Original Article

In vitro antimicrobial status of methanolic extract of *Citrus sinensis* Linn. fruit peel

Abstract

Aim: The present investigation evaluated the antimicrobial potential of methanolic extract of *Citrus sinensis* Linn. (Rutaceae) fruit peel. There is a basis for the traditional use of this plant for local health remedies. **Materials and Methods:** The antimicrobial activity of methanolic extract of *C. sinensis* fruit peel was tested against three bacterial and two fungal strains. Turbidimetric or tube dilution method and paper disc diffusion method were followed. Results are expressed as mean ± standard deviation. **Results:** The *C. sinensis* fruit peel methanolic extract exhibited antibacterial activity against *Escherichia coli* with minimum inhibitory concentration of 0.78 µg/ml and minimum bactericidal concentration of 6.25 µg/ml, and appreciable antifungal activity with minimum inhibitory concentration of 12.5 µg/ml. The phytochemistry of *C. sinensis* fruit peel methanolic extract revealed the presence of carbohydrates (reducing sugars, hexose sugars, non-reducing polysaccharides, gums, and mucilages), flavonoid glycosides, coumarin glycosides, volatile oils, organic acids, fats and fixed oils. **Conclusion:** Most of the organic chemical constituents reported are aromatic phenolic compounds, which are known for their wide spectra of antimicrobial activity. Therefore, the bacteriostatic and fungistatic action of the tested extract may be attributed to the presence of polyphenolic compounds. In short, *C. sinensis* fruit peel methanolic extract is a potential source of natural antimicrobials.

Key words:
Antimicrobial activity, Citrus sinensis Linn., paper disc diffusion method

Introduction

Antimicrobial compounds have many applications in medicines, food, agriculture, livestock, textiles, paints, and wood protectants. Microorganisms resistant to most antibiotics are rapidly spreading. Consequently, there is an urgent and continuous need for novel antimicrobial compounds. Most antibiotics have been developed from microorganisms. Plants also represent an important source for finding novel antimicrobial compounds, as plants in their permanent fight with microorganisms in their environment produce a wide spectrum of compounds with antimicrobial activity.[1] Because of the risks associated with the use of synthetic compounds, biological methods of control have been preferred. Resistance to biological control is rare and biological control agents are self-propagating and self-perpetuating.[2] Plant extracts have been used successfully to control diseases in plants and tuber crops.[1] Many medicinal plants are considered to be potential antimicrobial crude drugs as well as a source for novel compounds with antimicrobial activity, with possibly new modes of action. This expectation that some naturally occurring plant compounds can kill antibiotic-resistant strains of bacteria such as *Bacillus cereus*, *Escherichia coli*, *Micrococcus luteus*, and *Staphylococcus aureus* has been confirmed. The low molecular weight antimicrobial compounds called phytoalexins are produced by plants upon pathogen infection and have long been implicated as playing an important role in disease resistance.[3]

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Citrus fruits are well endowed with a variety of phytofungicides that are necessary to inhibit fungal growth and development.[4] The citrus peel essential oils are known to exhibit antimicrobial properties such as antifungal, antibacterial, antiviral and antiparasite.[5] Citrus oil has a lethal effect on fleas, fire ants, and houseflies due to the presence of 90–95% limonene. In the southeastern part of Nigeria, *Citrus sinensis* is used to manage malaria as well as skin diseases.[6]

The peel of citrus fruits is a rich source of flavanones and many polymethoxylated flavones, which are very rare in other plants. These compounds not only play an important physiological and ecological role, but are also of commercial interest because of their multitude of applications in the food and pharmaceutical industries.[7] There is growing interest in correlating phytochemical constituents of plant with its pharmacological activity.[8]

In earlier studies, the antimicrobial activity of flavonoids extracted from bergamot (*Citrus bergamia* Risso) peel, a by-product of essential oil industry, was evaluated against gram-negative bacteria (*E. coli, Pseudomonas putida, Salmonella enterica*), gram-positive bacteria (*Listeria innocua, Bacillus subtilis, S. aureus, Lactococcus lactis*), and the yeast *Saccharomyces cerevisiae*. Bergamot ethanolic fractions were found to be active against all gram-negative bacteria tested, and their antimicrobial potency increased after enzymatic deglycosylation.[9] In a study, the antioxidant and antimicrobial properties of methanol (100% and 80% aqueous) extracts of pummelo fruit's albedo (*Citrus grandis* Osbeck) were evaluated, the responsible components were purified, and the isolated compounds were tested for antioxidant and antimicrobial potential.[10] In another study, the antimicrobial properties and chemical composition of *Citrus paradisi, C. sinensis, Citrus nobilis* and *Citrus limon* fruit essential oils were determined to control *Paenibacillus* larvae by broth microdilution method. The antimicrobial assays showed that the oil of *C. paradisi* inhibited the bacterial strains at the lowest concentrations tested, with the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) averages of 385 mg/ml and 770 mg/ml, respectively.[11]

