CHAPTER II
ANTI MICROBIAL ACTIVITY

2.1 Introduction

Medicinal Plants are rich in secondary metabolites and are potential source for drugs and essential oils. Steroids, alkaloids, glycosides, insecticide, additives and related active metabolites found in plants are of great value in the drug and pharmaceutical industry (Khatune et al., 2005). Many herbal remedies, individually or in combination with different formulations such as leaf powder, pastes, decoctions and infusion, pills etc., have been recommended in various medicinal treaties (Zahra et al., 2000).

Plants have been utilized as medicines for thousands of years (Samuelsson, 2004). Over the last 40 years, intensive efforts have been made to discover clinically used antibacterial and antifungal drugs (Sofowora, 1984, Ahamed et al., 1998, Sardari et al., 1998, Werner et al., 1999, Kudi et al., 1999, Perumalsamy et al., 2008, Uma Devi et al., 2007 and Pandey et al., 2010). These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Balick and Cox, 1997 and Samuelsson, 2004). The specific plants to be used and the methods of application for particular ailments are passed down through oral tradition. Eventually information regarding medicinal plants was recorded in herbal phamacopoeias (Balunas, 2005). Plant derived medicines are widely used because they are relatively safe and cheaper (Iwu et al., 1999). Many plant species have been evaluated for their antimicrobial activity in the past 20 years (Castello et al., 2002 and Shajahan and Ramesh, 2004).

Although hundred of plant species have been tested for antimicrobial properties, the vast majority of have not been adequately evaluated (Balandrin et al., 1985 and Mahesh
and Sathish, 2008). Medicinal plants are valuable antimicrobial agents and are a source of many potent and powerful drugs (Srivastava, et al., 1996). The different parts used include root, stem, flower, fruit, twigs, exudates and modified plant organs, while some of these raw drugs are collected in smaller quantities by the local communities and folk healers for local uses, many other raw drugs are collected in larger quantities and treated in the market for many herbal industries (Uniyal et al., 2006).

The characteristics of the plants that inhibit micro-organisms and are important for human health have been researched in laboratories since 1926 (Erdogrul, 2002). Traditional medicinal ailments in daily life are being used with empirical methods. In recent years development of multidrug resistance in the pathogenic bacteria and parasites had created major clinical problems in the treatment of infectious disease (Davies, 1994). This and other problems such as toxicity of certain antimicrobial drugs on the host tissue (Idose et al., 1968 and Maddux and Barrere, et al., 1997) triggered interest in search of new antimicrobial substances/drugs of plant origin.

The clinical efficacy of many existing antibiotics has being threatened, due to the emergence of multidrug resistant pathogens (Bandow et al., 2003 and Lakshmi Naidu et al., 2006). To date, resistance in bacteria is most prevalent. For example, methicillin resistant Staphylococcus aureus (MRSA) has become a huge problem worldwide to treat nosocomial infections since 1990s (Lee et al., 2007). Strains of resistant food borne pathogens to a variety of antimicrobials have become a major health concern (Kiessling et al., 2002). Changes in the antimicrobial target, inactivation by enzymes, change in cellular permeability, antimicrobial active efflux and over production of target enzymes and by pass the antimicrobial of resistance have been the common mechanisms of
antimicrobial resistance (McKeegan et al., 2002). One of the reasons in the development of resistance to chemotherapeutic agent is due to abuse of these drugs (Reuters, 2005).

Drug discovery from medicinal plants has traditionally been lengthier and more complicated than other drug discovery methods. Therefore, many pharmaceutical companies have eliminated or scaled down their natural product research (Butler, 2004 and Koehn and Carter, 2005). Plant based antimicrobial have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Parekh and Chanda, 2007 and Kumaraswamy et al., 2008).

