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Anti-bacterial Effects of *Citrus medica* on Some Clinically Important Human Pathogens.

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ABSTRACT

The use of traditional herbal medicines in crude or refined form may help in the treatment of microbial infections with two advantages that they cure is achieved and the chances of microbes becoming resistant are minimized. The herbal medicines have the advantage of not producing major side effects as is found in case of usual antibiotics. Therefore this study was undertaken to focus on the in vitro antimicrobial effects of *Citrus medica* were tested for their antibacterial activities on selected strains of bacteria namely, *Bacillus subtilis (ATCC 6633), Shigella flexneri (ATCC 12022)* and *Pseudomonas aeruginosa (ATCC 27853).* These activities were compared with standard antibiotics such as Tetracycline and *Kanamycin*. Our results clearly indicate that the plant *Citrus medica* possess the antibacterial efficacy.

Keywords: Antimicrobial, *Pseudomonas aeruginosa, Shigella flexneri*, Resistance, Antibiotics, *Citrus medica.*

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INTRODUCTION

Many of the plants used today were known to the people of ancient cultures throughout the world. Practitioners of traditional medicine believe that the constituents of plants are unique as they contain both active ingredients and non-active components that play a role in enhancing the well-being of their patients. A rekindled interest in the pharmaceutical importance of plants has led to the discovery and adoption of plant extracts which were commonly used in traditional medicine, as alternative source of remedy [1]. The use of medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilization in China, India and the Near east, but it is doubtless an art as old as mankind. Neanderthals living 60,000 years ago in present day Iraq used plants such as holly back, these plants are still widely used in ethno medicine around the world [2].

The potential of higher plants as source for new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller [3]. Thus, any phytochemical investigation of a given plant will reveal only a very narrow spectrum of its constituents. Historically pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents. Random screening as tool in discovering new biologically active molecules has been most productive in the area of antibiotics [4]. Even now, contrary to common belief, drugs from higher plants continue to occupy an important niche in modern medicine. On a global basis, at least 130 drugs, all single chemical entities extracted from higher plants, or modified further synthetically, are currently in use, though some of them are now being made synthetically for economic reasons [5].

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs [6]. A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties. 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 used, many other raw drugs are collected in larger quantities and traded in the market as the raw material for many herbal industries [7]. Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of have not been adequately evaluated [8].

Citrus medica Linn. Commonly known as a Citron in English and bijapura in Ayurvedic literature is shrub or small tree. its leaflets are 3-6 inch long, elliptic-ovate or ovate-lanceolate with sort, wingless or nearly wingless petioles; flowers are 5-10 in a raceme, small or middle-sized; petals are generally more or less pink; fruit is globose ovoid or oblong often mamillate at the apex. This plant is found in all over india especially Tamil Nadu and Kumaon, Pachamarhi, Sikkim, Khasia Hills, Garo hills, Chittagong, Upper Yunzalin valley, the Western Ghats and Satpura range in Central India [9].

MATERIALS AND METHODS

Plant Materials

The aerial parts of the plant *Citrus medica* were collected from the authorized Ayurvedic store. The plant materials were identified and authenticated by reputed botanist Chennai, India.

Extraction from plants

The plant materials (Leaves and stem) were dried in shade and powdered in a mechanical grinder. The powder of the plant materials were initially de-fatted with petroleum Benzene (60^0C-80°C), followed by extraction with 1000 ml of chloroformic ethanol (1:1 ratio) by using a Soxhlet extractor for 72 hrs at a temperature not exceeding the boiling point of the solvent .The extract was filtered using Whattman filter paper (No.1) and then was concentrated and dried at 45°C for chloroformic ethanol elimination (1:1) , and the extracts were kept in sterile bottles, under refrigerated conditions until further use. The dry weight of the plant extracts was obtained by solvent evaporation and used to determine concentration in mg/ml. The extract thus obtained was directly used in the assay of antimicrobial activity.

Antibiotics

Broad spectrum antibiotics such as Tetracycline and Kanamycin were used as control drugs.

Bacterial Strains

The strains of microorganisms *Bacillus subtilis (ATCC 6633), Shigella flexneri (ATCC 12022)* and *Pseudomonas aeruginosa (ATCC 27853)* were used.

Determination of Antimicrobial Activity

Antimicrobial activity was measured using the standard method of diffusion disc plates on agar and the MIC was calculated using dilution method (Kirby- Bauer method) [16].

Dilution Methods

Dilution susceptibility testing methods were used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganisms. This was achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are generally tested in $\log_2$ serial dilutions.

Broth Dilution Method

The Broth Dilution Method is a simple procedure for testing a small number of isolates, even single isolates.

