ANTIMICROBIAL ACTIVITY OF THREE INDIAN MEDICINAL PLANTS – AN IN VITRO STUDY

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ABSTRACT
The alcoholic leaf extract of the three Indian medicinal plants Annona squamosa, Aegle marmelos and Citrus limon were screened for its antimicrobial activity using disc diffusion method. They were tested against four gram positive bacteria (Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Bacillus subtilis), three gram negative bacteria (Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia) and against three fungi (Aspergillus niger, Aspergillus fumigates, Candida albicans). It was observed that all the three alcoholic leaf extracts showed antibacterial and antifungal activity. The alcoholic leaf extract of Annona squamosa was found to be most active against S. aureus, B. cereus, K. pneumonia, A. niger and A. fumigates. The alcoholic leaf extract of Aegle marmelos was found to be active against B. subtilis, E. coli, P. aeruginosa and C. albicans. The alcoholic leaf extract of Citrus limon was most active against S. epidermidis and E. coli. The susceptibility of the microorganisms to the extracts of these plants was compared with each other and with the standard antibiotics ciprofloxacin and ketoconozole. The antimicrobial activities of the three alcoholic leaf extracts are discussed according to their phytochemical components. It is concluded that these three Indian medicinal plants may serve as a valuable source of compounds with therapeutic potential.

INTRODUCTION
Infectious diseases are the leading cause of death world-wide. For a long period of time plants have been valuable sources of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in India. Many works have been done which aim at knowing the different antimicrobial and phytochemical constituents of medicinal plants using them for the treatment of microbial infections as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become resistant. During the last ten years the pace of development of new antimicrobial drugs has slowed down while the prevalence of resistance has increased astronomically (Hugo and Russell, 1984). Literature reports and ethnobotanical records suggest that plants are the sleeping giants of pharmaceutical industry (Hostettmann and Hamdurger, 1991).

Natural products, either as pure compound or as standardized plant extracts, provide unlimited opportunities for new drug leads, because of the unmatched availability of chemical diversity. There is continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Rojas et al., 2003). Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections.

The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo and Bosisio, 1996).

In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents (Krishnaraju et al., 2005). Thus it is anticipated that phytochemicals with adequate antibacterial efficacy can be used for the treatment of bacterial infections (Balandrin et al., 1985). Since time immemorial, it has been suggested that ethanolic extract from plants used in allopathic medicines are potential sources of antiviral, antitumoral and antimicrobial agents (Negi et al., 1993).

Annona squamosa Linn, commonly known as Sugar apple, belonging to the family Annonaceae, is said to show varied medicinal effects, including insecticide, antivulatory and abortifacient. A bark is used to prevent diarrhoea, the root is used in the treatment of dysentery. A decoction of the leaves is used for cold and to clarify urine, to treat hysteria and fainting spells (Asolkar et al., 1992). The fruits of Annona are haematonic, cooling, sedative, stimulant, expectorant, maturant tonic. The bark and leaves contain annonaine, an alkaloid (Vohora et al., 1975) which is found to posses many of these properties.

Aegle marmelos, commonly known as bael, is a spiny tree belonging to the family Rutaceae. The leaves, roots, bark, seeds and fruits of Aegle marmelos are edible. The medicinal properties of this plant have been described in the Ayurveda. In fact, as per Charaka (1500 B.C.), no drug has been longer or
better known or appreciated by the inhabitants of India than the bael (Chemexcil, 1992). The leaves of bael are astringent, a laxative, and an expectorant and are useful in treating ophthalmia, deafness, inflammations, cataract, diabetes and asthmatic complaints. The leaves are bitter and are used as a remedy for ophthalmia, ulcers, dropsy, cholera and beri beri.

Fresh aqueous and alcoholic leaf extracts of Aegle marmelos are reported to have a cardiotonic effect (Nadkarni, 1976). An aqueous decoction of the leaves has been shown to possess a significant hypoglycemic effect (Karunanayake et al., 1984). Aegle leaf extract has been reported to regenerate damaged pancreatic β-cells in diabetic rats (Das et al., 1996). It is found to be as effective as insulin in the restoration of blood glucose and body weight to normal levels (Seema et al., 1996).