Recently there has been increasing interest in discovering new natural antimicrobials (Sagdic et al., 2003). Plant products with antimicrobial properties notably have obtained emphasis for a possible application in food production in order to prevent bacteria and fungal growth (Lanciotti et al., 2004). Antimicrobial activity of several plant metabolites have been extensively documented (Zahra et al., 2000; Singh et al., 2004; Basu et al., 2005 and Khatune et al., 2005). A. esculentus was also reported to possess antimicrobial activity (Amritpaul Singh et al., 2008). Reports related to antimicrobial activity of this plant using various organic solvents have not been thoroughly studied. Based on the thorough scrutiny of scientific literatures, no scientific information on bioactivity of tender fruits of A. esculentus was found. This study, thus presents the influence of various extracts of tender fruit of A. esculentus against bacterial pathogens.
2.2 MATERIALS AND METHODS

Pathogenic Strains:

The test organisms used were *Staphylococcus aureus*, *Streptococcus fecalis*, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aerogenosa* and *Bacillus subtilis*. The bacterial strains were collected from Arvind eye hospital lab, Tirunelveli, Tamilnadu, India. All the bacterial cultures were cultured in nutrient broth (Hi-media) and incubated at 37\(^\circ\) C for 24 hours.

2.2.1 Antibacterial screening:

The antibacterial activity of *A. esculentus* was screened using disc diffusion method, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Disc diffusion method:

Filter paper disc diffusion technique in agar was followed to determine antimicrobial activity by the procedure of Garg and Jain, (1998.) What man No.1 filter paper discs of 6-mm diameter, placed in dry Petri plates, were autoclaved. The test extracts in measured quantities were dissolved in minimum amount of acetone. Steriled filter paper No.1 discs were loaded with the extracts of *A. esculentus* using different solvents. The amount of extracts loaded in each disc was in the concentration *viz.*, 25\(\mu\)g/ml, 50\(\mu\)g/ml, 75\(\mu\)g/ml and 100\(\mu\)g/ml. Similarly discs were prepared for standard antibiotic tetracyline (w/v) and were impregnated in the filter paper discs in different concentrations (25 and 50 \(\mu\)g/ml).
The pathogenic strains were suspended with nutrient broth (Hi-media) by transferring a loop full of 24 hrs growth from agar slopes. The suspensions were vortexes and 0.1ml aliquots were spread over respective agar medium plates. The extracts and tetracycline loaded discs were then placed over the plates seeded with respective microorganisms. The plates were incubating at 37\(^{0}\)C for 24hrs. The antibacterial activity was determined by measuring the inhibition zone around the discs. The diameter of inhibition zones (including the diameter to the disc) was measured.

2.2.2 MIC and MBC test:

MIC and MBC test:

Minimum Inhibitory Concentration (MIC) of the extracts was determined from the culture plates that had the lowest concentrations and prevented the growth of bacterial strain. Minimum Bactericidal Concentration (MBC) was determined by using the method of Samy and Ignacimuthu (2001). The tender fruit extracts of *A. esculentus* were diluted to obtain concentration ranging from 10 µg -100 µg /ml. The test tube containing 3ml of Muller Hinton broth and 0.1 ml bacterial suspensions and 0.1 ml plant extract were incubated at 37\(^{0}\)C for 24h. Bacterial turbidity was measured at 650 nm to determine bacterial inhibition. Streptomycin at 20 and 40µg /ml was used as a reference for determination of minimum inhibitory and bactericidal concentrations respectively. The tubes containing only the growth medium were used as control. The minimum bactericidal concentration that showed the reduction of the bacterial colony as measured from the turbidity of the culture by optical density value.
Total bacterial count of each bacterial species was estimated by counting the number of bacteria in each test tube incorporated with different concentrations of plant extracts and control. The average of three counting was taken as the total number of colony forming bacterial suspensions. All determinations were made in triplicate of extracts.

