus glomerata* bark extract against certain bacteria

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**Abstract:** *Anthocephalus cadamba, Butea monosperma, Diospyrous melanoxylon* and *Ficus glomerata* bark are used for the preparation of ethnic skin ointments. This ointment in turn is deployed over wounds for healing purposes and to check abscess formation. To access their anti microbial efficacy these plants were evaluated *in vitro* against five test bacteria viz. *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae Proteus vulgaris* and *Salmonella typhi.* Zone of inhibition, minimum inhibitory concentration and MBC/MIC ratio for methanolic and aqueous extract was studied as chief antibacterial parameters. *Butea monosperma* was found to be nullified inhibitory for all the test bacteria while *Anthocephalus cadamba* and *Ficus glomerata* inhibited partially and results when compared to reference antibiotic were not statistically significant. *Diospyrous melanoxylon* showed significantly inhibition for *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi.* None of the experimented plant was inhibitory to *Proteus vulgaris.*

**Key words-** *Anthocephalus cadamba, Butea monosperma, Diospyrous melanoxylon* and *Ficus glomerata,* MBC/MIC ratio

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## I. INTRODUCTION

Since the inception of civilization man has dwelled upon natural resources for it basic and preferential necessities. Among all requisites the health care practices has efficiently proved its efficacy since time immemorial. These therapeutic practices have been questioned in current scenario because of five unscientific approaches i.e. prevalence of ambiguity in their identification, lack of availability in urban localities, lack of clinical authentication for the alimental parameters, lack of standard pharmaceutical dosages and practices by forged local practitioners. Researchers are validating these practices through clinical parameters. In continuation with same, current research was carried out to investigate antimicrobial efficacies of plants deployed as ointment over infected wounds by tribal's of south east Rajasthan. Wound term generally refers to an injury to living tissue caused by a cut, blow or other impact, typically one in which the skin is cut or broken and healing refers to an intricate process in which the skin repairs itself after injury through different phases as blood clotting (hemostasis), inflammation, tissue growth (proliferation) and tissue remodeling (maturation). The healing process is hindered due to various physiological and pathological factors among which microbial infections are more common. Ethnic peoples of Rajasthan deploy various plants as ointment, poultice or paste to heal the wounds [1]. Plant parts applied over wounds may function on two arrays-

1. They may have healing properties as they may direct rapid hemostasis, angiogenesis and reepithelialization.
2. They may check microbial growth and in turn prevent abscess formation.

Therefore, plants that are specialy used to check abscess formation can be broadly termed as plants with antimicrobial efficacy. Perusal of literature reveals that bark of *Anthocephalus cadamba* Roxb. (Rubiaceae), *Butea monosperma* (Lam.)Taub. (Fabaceae), *Diospyrous melanoxylon* Roxb. (Ebenaceae) and *Ficus glomerata* Roxb. (Moraceae) is used for ointment preparation that is deployed topically over chronic wounds. For ointment preparation, approximately 100 g of powder of dried bark is a added to 100 ml of sesame (*Sesamum indicum*) oil and sun heated for seven days. After filtration, molten bee wax is added and the resultant is used over wounds to check infections [2,3]. To evaluate the efficacies of these ethnical usages, these plants were tested for their antimicrobial efficacy against *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae Proteus vulgaris* and *Salmonella typhi.*

## II. MATERIALS AND METHODS

2-1 **Preparation of crude extract**

- The bark of selected four plant material/s viz. *Anthocephalus cadamba*, *Butea monosperma*, *Diospyrous melanoxylon* and *Ficus glomerata* was obtained from Germplasm Resource centre and was air dried under shade for two weeks and then oven dried at 40 °C for 18-24h. The dried plant material was grinded to a powdered with a mortar and pestle. The powder was weighed (50 g for each plant sample) and extracted using Soxhlet's method. The two solvents (250 ml for each sample): methanol and water were used for extract preparation. The suspensions were filtered twice, first under vacuum through a double layer of Whatman filter paper and then by gravity through a single sheet of Whatman filter paper. The solvents were removed from the clear supernatant by means of vacuum distillation at 30-35° C using a Buichi Rotary Evaporator.

2-2 Test Bacteria

Antimicrobial activity was investigated against five registered Microbial Type Culture Collection (MTCC) bacterial isolates viz. *Escherichia coli* (MTCC433), *Klebsiella pneumoniae* (MTCC3384), *Proteus vulgaris* (MTCC426), *Bacillus subtilis* (MTCC441) and *Salmonella typhi* (MTCC531) which were obtained from the from Institute of Microbial Technology, Chandigarh.