Citrus limon originated in Southeast Asia, China, and the Malayan Archipelago. The fruit is rich in vitamin C which helps the body to fight off infections and also to prevent or treat scurvy (Chopra et al., 1986). Lemon juice is also a very effective bactericide (Chiey, 1984). It is also a good antiperiodic and has been used as a substitute for quinine in treating malaria and other fevers, in the treatment of rheumatic conditions (Chevalier, 1996). The skin of the ripe fruit is carminative and stomachic (Chopra et al., 1986). The essential oil from the skin of the fruit is strongly rubefacient and when taken internally in small doses has stimulating and carminative properties (Grieve, 1984). The stem is bitter, stomachic and tonic (Duke and Ayensu, 1985). An essential oil from the fruit rind is used in aromatherapy. They are rich in vitamin C, bioflavonoids, acids and volatile oils. They also contain coumarins such as bergapten which sensitizes the skin to sunlight. Main properties of Citrus limon are antiseptic, anti-rheumatic, antibacterial, antioxidant, reduces fever.

The purpose of the present study was to screen the alcoholic leaf extracts of these medicinal plants that could be useful for the development of new tools as antimicrobial agents.

**MATERIALS AND METHODS**

**Plant material**

Fresh leaves of Annona squamosa, Aegle marmelos and Citrus limon plants were collected locally during the month of November to January and were authenticated.

**Preparation of crude extract**

10g of air dried powder was macerated with 100 mL of absolute alcohol and stored for 72 hr in ice cold condition. After 72 hr the miscella was filtered using Whatmann No. 1 filter paper and the organic layer was allowed to evaporate. The resulted dark green extract was concentrated up to 100 mL on Rota vapour under reduced pressure. The concentrated crude extracts were lyophilized into paste (5 and 15 g respectively) and were taken for the screening of antimicrobial activity.

**Microorganisms**

In vitro antimicrobial studies were carried out against the strains purchased from American Type Culture Collection (ATCC). The four Gram-positive organisms studied were Staphylococcus aureus (ATCC 9144), Staphylococcus epidermidis (ATCC 155), Bacillus cereus (ATCC H778), Bacillus subtilis (ATCC 6633) and the three Gram-negative organisms studied were Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 1688) Klebsiella pneumonia (ATCC 11298). The fungi strains studied were Aspergillus niger (ATCC 9029), Aspergillus fumigates (ATCC 46645) and Candida albicans (ATCC 10231).

**Disc diffusion method**

The disc diffusion method that followed by National Committee for Clinical Laboratory Standards (NCCL) protocol was used to evaluate the antimicrobial activities. For susceptibility testing, 100 mg/mL crude ethanolic extract was prepared in dimethyl sulfoxide (DMSO). Sterile Antibiotic Assay (AA) Discs (Whatman, 6mm) were impregnated with test compounds (25, 50 100 μL and μg of the reconstituted extract and were dried completely. The Discs were placed on the surface of the agar dispersion plates inoculated with the suspension (105 CFU/mL) of microbes that was matched to McFarland barium sulphate standard. The extracts were tested in triplicates. DMSO saturated assay disc and blank assay discs were used as negative controls. Standard antibiotic disc such as Ciprofloxacin (50 μg/disc) and Ketoconazole (50 μg/disc) were used as positive controls. The plates were incubated at 37°C for 24 hr and 48 hr for bacteria and fungi. Inhibition zones were recorded as the diameter of growth-free zones, including the diameter of the disc, at the end of the incubation period.

**RESULTS**

The antimicrobial activity of three Indian medicinal plants Annona squamosa, Aegle marmelos, Citrus limon, were tested against four Gram-positive, three gram-negative and three fungi as reported in Figs. 1, 2 and 3.

