ASSAY OF ANTIMICROBIAL ACTIVITY

7.1. Introduction

Plants are one of the most important sources of medicine and plant derived compounds (phytochemicals) have been attracting much interest as natural alternatives to synthetic compounds. Now a days herbal drugs are prescribed widely even when their biologically active compounds are unknown because of their effectiveness, minimal side effects in clinical experience and relatively low cost (Valiathan, 1998). The studies of World Health Organization (WHO) indicate that over 30% of world’s plant species have at one time or another been used for medicinal purposes. The medicinal value of plant is due to the presence of certain secondary metabolites. The application of plants as medicine dates back to prehistoric period. The early civilization reveals that a considerable number of drugs used in modern medicine have figured in ancient manuscripts such as the Rig Veda, the Bible, the Quran, the Iliad, the Odyssey and the History of Herodotus. Over 600 years ago, the ancient Chinese were the first to use the plants of natural vegetation as the source of medicine. In India, the ayurvedic system bark of medicine has been in use for over 3000 years. Charaka and Susruta, two of the earliest Indian authors had sufficient knowledge on the proprieties of the Indian medicinal plants.

Owing to the local medicinal usage of the two studied species, *Acacia caesia* and *Acalypha fruticosa*, studies on their antimicrobial properties are needed to confirm their healing properties. Hence, in the present study, the extracts of the useful parts of these two species viz., leaf, stem bark bark and root by using various solvents were tested against certain pathogenic bacteria and fungi.

7.2. Materials and methods

The informations collected from local public and through literature showed that in the two study species, *Acacia caesia* and *Acalypha fruticosa*, the leaf, stem bark and root parts hold medicinal uses and hence used for medicinal purposes. Therefore, in the present study antimicrobial properties were analyzed by using these parts against the microbes selected.
7.2.1. Collection and processing of plant parts

Fresh leaf, stem bark and root parts of the two study species were collected from the Maruthamalai hills of Coimbatore district and brought to the laboratory by keeping them in ice box. These fresh materials were washed under running tap water, air dried and then homogenized to fine powder and stored in air tight bottles.

7.2.2. Preparation of plant extracts

To know the medicinal importance, the shade dried plant parts of the study species were made into a fine powder of 40 mesh size using the pulverizer separately. Following that, 100 g of the powder was filled in the filter paper and successively extracted using 500 mL solvents viz. petroleum ether, ethylacetate and methanol separately using the soxhlet extractor for 8 – 10 hours (Gafner et al., 1985). Then the extracts were filtered separately through Whatman No.1 filter paper to remove all undissolved matter, including cellular materials and other constituents that are insoluble in the extraction solvents.

7.2.3. Antimicrobial activity of the plant extracts

A vast number of experiments were carried out to show the antimicrobial efficacy of the plant extracts to cure large number of pathogenic diseases. Antimicrobial activity of petroleum ether, ethyl acetate and methanol extracts of leaf, stem bark and root parts of the two study species were determined by disc diffusion method (Bauer et al., 1966).

Collection and maintenance of microorganisms

The following microorganisms were used in the present study:

Bacterial strains

G (+) ve bacteria

*Bacillus subtilis*, *B. thuriengensis*, *Micrococcus* sp. and *Lactobacillus* sp.

G (-) ve bacteria

*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *P. stutzeri*, *Serratia* sp. and *Moraxetta* sp.
Fungal strains

Aspergillus niger, A. flavus, A. baumanii, Fusarium oxysporum, F. solani, Mucor rouxii, Alternaria alternata, Candida albicans, Cladosporium sp. and Rhizopus sp.

These microbes were obtained from the Department of Microbiology, Tamil Nadu Agricultural University, Coimbatore. The bacterial and fungal strains were maintained at 4°C on nutrient agar slants and potato dextrose agar slants respectively and kept in refrigerator prior to subculture.

7.2.4. Media used

Freshly prepared nutrient agar medium and potato dextrose agar (PDA) medium were used for the culture of bacteria and fungi respectively.

Composition of Nutrient agar medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Composition of PDA medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200.0g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000mL</td>
</tr>
<tr>
<td>pH</td>
<td>6.2</td>
</tr>
</tbody>
</table>

7.2.4.3. Method

The culture media were prepared and autoclaved at 121°C at 15 p.s.i. for 20 minutes and stored in refrigerator. The media were melted before the process of inoculation. The clean dry sterile Petri dishes were poured with nutrient agar medium (for bacteria) and potato dextrose agar medium (for fungus). Ten numbers of 10 mL
broths were prepared separately for nutrient agar medium and potato dextrose agar medium in test tubes and plugged with cotton and autoclaved. The test tubes were labeled with the microbes to be inoculated. The bacterial strains were inoculated onto the nutrient broth, and fungi were inoculated onto potato dextrose broth under aseptic conditions and incubated at 37 ± 0.5°C for 18 hours. After incubation, the bacteria and fungi were smeared on the nutrient agar and potato dextrose agar plate respectively using a sterile cotton swab. A sterile disc of 6 mm diameter was loaded with known quantity of 10 mg of dried crude extracts. These discs were placed on the surface of the media. The positive control antibiotic, tetracycline was used at the concentration, 0.1 mg/10 mL distilled water and maintained by loading on discs. Then the Petri dishes were incubated at 37 ± 0.5 ºC for 24 to 48 hours. The diameter of inhibition zone was measured. Triplicates were maintained for all tests.