2-3 Maintenance of Bacterial Lineages

- Luria broth was used to culture bacteria. This method ensures usage of uniform number of bacteria; a set of graphs of killing/viability curves for each strain of bacterial species was prepared. A final concentration of $5 \times 10^6$ CFU (Colony Forming Unit) /ml was adopted for this assay.
- Using aseptic techniques, a single colony was transferred into a 100 ml bottle of Luria broth capped and placed in incubator overnight at 35°C. After 12–18 h of incubation, using aseptic preparation and the aid of a centrifuge, a clean sample of bacteria was prepared. The culture was centrifuged at 4000 rpm for 5 min with appropriate aseptic precautions. The supernatant was discarded and the pellet was resuspended using 20 ml of sterile normal saline and centrifuged again at 4000 rpm for 5 min. This step was repeated until the supernatant was clear. The pellet was then suspended in 20 ml of sterile normal saline and was labelled as bacteria. The optical density of the bacteria was recorded at 500 nm and serial dilutions were carried out with appropriate aseptic techniques until the optical density was in the range of 0.5-1.0. The actual number of colony forming units was calculated from the viability graph. The dilution factor needed was calculated and the dilution was carried out to obtain a concentration of $5 \times 10^6$ CFU/ml.

2-4 *In vitro* antibacterial assay

- The *in vitro* antibacterial activity was studied by Resazurin based Microtitre Dilution Assay and Disc Diffusion assay through three parameter-
  1. Zone of inhibition
  2. Minimum inhibitory concentration and
  3. Ratio of Minimum Bactericidal concentration/ Minimum inhibitory concentration
- The resazurin solution was prepared by dissolving 300 mg resazurin powder in 50 ml of sterile distilled water. A vortex mixer was used to ensure its homogenioty. Resazurin based MDA was performed in 96 well plates under aseptic conditions. A volume of 100 µl of test materials in 10% (v/v) DMSO or sterile water (usually a stock concentration 12 mg/ml of crude extracts) was added into the first row of the plate. Serial dilutions were performed using a multichannel pipette such that each well had 100 µl of the test material in serially descending concentrations.
  10 µl of resazurin indicator solution was added in each well. Finally 10 µl of bacterial suspension was added to each well to achieve a concentration of $5 \times 10^6$ CFU/ml. Each plate was wrapped loosely with cling film to prevent dehydration of bacteria. Each plate had a set of controls: a column with tetracycline as positive control. The plates were prepared in triplicate and placed in an incubator at 37°C for 18–24 h. The colour change was then assessed visually. Any colour change from purple to pink or colorless was recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC (minimum inhibitory concentration) value.
- The disc diffusion test was performed in radiation sterilized petri plates of 10.0 cm diameter (Tarson). The disc of the sample was placed on the surface of the agar plates already inoculated with bacterial culture. The plates were incubated at 37°C and examined after 48 h for zone of inhibition around the discs.

## III. OBSERVATIONS

**Table 1.1: Evaluation of antibacterial efficacy of experimented plants methanolic extracts (100 µg /ml ) against bacterial species tested by disc diffusion assay. (Antibiotic reference : Tetracycline (10 µg /ml ) ZOI for *Bacillus subtilis* =16.00 ± 0.33 ; *Escherichia coli* =18±0.33 ; *Klebsiella pneumoniae* = 14 ± 0.33; *Proteus vulgaris* = 17.5 ± 0.33; and *Salmonella typhi* = 16 ± 0.33) [diameter of well -6 mm]**

| Test Plant Species | Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | *Escherichia coli* | *Salmonella typhi* | *Bacillus subtilis* | *Proteus vulgaris* | *Klebsiella pneumoniae* |
| *Anthocephalus cadamba* | 16.80 ± 0.00 | 14.70 ± 0.57 | 21.00 ± 0.33* | 13.50 ± 0.00 | 23.50 ± 0.88* |
| *Butea monosperma* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Diospyrous melanoxylon* | 18.00 ± 0.00* | 0.00 | 0.00 | 14.00 ± 0.88 | 16.00 ± 0.66* |
| *Ficus glomerata* | 8.70 ± 0.66 | 0.00 | 0.00 | 0.00 | 0.00 |
| Values are mean inhibition zone (mm) ± S.D of three replicates; * Significant | | | | | |