Preparation of microorganisms for experiment

The pure cultures of organisms *(Bacillus subtilis¸ Pseudomonas aeruginosa and Shigella flexneri)* were sub-cultured in nutrient broth. They were inoculated separately into nutrient broth and kept at 37°C for 24 hours. Then, they were kept at 4°C until use.

Growth Method

At least three to five well-isolated colonies, of the same morphological type, were selected from an agar plate culture of a particular microorganism. The top of each colony was touched with a loop, and the growth was transferred into a tube, containing 4 to 5 ml of Nutrient broth medium. The broth culture was incubated at 35°C for 8 hours. After the incubation period broth culture became turbid.

Disc Diffusion Method:

(a) **Mueller-Hinton Agar Medium**

Mueller-Hinton Agar is considered to be the best for routine susceptibility testing of non- fastidious bacteria for the following reasons; it shows acceptable batch-to-batch reproducibility for susceptibility testing. Medium is transparent, so that the inhibition zone can be visualized clearly. It gives satisfactory growth of most non fastidious pathogens. A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

(b) Preparation of Mueller-Hinton Agar

Mueller-Hinton Agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions. Immediately after autoclaving, it was allowed to cool in a 45 to 50°C water bath. The freshly prepared and cooled medium was poured into glass or plastic, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm. The agar medium was allowed to cool to room temperature and unless the plate is used the same day, stored in a refrigerator. Plates were used within seven days after preparation unless adequate precautions, such as

wrapping in plastic, have been taken to minimize drying of the agar. A representative sample of each batch of plates was examined for sterility by incubating at 30 to 35°C for 24 hrs or longer [10].

A) Preparation of antibiotic stock solutions

Discs of the two antibiotics such as Tetracycline and Kanamycin were used for this study.

B) Preparation of plant extracts solution for the experiment

The dried plant extracts were weighed and dissolved in sterile distilled water to prepare appropriate dilution to get required concentrations (1.0mg/ ml, 1.5mg/ ml, 2.0 mg/ ml, and 2.5 mg/ ml). They are kept under refrigeration.

C) Preparation of dried filter paper discs

Whattman filter paper (No.1) was used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven. After the sterilization, the discs were poured into the different concentration of broad spectrum antibiotics and into the prepared plant extract solutions and again kept under refrigeration for 24 hrs [11].

RESULTS AND DISCUSSIONS

Reading of Minimum Inhibition Concentration

Minimum inhibition concentration was expressed as the lowest dilution which inhibited growth judged by lack of turbidity in the tube, because very faint turbidity might be given by the inoculums itself. The inoculate tube was kept in the refrigerator overnight and was used as the standard for the determination of complete inhibition. The plant extracts were found to be effective against the three selected bacterial species.

Reading Zone of Inhibition and Interpreting Results

After 16 to 18 hrs of incubation each plate was examined. Once the resulting zones of inhibition came uniformly circular and in a confluent lawn of growth, the diameters of the zone of complete inhibition are measured, including the diameter of the disc. Zones are measured to the nearest mm using a ruler, which was held on the back of the inverted Petri plate.

Figure 1: Shows that the MIC value of Tetracycline and Kanamycin on *Bacillus subtilis, Pseudomonas aeruginosa and Shigella flexneri.*

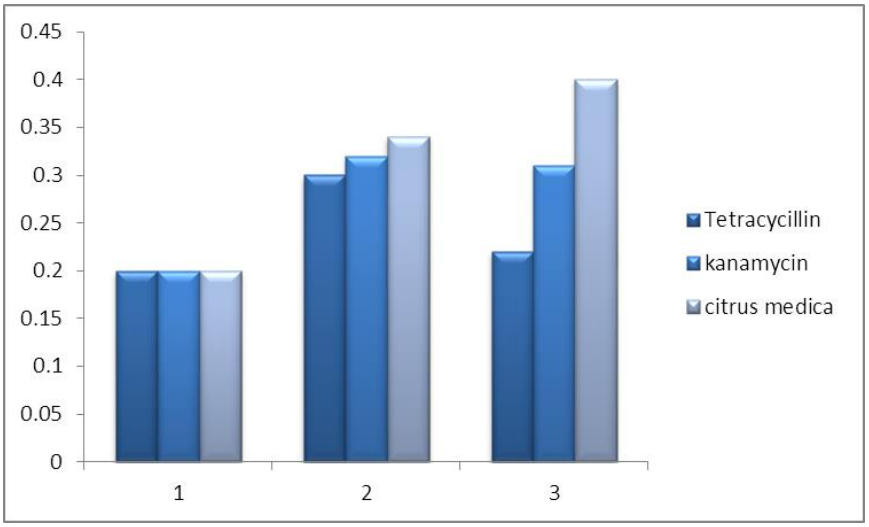

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Figure 2: Shows that Zone of inhibition (mm) on Tetracycline, Kanamycin and chloroformic ethanol extract of *C. Medica* against *Bacillis Subtilis*.