7.3. Results

7.3.1. Antibacterial activity

The antibacterial activity of the extracts of the two studied species was assayed in vitro by disc diffusion method against ten bacterial pathogens. Tables 31-36 exhibited the data on bacterial growth inhibition by various alcoholic extracts of leaf, stem bark bark and root parts of two studied plant species.

The antibacterial activity of the all the alcoholic leaf extracts of the study species, Acacia caesia generally showed inhibitory activity against the growth of Bacillus thuringiensis and Lactobacillus sp. (Plate – XIIIa). However, towards Pseudomonas aeruginosa, P. stutzeri, Escherichia coli, Micrococcus sp., Serratia sp., Moraxetta sp., Bacillus subtilis and Klebsiella pneumoniae, all these extracts showed activity with less pronounced manner (Table 31). The antibacterial activity of certain alcoholic stem bark extracts of Acacia caesia is given in Table 32. It shows that generally, all extracts have significant activity against the four bacteria viz., Bacillus subtilis (Plate – XIIIb) B. thuringiensis, Klebsiella pneumoniae and Moraxetta sp. and it was less against the other bacteria, Pseudomonas aeruginosa, P. stutzeri, Escherichia coli, Micrococcus sp., Serratia sp. and Lactobacillus sp. On the other hand, the root extracts of this species has showed significant inhibitory activity against the five bacteria viz., Bacillus thuringiensis, Micrococcus sp. Escherichia coli, Serratia sp. and
*Moraxetta* sp. (Table 33 and Plate XIIIc). Further, it was noted that the inhibitory activity was noteworthy against the bacteria, *Pseudomonas aeruginosa, P. stutzeri, Lactobacillus sp. Bacillus subtilis* and *Klebsiella pneumoniae* also.

The antibacterial activity of certain alcoholic leaf extracts of the other study species, *Acalypha fruticosa* is exhibited in Table 34. The study revealed that the methanol extract showed higher inhibitory activity against the growth of the bacteria, *Bacillus subtilis* (21.97mm diameter inhibitory zone) and *Moraxetta* sp. (17.97mm diameter inhibitory zone) (Plate – XIVa). Petroleum ether and ethyl acetate extracts showed higher inhibitory zone against the bacterium, *Moraxetta* sp. The petroleum ether and methanol stem bark extracts of this study species produced maximum inhibitory zone against the bacteria, *Escherichia coli* and *Bacillus subtilis* (Plate – XIVb) (8.82 mm and 12.17 mm diameter inhibitory zone respectively) (Table 35). In addition, it has been noted that the inhibitory activity against the bacterium, *Bacillus subtilis* was highest (32.67 mm diameter inhibitory zone) by the ethyl acetate stem bark extract of this species than the any other extracts examined (Table 35). The inhibition effect of alcoholic root extracts of this species is given in Table 36. The study reports that the methanol extract showed highest inhibitory activity against the growth of the bacteria, *Bacillus subtilis* (21.83 mm diameter inhibitory zone) and *B. thuringiensis* (20.63mm diameter inhibitory zone) (Plate – XIVc). Petroleum ether and ethyl acetate extracts also showed higher inhibitory zone against the bacterium, *B. subtilis* by producing 9.67 mm diameter inhibitory zone and 16.13 mm diameter inhibitory zone respectively.

### 7.3.2. Antifungal activity

The antifungal activity of various alcoholic leaf extracts of the study species, *Acacia caesia* against the ten studied fungal species is given in Table 37 and Plate – XIIIId. The results of the study report that the ethyl acetate extract has the highest inhibitory activity (20.67 mm diameter inhibitory zone) against the fungus, *Mucor rouxi*. The petroleum ether and methanol extracts were also found to be better with respect to inhibitory function against the two fungal species, *Mucor rouxi* and *Rhizopus* sp. (17.73 and 16.75 mm diameter inhibitory zone respectively). The petroleum ether and methanol stem bark extracts of this species showed maximum inhibitory zone against the fungus, *Mucor rouxi* (Plate – XIIIe) (20.73 mm diameter inhibitory zone)
and 24.73 mm diameter inhibitory zone respectively) (Table 38). The inhibitory activity against the fungus, *Mucor rouxii* was highest (30.77 mm diameter inhibitory zone) by the ethyl acetate stem bark extract than the other extracts examined. The inhibition effect of alcoholic root extracts of this species is given in Table 39. The study exhibited that the ethyl acetate extract showed highest inhibitory activity against the growth of the fungus, *Mucor rouxii* by producing 28.53 mm diameter inhibitory zone (Plate – XIIIif). Petroleum ether and methanol extracts showed higher inhibitory zone against the fungi, *Alternaria alternate*, *Mucor rouxii* and *Rhizopus* sp. (between 10.73 and 20.77 mm diameter inhibitory zone).