**Table 1.2:** Evaluation of antibacterial efficacy of experimented plants aqueous extracts (100 µg /ml ) bacterial species tested by disc diffusion assay. (Antibiotic reference : Tetracycline (10 µg /ml ) ZOI for *Bacillus subtilis* =16.00 ± 0.33 ; *Escherichia coli* =18±0.33 ; *Klebsiella pneumoniae* = 14 ± 0.33; *Proteus vulgaris* = 18.0 ± 0.33; and *Salmonella typhi* = 16 ± 0.33) [diameter of well -6 mm]

| Test Plant Species | Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | *Escherichia coli* | *Salmonella typhi* | *Bacillus subtilis* | *Proteus vulgaris* | *Klebsiella pneumoniae* |
| *Anthocephalus cadamba* | 17.60 ± 0.66 | 15.00 ± 0.33 | 14.80 ± 0.21 | 11.50 ± 0.79 | 13.40 ± 0.66 |
| *Butea monosperma* | 0.00 | 0.00 | 0.00 | 11.00 ± 0.57 | 0.00 |
| *Diospyrous melanoxylon* | 23.00 ± 0.66* | 21.00 ± 0.57* | 18.00 ± 0.79* | 17.00 ± 1.00 | 19.50 ± 0.66* |
| *Ficus glomerata* | 8.00 ± 0.33 | 7.00 ± 0.79 | 9.20 ± 0.33 | 0.00 | 12.60 ± 0.33 |
| Values are mean inhibition zone (mm) ± S.D of three replicates * Significant | | | | | |

**Table 1.3:** Evaluation of minimum inhibitory concentration of methanolic extracts of experimented plants against selected test bacteria (MIC activity of tetracycline against test bacteria i.e. Escherichia coli (0.0800 mg/ml), Salmonella typhi (0.0152 mg/ml), Bacillus subtilis (0.0172 mg/ml), Proteus vulgaris (0.005 mg/ml) and Klebsiella pneumoniae (0.005 mg/ml ))

| Test Plant Species | Minimum Inhibitory Concentration (mg/ml) | | | | |
|---|---|---|---|---|---|
| | *Escherichia coli* | *Salmonella typhi* | *Bacillus subtilis* | *Proteus vulgaris* | *Klebsiella pneumoniae* |
| *Anthocephalus cadamba* | 2.25 ± 0.66 | 6.00 ± 1.00 | 1.50 ± 0.33 | 1.50 ± 0.60 | 5.25 ± 0.10 |
| *Butea monosperma* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Diospyrous melanoxylon* | 0.75 ± 1.00 | 0.00 | 0.00 | 3.00 ± 0.50 | 4.50 ± 0.60 |
| *Ficus glomerata* | 6.00 ± 0.50 | 0.00 | 0.00 | 0.00 | 0.00 |
| Values are mean ± S.D of three replicates | | | | | |

**Table 1.4:** Evaluation of minimum inhibitory concentration of aqueous extracts of experimented plants against selected test bacteria (MIC activity of tetracycline against test bacteria i.e. *Escherichia coli* (0.0800 mg/ml), *Salmonella typhi* (0.0152 mg/ml), *Bacillus subtilis* (0.0172 mg/ml), *Proteus vulgaris* (0.005 mg/ml) and *Klebsiella pneumoniae* (0.005 mg/ml ))

| Test Plant Species | Minimum Inhibitory Concentration (mg/ml) | | | | |
|---|---|---|---|---|---|
| | *Escherichia coli* | *Salmonella typhi* | *Bacillus subtilis* | *Proteus vulgaris* | *Klebsiella pneumoniae* |
| *Anthocephalus cadamba* | 0.75 ± 0.10 | 6.00 ± 0.50 | 4.50 ± 0.66 | 5.25 ± 0.75 | 4.50 ± 0.66 |
| *Butea monosperma* | 0.00 | 0.00 | 0.00 | 6.00 ± 1.00 | 0.00 |
| *Diospyrous melanoxylon* | 4.50 ± 0.33 | 1.50 ± 0.75 | 6.00 ± 0.66 | 3.00 ± 0.00 | 6.00 ± 0.33 |
| *Ficus glomerata* | 6.00 ± 0.66 | 0.75 ± 0.50* | 6.00 ± 0.10 | 0.00 | 3.00 ± 0.60 |
| Values are mean ± S.D of three replicates | | | | | |

**TABLE 1.5:** Mbc / Mic Ratio Of Methanolic Extracts Of Experimented Plants Against Selected Test Bacteria

| Test Plant Species | *Escherichia coli* | *Salmonella typhi* | *Bacillus subtilis* | *Proteus vulgaris* | *Klebsiella pneumonia* |
|---|---|---|---|---|---|
| *Anthocephalus cadamba* | 1.5 | 2.5 | 1.75 | 2.7 | 4.25 |
| *Butea monosperma* | 0 | 0 | 0 | 0 | 0 |
| *Diospyrous melanoxylon* | 1 | 0 | 0 | 3.5 | 3.65 |
| *Ficus glomerata* | 4 | 0 | 0 | 0 | 0 |