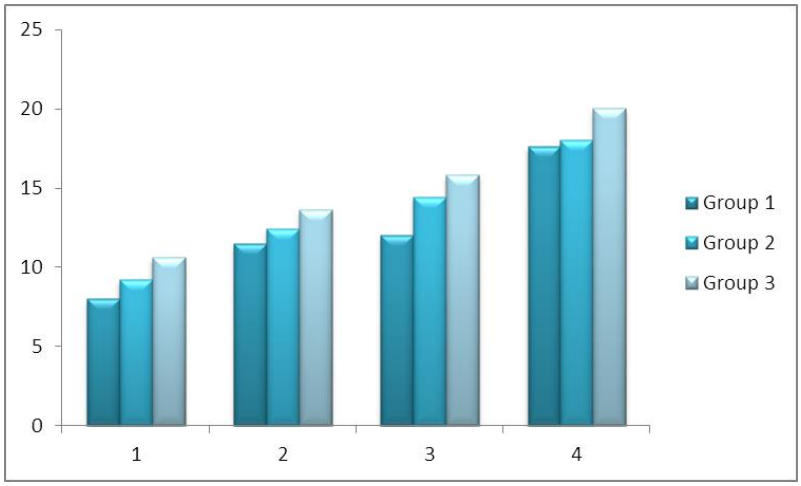


Figure 3: Shows that Zone of inhibition (mm) on *Tetracycline* , *Kanamycin* and chloroformic ethanol extract of *C. Medica* against *Pseudomonas Aeruginosa* .

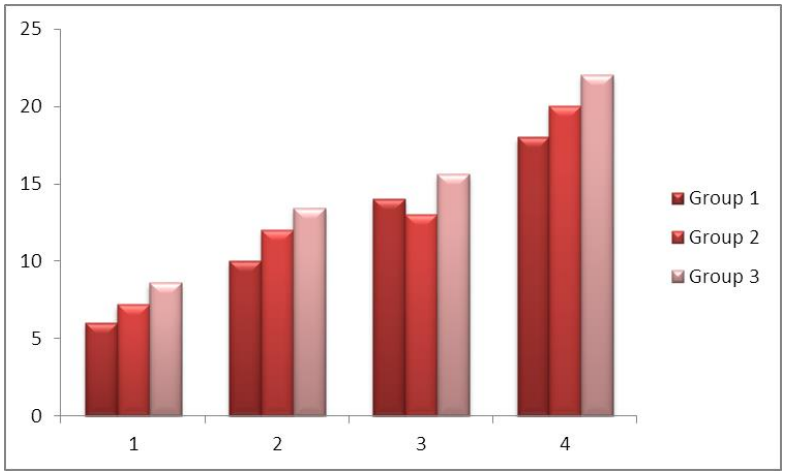
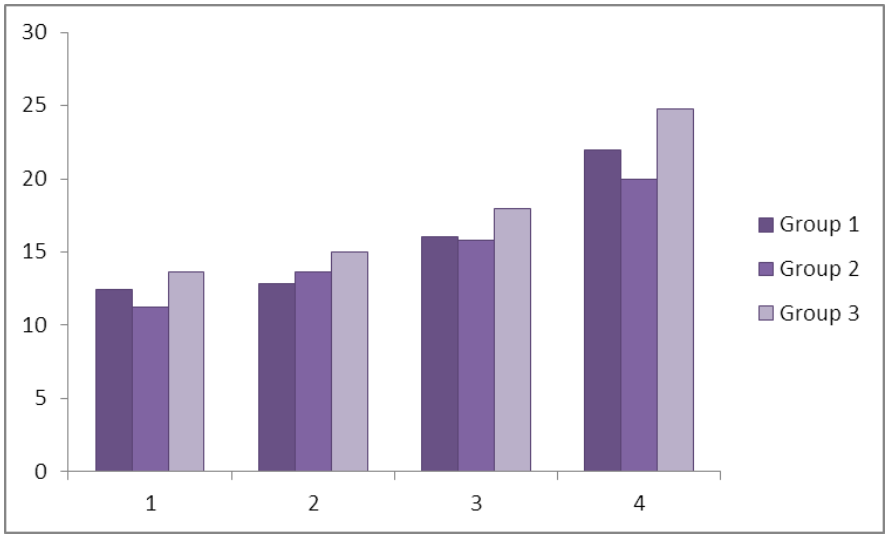


Figure 4: Shows that Zone of inhibition (mm) on Tetracycline, Kanamycin and chloroformic ethanol extract of *C. Medica* against *Shigella flexneri*.