It was observed that all the three alcoholic leaf extracts showed antibacterial and antifungal activity. Alcoholic leaf extract of Annona squamosa was found to be most active against Staphylococcus aureus, Bacillus cereus, Klebsiella pneumonia, Aspergillus niger and Aspergillus fumigates. Alcoholic leaf extract of Aegle marmelos was found to be most active against Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and Candida albicans. Citrus limon was found to be most active against Staphylococcus epidermidis, and E. coli. The activity of all the leaf extracts were found to be dose dependent i.e., 100 μg/mL showed greater inhibition. The susceptibility of the microbes to the extracts of the plants was compared with standard antibiotics, ciprofloxacin and ketoconazole.

**DISCUSSION**

Medicinal plants have provided a source of inspiration for novel drugs compounds as since plant derived medicines have made large contributions to human health. The traditional healers make use of water primarily as solvent but alcoholic extracts of these plants were certainly much better and powerful. This may be due to the better solubility of the active components in organic solvents (Yoganarasimhan, 2000; Kumar et al., 2009).

Annona squamosa was found to be more active against S. aureus, B. cereus, S.epidermidis, K. pneumonia, A. niger, A.fumigates and showed least activity against C. albicans, B.
Alcoholic leaf extract of *Aegle marmelos* was found to be active against *B. subtilis*, *E. coli*, *P. aeruginosa* and *C. albicans*, and the activity was found to be less against *S. aureus*, *B. cereus*, *S. epidermidis*, *K. pneumonia*, *A. niger*, *A. fumigates*. Furthermore, *E. coli*, which is a gram-negative bacterium, was also inhibited by bael leaf extract at low concentration. Although *E. coli* belongs to the normal flora of humans, an enterohaemorrhagic strain of *E. coli* has caused serious food poisoning, and preservatives to eliminate its growth are needed (Buchanan and Doyle, 1997 and Gulcin et al., 2003) the extract of *Aegle marmelos* might therefore be of use. *E. coli* was found to be more susceptible to *Aegle marmelos* and this may be due to the presence of tannins and alcohols which inhibits the growth of microorganisms. Thus it is evident that bael has antibacterial activity and the mechanism of action may be due to the blockage of protein synthesis either at transcription or translation level and its synthesis at membrane level. The antimicrobial activity of bael leaf extract may be due to the presence of cuminaldehyde and eugenol as these compounds have already shown their activity against various bacterial strains (Katayama and Nagai, 1960; Duke, 1992).

Alcoholic leaf extract of *Citrus limon* was found to be active against *S. epidermidis* and *E. coli*. Citric acid is a major organic acid and malic acid is a minor constituent as reported by Nii et al., 1997. These organic acids present in higher concentration may be the causative factor for the antimicrobial activity against *S. epidermidis* and *E. coli*. Vinegar has been reported to exhibit antimicrobial activity against number of gram positive and gram negative bacteria (Entani et al., 1998). The antimicrobial activity of citric acid could be due to the presence of high concentration of citric acid and malic acid. The antimicrobial activity due to citric acid is because of its undissociated structure and cytotoxic effect.

The results of the present study support the traditional usage of the assessed plants and suggest that some of the plant extracts possess compounds with antimicrobial properties. These plants are used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. It is observed from the present study that *Annona squamosa* is most active against *S. aureus*, *B. cereus*, *S. epidermidis*, *K. pneumonia*, *A. niger*, *A. fumigates* while *Aegle marmelos* is active against *B. subtilis*, *E. coli*, *P. aeruginosa* and *C. albicans* yet *Citrus limon* was found to be least inhibitory towards bacterial and fungal growth. However further work may be carried out to isolate the active principle and study the nature of the compound. Thus it may be concluded that the alcoholic leaf extracts of *Annona squamosa*, *Aegle marmelos* and *Citrus limon* possess significant *in vitro* antimicrobial activity and these extracts can be subjected to isolation of therapeutic antimicrobials to carry out further

**Figure 1:** Screening of antibacterial activity of three medicinal plants against gram positive organisms

**Figure 2:** Screening of antibacterial activity of three medicinal plants against gram negative organisms

**Figure 3:** Screening of antibacterial activity of three medicinal plants
pharmacological evaluation.

REFERENCES