In precedent reports about *C. sinensis*, the antimicrobial activity was demonstrated for its essential oil against several microbial species, but no report was found about the methanolic fruit peel extract of *C. sinensis* against the bacterial strains *E. coli, B. subtilis, S. aureus* and the pathogenic fungal strains, *Aspergillus niger* and *Candida albicans*. In a recent study, *C. sinensis* was screened for its phytochemical composition and evaluated for its larvicidal and antimicrobial activity using agar well diffusion method,[12] while no data were given about its MIC or MBC/ minimum fungicidal concentration (MFC).

In our study, *C. sinensis* fruit peel methanolic extract has been evaluated against *B. subtilis, S. aureus*, *E. coli* and fungal strains *C. albicans* and *A. niger* using turbidimetric assay method and paper disc diffusion method. MIC, MBC and MFC were also determined with reference to the tested gram-positive, gram-negative bacterial strains and pathogenic fungal strains. The antimicrobial activity can be determined using different methods like approved standard Clinical and Laboratory Standard Institute (CLSI) method for agar dilution technique, serial dilution method, paper disc diffusion method, cup-plate method etc. CLSI method may be used for greater consistency in antimicrobial testing and serves as the gold standard for clinical studies.[13] In the present study, we have selected serial dilution/turbidimetric assay method and paper disc diffusion method for screening the antimicrobial spectrum of the test extract.

Materials and Methods

Plant collection and identification

The fresh peels of *C. sinensis* were collected from Bahadurgarh, India, in December 2009. The plant was identified and authenticated for taxonomic identity in Department of Biosciences, M. D. University, Rohtak, India, by Prof. J. P. Yadav, and a voucher specimen was deposited in Herbarium of Pharmaceutical Sciences, M. D. University (voucher specimen number DPS 0014).

Processing and extraction of plant material

The peels were chopped and shade-dried at room temperature for 2 weeks and then grounded to a coarse powder for ease of extraction of active compounds. The powdered plant material (100 g) was packed into a Soxhlet apparatus (1 l) and extracted with methanol for 4 h. The extract was filtered and the solvent was evaporated under reduced pressure using rotary vacuum evaporator, lyophilized into dry powder, and kept in desiccator.

Concentration of extract

The stock solution of the methanolic extract was prepared by dissolving 10 mg of the *Citrus* fruit peel extract in 10 ml dimethyl sulfoxide (DMSO). The following concentrations were prepared: 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 μg/ ml of the crude extract for determination of MIC, MBC and MFC. For antimicrobial sensitivity testing, concentrations taken were from 1 to 20 μg/ml. Standard antibacterial agent, Norfloxacin, and antifungal agent, Fluconazole (Belco Pharmaceuticals, Bahadurgarh, India) served as positive control. DMSO (HPLC grade) was used as negative control.

Phytochemical analysis

The crude methanolic extract of *C. sinensis* fruit peel was subjected to qualitative chemical tests for identification of various categories of organic chemical constituents such as carbohydrates, tannins, saponins, glycosides, steroids/ triterpenoids, flavonoids, organic acids, alkaloids,

fats and fixed oils. The phytochemical analysis was done according to standard methods.[14,15]

Test microorganisms

The tested bacterial strains (*B. subtilis, S. aureus*, *E. coli*) and fungal strains (*C. albicans* and *A. niger*) were obtained as gift samples from the Department of Microbiology, Guru Jambeshwar University, Hissar, India.