2.3 RESULT AND DISCUSSION

2.3.1 Antibacterial assay

Antibacterial activity of fruit extracts of *A. esculentus* were evaluated by measuring the zone of inhibition against human bacterial pathogen and the results were presented in Figure 2.1. In the case of ethanolic extract of *A. esculentus* tender fruits showed high antimicrobial activity against all the test pathogens while other extracts showed comparatively moderate activity. The ethanolic extract (100 mg/ml) of *A. esculentus* tender fruits showed high antimicrobial activity against *S. feacalis* (4.6 cm), (Plate 2.1), *E. coli* (4.5 cm), (Plate 2.2) while with *B. subtilis* (4.4 cm), (Plate 2.3), *S. aureus*, *S. typhi* (4.2 cm) and *P. aerugenosa* (3.3 cm) (Plate 2.1). Sharma and Sharma, (2010) studied the antibacterial activity of ethanolic and aqueous extracts of *Tridax procumbens* and *Mimosa pudica* and showed significant activity against *Klebsiella sp.*, and least activity against *Staphylococcus aureus*. The chloroform extract of *A. esculentus* tender fruits showed less antimicrobial activity against all the test pathogens. The potentiality of *A. esculentus* fruit extracts was conformed to antibiotic treated.
Figure 2.1. The antibacterial effect of various extracts of tender fruits *A. esculentus* against human pathogenic bacterial organisms in disc diffusion method.
According to the method of Bauer et al. (1966) the antimicrobial activity is classified into resistant if zone of inhibition in millimeter is less than 2, if it is 3-5 mm intermediate and if the inhibition is 6 or more it is sensitive. In the present investigation the disc susceptibility test on test pathogens showed the concentration of extract posses at 100 mg/ml high antimicrobial activity than other concentrations. A variety of biologically active compounds, such as alkaloids, flavanoids, saponins, glycosides and steroids were seen in the tender fruits of A. esculentus. It was coincided with the report of Nudrat et al., (2005). L. camara extract exhibited remarkable antibacterial activity was recored by its phytochmical constituents (Mello et al., 2005; Verma and Verma, 2006 and Ganjewala et al., 2009).

Flavanoids in plants are shown to have antimicrobial properties (Linuma et al., 1994 and Tullanithi et al., 2010) Saponins are of wide interest because of their medicinal properties, antimicrobial activity and their likely role as determinants of plant disease resistance (Haralampidis et al., 2002). Tannins are reported to have various physiological effects like anti irritant, antimicrobial and antiparasitical effects. Phytotherapeutically tannin containing plants are used to treat non specific diarrhea, inflammation of mouth and throat and slightly injured skins (Westendarp, 2006). The above mentioned phyto constituents reported in the present study and confirmation of flavanoid group of compounds by NMR studies strengthen the anti microbial activity exhibited by the ethanol extract of A. esculentus.

Based on the findings of the present study, and with the earlier reports, among the various solvent extracts (hexane, butanol, ethanol, chloroform and water), the ethanol extract of A. esculentus showed strong antibacterial activity against the tested pathogens.
The secondary metabolites identified in *A. esculentus* of the present study might be responsible for antimicrobial activity exhibited by this plant against tested pathogen. The result of the antimicrobial susceptibility test showed that the fruit of *A. esculentus* is easily available and safe therapeutics for the various infectious diseases.

**Table 2.1.** Minimum inhibitory concentration of various extracts of tender fruits

*A. esculentus* extracts against human pathogenic bacteria organisms.

<table>
<thead>
<tr>
<th>Solvent</th>
<th><strong>Minimum inhibitory Concentrations (mg/ml)</strong></th>
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<tbody>
<tr>
<td></td>
<td><em>S.aureus</em></td>
</tr>
<tr>
<td>Hexane</td>
<td>0.6</td>
</tr>
<tr>
<td>Butanol</td>
<td>0.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.3</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.9</td>
</tr>
<tr>
<td>Aqueous</td>
<td>0.3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Figure 2.2.** Minimum bactericidal concentration of various extracts of tender fruits

*A. esculentus* against human pathogenic bacterial organisms.
It is observed that a single plant is known to contain several active principles of biological significance (Ming et al., 2005). The present study is successful in identifying candidate folkloric plant with heat stable constituents, since, all the extracts were subjected to heat treatment and then subjected to antibacterial activity. Constituents were also found to be bactericidal since no growth was observed even after 48 hour of incubation in zone of inhibition area (Table 2.1). The antimicrobial activity of tender fruits of *A. esculentus* observed in the present study may be due to the presence of flavonoid. (Linuma et al., 1994 and Tullanithi et al., 2010).