**Table 1.6: MBC / MIC ratio of aqueous extracts of experimented plants against selected test bacteria**

| Test Plant Species | *Escherichia coli* | *Salmonella typhi* | *Bacillus subtilis* | *Proteus vulgaris* | *Klebsiella pneumonia* |
|---|---|---|---|---|---|
| *Anthocephalus cadamba* | 2.5 | 1 | 2 | 1 | 1 |
| *Butea monosperma* | 0 | 0 | 0 | 3.75 | 0 |
| *Diospyrous melanoxylon* | 2 | 2.25 | 1.5 | 3 | 2 |
| *Ficus glomerata* | 2.5 | 1.75 | 1.75 | 0 | 1.25 |

## IV. RESULT AND DISCUSSION

*In vitro* antimicrobial assay in both methanolic and aqueous extract of *Anthocephalus cadamba* depicted inhibitory zone for bacterial growth but the results were found only to be significant against test bacteria *Bacillus subtilis* and *Klebsiella pneumonia. Butea monosperma* reveals no methanolic as well aqueous extract activity against any test bacteria except *Proteus vulgaris* in aqua media.

*Diospyrous melanoxylon* showed significant inhibitory activity for both *Escherichia coli* and *Klebsiella pneumonia* in methanolic extract while its aqueous extract was effective against *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi.* Except *Escherichia coli* no zone of inhibition was observed when test microbes were projected to methanolic extract of *Ficus glomerata* while in its aqueous conditions except *Proteus vulgaris* all other test microbe showed inhibited growth. The antibacterial action involves disruption of membrane potential, inner membrane permeabilization, blebbing and leakage of cellular contents [4-7]. The zone of inhibition when compared to that of reference antibiotic it was insignificant and therefore its efficacies against test bacteria is questionable (Table 1.1 and 1.2).

The novel therapeutic source can be termed as effective against the maladies if its dose is not greater than four in terms of synthetic compound and four to six in terms of natural unprocessed material [8,9]. In current study the minimum inhibitory concentration of reference tetracycline has been 0.0800 mg/ml for *Escherichia coli*, 0.0152 mg/ml for *Salmonella typhi* , 0.0172 mg/ml for *Bacillus subtilis*, 0.005 mg/ml for both *Proteus vulgaris* and *Klebsiella pneumoniae*. Comparing the minimum inhibitory concentration of experimental plants the MIC values of *Anthocephalus cadamba* (aqueous extract) and *Diospyrous melanoxylon* (mathanolic extract) for *Escherichia coli* and *Ficus glomerata* (aqueous extract) for *Salmonella typhi* were found to be significant while in other group and sub group the values were too high (Table 1.3 and 1.4).

The MBC and MIC values were equal for *Diospyrous melanoxylon* (methanolic extract) for *Escherichia coli* and *Anthocephalus cadamba* (aqueous extract) for *Salmonella typhi, Proteus vulgaris* and *Klebsiella pneumonia* [10-14]. When MBC/MIC values are higher than 4 the phyto activity cannot be termed as

antimicrobial. The ratio of MBC/MIC exceeds 4 for *Anthocephalus cadamba* (methanolic extract) in respect to *Klebsiella pneumonia.* Therefore this activity cannot be termed as an antimicrobial (Table 1.5 and 1.6).

The prior studies on *Diospyros melanoxylon* [15, 16], *Anthocephalus cadamba* [17, 18], *Ficus glomerata* [19] and *Butea monosperma* [20-22] and present findings divulge application of *Diospyros melanoxylon* for broad array of bacterial strain while *Butea monosperma* to be highly specific in its antimicrobial strains.

## V. CONCLUSION

*Ficus glomerata* shows differential activity in both methanolic and aqueous extract. In methanolic extract it did not inhibit any test bacteria except *E.coli* which was also found to be comparatively insignificant while in aqueous median it insignificantly but partially inhibited *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi. Diospyros melanoxylon* effectively inhibited growth of *Escherichia coli* and *Klebsiella pneumonia* in methanolic and *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi* in aqueous extract respectively. *Anthocephalus cadamba* inhibited all the test bacteria in both the extracts but the zone of inhibition when compared to reference drug none of the resultant activity was significant except that for *Klebsiella pneumonia* in methanolic phase. *Butea monosperma* didn't reveal any inhibitory activity in either extracts against any test bacteria. None of the experimented plants was inhibitory to *Proteus vulgaris.* MBC/MIC values for all inhibitory sets were either below or equal to four revealing their bactericidal properties. *Diospyros melanoxylon* can be investigated further for broad spectrum herbal antibiotic.

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