For the other species, *Acalypha fruticosa*, the inhibitory effect of various alcoholic leaf extracts against the ten studied fungal species is presented in Table 40. The petroleum ether, ethyl acetate and methanol extracts of this species have some considerable inhibitory effect on the growth of the fungal species (Plate – XIVd). All the three alcoholic extracts were also have significant inhibitory effect against all fungal species tested except the fungal species, *Candida albicans*. The inhibitory effect of various alcoholic stem bark extracts of *A. fruticosa* is exhibited in Table 41. It was noted that generally the petroleum ether, ethyl acetate and methanol extract did the inhibitory function in a considerable manner against all the fungal species tested (Plate – XIVe). The fungus, *Rhizopus* sp. was highly sensitive against the ethyl acetate extract of this plant species. It is further noted that the root extracts of petroleum ether, ethyl acetate and methanol had significant inhibitory effect against the tested fungal species (Table 42). In addition, the ethyl acetate root extract of this species was highly effective with respect to the inhibition of growth of the two fungal species, *Rhizopus* sp. and *Aspergillus niger* (Plate – XIVf). The inhibitory effect of other alcoholic extracts viz., petroleum ether and methanol extracts against the fungal species, *Aspergillus niger* and *Fusarium solani* respectively was also considerable.

### 7.4. Discussion

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Traditional healers use primarily water as the solvent but in the present study it was found that plant extracts in organic solvents provided more consistent antimicrobial activity. These
observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and in addition to their intrinsic bioactivity by their ability to dissolve or diffuse in the different media used in the assay.

The results of the present study on the antimicrobial activities of various organic chemical extracts of leaf, stem bark and root parts of two studied species against the colonial growth of ten bacterial species and ten fungal species are presented in Tables 31-42. The study revealed that generally the inhibitory activity is pathogen specific and depends on the solvent and plant parts used for the extraction of secondary metabolites of inhibition property.

For the species, *Acacia caesia* almost all extracts of leaf, stem bark and root parts in general have the considerable antibacterial activity against the ten bacterial species investigated (Tables 31-33). However, the leaf, stem bark and root parts of the ethyl acetate extract were found to be effective against certain specific fungi (Tables 37-39). For the species, *Acalypha fruticosa* the methanol extracts of leaf and root parts and ethyl acetate extract of stem bark parts showed higher inhibitory activity against the bacterial species (Tables 34 - 36). For antifungal activity, petroleum ether, ethyl acetate and methanol extracts of leaf part of this species were determined to be highly effective (Table 40). The alcoholic extracts of stem bark and root parts of this plant were also found to be fit with respect to control of fungal growth (Tables 41 and 42).

The overall study on antimicrobial activity reports that the two plant species containing active compounds of inhibitory action substantially. The beneficial medicinal effects of these plant materials typically results from the combinations of secondary products present in the plant. These compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, phenol compounds *etc.* which are synthesized and deposited in specific parts of the study species. The heterogeneity of these secondary compounds in wild species is reported to be wide (Balandrin *et al.*, 1985). Based on this concept, it is explained that the two study species due to the heterogeneity of secondary compounds owing to their wildness could be with higher antimicrobial activity. The higher antimicrobial activity of alcoholic extracts of the present study species may further indicates that the antimicrobial principles/chemical constituents which are either polar or non polar can be effectively extracted only through the organic solvent medium (Eseawi and Srour, 2000; Raskin *et al.*, 2002; Aiyelaagbe *et al.*, 2007; Zakaria *et al.*, 2007).
2010 and Rahul et al., 2011). Many early studies also reported the effective inhibitory activity of alcoholic solvents against the growth of the pathogenic microbes (Thomas et al., 1999; Reddy et al., 2001; Erdogru, 2002; Ates and Erdogru, 2003; Nair et al., 2005; Mohan et al., 2005; Poonkothai and Saravanan, 2008). The poor antimicrobial activity of some extracts might be attributed to the extracting capacity of solvent and the concentration of the active ingredients in the extracts (Akpomie and Olorungbon, 2011).

From the present investigation, the results obtained confirm the therapeutic potency of the two studied plant species of Maruthamalai hills, Acacia caesia and Acalypha fruticosa used in traditional medicine by local people. Further, it supports the folkloric usage of these plants and suggests that their alcoholic extracts possess compounds of microbial growth inhibitory properties and they can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. The most active extracts can be subjected to isolation of the therapeutic antimicrobial compounds and undergo further pharmacological evaluation.