Preparation of media

A double strength nutrient medium was prepared for antibacterial studies using peptone (1 g), yeast (0.3 g) and sodium chloride (0.5 g), and dissolving these ingredients in water q.s. to make 50 ml. The media was then sterilized by autoclaving at 15 lb/psi pressure for 15 min. Double strength Sabouraud's glucose broth used for antifungal studies was prepared by dissolving glucose (8 g) and peptone (2 g) in distilled water in a quantity sufficient to make 100 ml with the aid of heating. The medium was cooled and filtered and pH was adjusted to 5.4 with 10% lactic acid. The media was sterilized by autoclaving at 15 lb/psi pressure for 15 min.[16]

Antimicrobial study

For antimicrobial study, cultures were maintained in refrigerated conditions. For culture studies, fresh 24-h-old cultures were prepared in case of *E. coli, S. aureus* and *B. subtilis*, 48-h culture for *C. albicans*, and 7-day culture for *A. niger*. The bacterial/fungal suspensions were prepared in normal saline by transferring the organism from fresh cultures (1×10^8 cells/ml).

Paper disc diffusion method

In this method, 0.2 µl of individual bacterial and fungal cultures was poured with nutrient agar medium (30 ml) in petri plates (90 mm). Sterilized filter paper discs (Whatman no. 1; 6 mm in diameter) soaked in different beakers containing the dissolved extracts of different concentrations were taken out with sterilized forceps and air-dried and placed on plates with the different organisms. The plates were incubated at 37°C for 24 h for bacterial strains, for 2 days at 37°C for *C. albicans*, and for 7 days at 25°C for *A. niger*. After incubation, the inoculated plates were observed for zones of inhibition in millimeter diameter using a transparent ruler. The susceptibility of the test bacteria and fungi to standard drug was tested using inoculated agar plate and norfloxacin and fluconazole as positive control at different concentrations. The zones of inhibition were measured and compared with those of the plant extract.[17] Evaluation of the inhibitory properties was carried out in triplicates.

Minimum inhibitory concentration, minimum bactericidal concentration and minimum fungicidal concentration

Turbidimetric method or tube dilution method was used for the determination of MIC, MBC, and MFC. One milliliter of the sterilized media poured in the concentration of 50 µg/ml was used. The extract was serially diluted to give concentrations of 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 µg/ml in test tubes containing 1 ml sterile nutrient broth. Then, the tubes were inoculated with 100 µl of bacterial suspension in saline and incubated at 37°C for 24 h (for plates containing bacterial cultures), at 37°C for 2 days (for plates containing *C. albicans* culture), and at 25°C for 7 days (for plates containing *A. niger* culture). A tube containing nutrient broth only was seeded with the test organism to serve as control. All the tubes were then incubated at 37°C for 24 h and then examined for growth by observing turbidity. The MBC of the plant extract on the clinical bacterial isolates was determined by pipetting out 0.1 ml bacterial culture from the mixture obtained in the determination of MIC tubes which did not show any growth and subcultured onto nutrient media and incubated at 37°C for 24 h. After incubation, the concentration at which there was no single colony of bacteria was taken as MBC. MFC was also determined similar to MBC.[18]

Results

The antimicrobial effect of *C. sinensis* fruit peel study involved a comparison of the inhibition zones of its methanolic extract [Tables 1 and 2] with those of commercially developed antibiotics. The activity of *C. sinensis* fruit peel extract against both gram-positive and gram-negative bacteria is an indication of its broad spectrum of activity, and thus can be used to source antibiotic substances for drug development that can be used in the control of these bacterial infections.[19] Results of zone of inhibition (mm) of different bacterial and fungal agents at various concentrations of *C. sinensis* are expressed as mean ± standard deviation [Tables 1 and 2, respectively]. As per the antimicrobial investigations, the methanolic extract exhibited antibacterial activity against *E. coli* with MIC of 0.78 µg/ml and MBC of 6.25 µg/ml, and appreciable antifungal activity with MIC of 12.5 µg/ml [Table 3]. The phytochemical screening results indicated the presence of carbohydrates (reducing sugars, hexose sugars, non-reducing polysaccharides, gums, and mucilages), flavonoid glycosides, coumarin glycosides, volatile oils, organic acids, fats and fixed oils as the main constituents.

Discussion

As new drug-resistant bacterial strains emerge, herbal drugs are being looked as very important source for discovery of new agents for treating various ailments related to bacterial infections. Plants belonging to the genus *Citrus* are well-known herbs used in ayuverdic traditional medicine for their effectiveness against a wide range of diseases, including skin infections, due to the advantage of the diversity of secondary metabolites responsible for their antibacterial activity. There has been increasing interest in the development

Table 1: Zone of inhibition (mm) of different bacterial agents at various concentrations of the test extract of *C. sinensis* and the standard Norfloxacin