Medicinal plants have been used to cure specific ailments. Today, there is wide spread interest in drugs derived from plants. This interest primarily stems from the belief that green medicine is safe and dependable, compared with costly synthetic drugs that have adverse effects. Natural antimicrobials can be derived from plants, animal tissues, or microorganisms. The use of medicinal plants in the world and especially in India, contribute significantly to primary health care. The antimicrobial medicinal plants are well documented. The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new antibacterial agent even against some antibiotic resistant strains. Except for *Schigella flexineri, Bacillus* anthracis and *Pseudomonas aeruginosa, Enterococci* may be resistant to Kanamycin and Tetracycline because of production of low affinity Tetracycillin binding protein (PBPs) or less commonly because of the production of β Lactamase [12]. In the present study the results show that the extract from *C. Medica* possess antimicrobial activities against *Bacillus subtilis, Shigella flexneri* and *Pseudomonas aeruginosa*. The extract compared favorably with the standard antibiotics *Tetracycline* and *Kanamycin*. The plant extract showed more activity that broad spectrum antibiotic activity.

The MIC of Tetracycline, Kanamycin and *C. Medica* were shown in Fig 1-4. The standard Tetracycline, Kanamycin and chloroformic ethanol extract (1:1) had MIC values varying between The *Bacillus Subtillis* value for Tetracycline was 0.200 mm, for Kanamycin was 0.200 mm and for chloroformic ethanol extract (1:1) of *C.Medica* was 0.200 mm respectively. The *Pseudomonas aeruginosa* value for Tetracycline , Kanamycin and chloroformic ethanol extract (1:1) of *C.Medica* was 0.300 , 0.320 , 0.340 mm Respectively. And for *Shigella flexneri* the values for Tetracycline was 0.220mm Kanamycin was 0.310 mm and chloroformic ethanol extract (1:1) of *C.Medica* was 0.400 mm The results indicated that the extract of *C. Medica* has stronger activity than that of standard antibiotics. Since ancient times, herbs and /or their essential oils have been known for their varying degrees of antimicrobial activities [13].

Zone of inhibition (mm) on Tetracycline, Kanamycin and chloroformic ethanol extract of *C. Medica* against *Bacillis Subtilis* , *Pseudomonas aeruginosa* and *Shigella flexneri*. *Bacillus Subtillis* concentration drug shows More recently medicinal plant extracts were developed and proposed for use in food as natural antimicrobials. Antimicrobials are powerful but controversial tools. Food animals are often exposed to antimicrobial compounds to treat or prevent infectious diseases and or to promote growth. On the basis of the results obtained in the present study, we conclude that the extract has significant antimicrobial activity. Further studies are on to isolate the active principles responsible for their antimicrobial activities [14].

The microorganism *Bacillus Subtilis* shows higher value for Tetracycline Chloroformic ethanol extract of *C.Medica* when compared to two different antibiotics having variance in concentration of drug (mg/ml). For concentration 1.0 mg/ml Chloroformic ethanol extract of *C.Medica* shows higher value 10.6 mm where as Tetracycline and Kanamycin shows lower value 8.0 and 9.2 mm respectively. In case of concentration drug value 1.5 mg/ml Chloroformic ethanolic extract of *C.Medica* shows 13.6 mm as higher value and Tetracycline and Kanamycin shows lower value 11.2 and 12.4 mm. Taking concentration drug 2.0 mg/ml the Chloroformic ethanol extract shows higher value 15.8 mm and for Tetracycline it is 12.0 mm and for Kanamycin it was 14.4 mm. For concentration drug 2.5 mg/ml again chloroformic ethanol extract shows higher value 20.0 mm whereas Tetracycline shows 17.6 mm and Kanamycin shows 18.0 mm respectively. The *pseudomonas aeruginosa* was also slow and higher value of ethanolic when compared with antibiotics Tetracycline and Kanamycin for different concentration for conc. 1.0, 1.5, 2.0 and 2.5. Ethanolic shows higher value of 8.6, 13.4, 15, 6 and 22.0 mm respectively. Antibiotics Tetracycline shows 6.0, 10.0, 14.0 and 18.0 mm respectively. In case of antibiotics Kanamycin it is 7.2, 12.0, 13.0 and 20.0 mm respectively. Similarly microorganism *shigella flerneri* also shows higher value for the Chloroformic ethanolic extract when compared with those two antibiotics. The value for chlroformic ethanol extract concentration of 1.0,1.5,2.0 and 2.5 mg/ml was 13.6,15.0,18.0 and 24.8 mm respectively .Whereas *Tetracycline* show lower value of 12.4, 12.8, 16.0 and 22.0 mm. In case of *Kanamycin* it was 11.2, 13.6, 15.8 and 20.0 mm respectively. Hence, it is of interest to compare the extract with these two antibiotics to check the antibacterial efficacy of *C-media* in different conc. (1.0, 1.5, 2.0 and 2.5mg/ml) .Our results proved the antibacterial efficacy of the plant [15-16].

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