2.3.2 MIC and MBC

When comparing the influence of various extracts of *A. esculentus* on MIC and MBC values against bacterial pathogens ethanol extract exhibited a significant result *viz.*, 0.2 to 0.4 and 0.6 to 0.9 mg/ml respectively. Comparatively a better result was observed with aqueous extract where as it was moderate in all other extracts (Table 2.1 and figure 2.2).

Among the various extracts, the minimal inhibitory concentration of *A. esculentus* against bacterial pathogens, the ethanol extract showed highest activity than all other solvent extracts. The MIC value was found to be 20-40 µg/ml against tested bacterial pathogens.

In the present study the MIC of hexane extract of *A. esculentus* showed the highest effect in *B. subtilis, E.coli* and *Pseudomonas aeruginosa* around 40 µg/ml and the lowest effect in and *Staphylococcus aureus, S. feacalis* and *S. typhi* around 60 µg/ml. The findings of highest MIC in *Pseudomonas aeruginosa* and the lowest MIC in
*Staphylococcus aureus* with *Acacia nilotica* reported by Raghavendra *et al.* (2006) coincided the results of the present study.

However, the MIC of butanol extracts showed the highest effect in *Pseudomonas aeruginosa* (30 µg/ml) followed by *S. typhi* (40 µg/ml), *B. subtilis* and *E. coli* (50 µg/ml) and *Staphylococcus aureus* and *S. feacalis* (60 µg/ml). Similar observations due to different plants were reported by various workers (Akunyili *et al.*, 1993 and Nascimento *et al.*, 2000).

Regarding the minimal inhibitory concentration of chloroform extract of *A. esculentus*, the highest effect was seen in *Pseudomonas aeruginosa* (30 µg/ml) and lowest effect was found to occur in *Staphylococcus aureus* (90 µg/ml). The highest MIC effect was seen in *Pseudomonas aeruginosa* and the least activity in *Staphylococcus aureus* due to ethanol and aqueous extract of *Tamarindus indica* reported by Doughari, (2006) corroborated the result of the present study.

The MIC activity of water extract the of *A. esculentus* highest effect in *Pseudomonas aeruginosa* (20 µg/ml) and least effect in *Staphylococcus feacalis* (50 µg/ml) were noticed. Similar findings due to leaf extract of *A. aspera* were reported by Mohana *et al.*, (2008) and Alam, (2009.)

Hugo and Russel (1984) have reported that the MBC values can either be the same or higher than the MIC values. In this study, the MIC values were either the same or slightly lower than the MBC values, similar to the results of Karou *et al.* (2006). It is evident from the present investigation that the MBC values which are obtained after
plating various dilutions of the extracts, is more reliable than the MIC values as reported by Junaid et al. (2006).

Bacterial pathogens were subjected to *Canavalia rosea* leaf extracts and varying degrees of susceptibility of bacteria to plant extracts was reported. The basis of inhibition zone (diameters) varied according to strains and species (Karou et al., 2006). Similar data obtained in the present study too. *A. esculentus* tender fruit extract the highest zone of inhibition was by ethanol extract of tender fruits of *A. esculentus* against the tested microorganisms as in the case of *Canavalia rosea*. Among the various extracts of *A. esculentus* a better antibacterial activity was exhibited by ethanol extract and that can be substantiated by the MIC and MBC values obtained against various pathogens by the same extract. These results show considerable activity against gram negative and gram positive bacterial pathogens. As mentioned earlier, the phytoconstituents, particularly flavanoid group of compounds could be the reason for the better anti microbial activity of the present study.