Concentration (μg/ml)	Bacterial agent					
	E. coli		*S. aureus*		*B. subtilis*	
	C. sinensis	Norflox	*C. sinensis*	Norflox	*C. sinensis*	Norflox
20	12.6±0.5	30±1.0	11.6±0.5	20.6±0.5	8.3±1.1	24±1.0
10	10±1.0	25±1.0	11±1.0	17.6±0.5	8±1.0	22.6±0.5
5	9±1.0	20.6±1.1	11±0.0	17±0.0	8±0.0	19.6±0.5
3	7.6±0.5	15.6±0.5	9.6±0.5	14.6±0.5	–	9±0.0
1	–	–	7.6±0.5	13±1.0	–	8.3±1.5

Observations are expressed as mean±standard deviation, $n=3$, The above-mentioned readings are inclusive of paper disc diameter

Table 2: Zone of inhibition (mm) of different fungal agents at various concentrations of the test extract of *C. sinensis* and the standard Fluconazole

Concentration (μg/ml)	Fungal agent			
	A. niger		*C. albicans*	
	C. sinensis	Fluconazole	*C. sinensis*	Fluconazole
20	10±1.0	14±0.0	8.6±0.5	13±0.5
10	9±0.0	ND	8.3±1.5	ND
5	8.3±0.5	ND	8±0.0	ND
3	6.3±1.5	ND	–	ND
1	–	ND	–	ND

ND - Not detected;Observations are expressed as mean±standard deviation, $n=3$, The above-mentioned readings are inclusive of paper disc diameter

Table 3: MIC and MBC/MFC of *C. sinensis* methanolic leaf extract against microbial strains

C. sinensis methanolic extract	MIC (μg/ml)	MBC/MFC (μg/ml)
E. coli	0.78	6.25
S. aureus	25	50
B. subtilis	3.125	6.25
C. albicans	12.5	>50
A. niger	12.5	>50

MIC - Minimum inhibitory concentration; MBC - Minimum bactericidal concentration; MFC - Minimum fungicidal concentration

of new types of effective and nontoxic antimicrobial compounds. The phytochemistry of *C. sinensis* methanolic extract revealed the presence of carbohydrates (reducing sugars, hexose sugars, non-reducing polysaccharides, gums, and mucilages), flavonoid glycosides, coumarin glycosides, volatile oils, organic acids, fats and fixed oils. Most of the organic chemical constituents reported are known to possess aromatic phenolic compounds, which are known for their wide spectra of antimicrobial activity. A drug candidate is said to be bacteriosatic/fungistatic when its MFC and MBC values are threefold higher than its MIC values.[20]

Therefore, the bacteriostatic and fungistatic action of the test extract may be attributed to the presence of aromatic phenolic compounds.

Flavonoids are hydroxylated phenolic substances and occur as a C_3–C_6 unit linked to an aromatic ring. They are known to be synthesized by plants in response to microbial infection. Their activity is probably due to their ability to complex with extracellular and soluble proteins, and to complex with bacterial cell walls. More lipophilic flavonoids may disrupt the microbial membranes.[21] The flavonoids present in the citrus fruit peel methanolic extract may be responsible for the antimicrobial action. Volatile oils have been reported to have insecticidal or insect repellent effects, while there are some with potent antimicrobial effects against both fungi and bacteria.[22] Inhibitory effects of organic acids against microbes are pH dependent. At low pH, most of the organic acids are in the undissociated form. Undissociated organic acids are lipophilic and can diffuse across the cell membrane. Once in the bacterial cell, they dissociate at pH of the cytoplasm (>7), causing metabolic uncoupling.[23] Various essential oils obtained from the plants also showed antimicrobial activity against a range of microorganisms including gram-positive bacteria gram-negative bacteria, and fungi. However, the differences may be explained by susceptibility, testing conditions, physico-chemical characteristics of the oil and strain differences.[24] Essential oils are aromatic phenolic compounds and found to possess a wide spectra of antimicrobial activity,[25] and the observed antimicrobial action would tend to further validate the medicinal properties of this commonly used endemic medicinal and food plant. Therefore, the presence of oils and organic acids along with flavonoids might have contributed toward high antimicrobial activity of methanolic extract of *C sinensis* fruit peel.

The results showed that the methanolic extract of *C. sinensis* fruit peel was able to inhibit all of the bacteria and fungi used in this study with different degrees of inhibition. This suggested that the phenolic compounds might significantly contribute to the antibacterial activity of the test extract. Further, pharmacological and clinical studies are required to understand the mechanism and the actual efficacy of this herbal extract in treating various infections and skin diseases.

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