Research Article

Antibacterial Efficacy of *Citrus sinensis* (Sweet Orange) against *Enterococcus faecalis*-An *In vitro* Study

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Abstract

Background: Resistant microbes always represent a challenge in the treatment of various well-known infections and urges the need for substances with potent antimicrobial properties. *Citrus sinensis* is one of the most important and widely known among various fruits for its medicinal value. Many medicinal properties of orange peel extract, such as against colic, upset stomach, cancer, diuretic, immune-enhancing, stomachic, tonic to digestive system and it is known to fight viral and bacterial infections. Thus this study aims to evaluate the antibacterial efficacy of *Citrus sinensis* against *E. faecalis* biofilm. **Methodology:** Aqueous and ethanol extracts prepared from peel of *Citrus sinensis* were screened for *in vitro* antimicrobial activity against *E. faecalis* using agar well diffusion method. The lowest concentration of every extract considered as the minimal inhibitory concentration (MIC) values were determined for both test organisms. One way ANOVA test was applied for statistical analysis. **Results:** *E. faecalis* were inhibited most by extracts of *Citrus sinensis* peel. MIC of *Citrus sinensis* peel was 37.5 µg/ml and MBC was 59.42 µg/ml. **Conclusion:** *Citrus sinensis* peels extract demonstrated antimicrobial activity against *E. faecalis in vitro* warranting further *in vivo* clinical studies to determine the exact dosages and its effectiveness in practical situations.

Keywords: *Citrus sinensis*; Sweet orange; Agar well diffusion assay; Minimum inhibitory concentration; Minimum bacterial concentration; Orange peel

Introduction

Sweet orange belongs to the family Rutaceae is botanically known as "*Citrus sinensis*". *Citrus sinensis* is one of the widely known fruits with total global production reported to be around 120 million tons.

Orange trees are widely cultivated in tropical and subtropical climates for its tasty juice and medicinal value. [1,2]

Major medicinal properties of orange peel extract are immune-enhancing, stomachic, tonic to digestive system, immune system and proven against colic, upset stomach, and cancer.

It is mainly used to treat and prevent vitamin deficiencies, colds, flu, and scurvy and help to fight viral and bacterial infections.

The fruit has major core which contains sweet pulp and numerous seeds. The fruit pulp is mainly formed of segments of juice filled with flavor that goes from sour to sweet.

The fruit is perennial and it has adapted to a variety of climates. [3,4]

C. sinensis is consumed all over the world as an excellent source of vitamin C. It is a rich source of secondary metabolites containing pharmacologic activities.

Several types of chemical compounds have been identified in fruits, peel, leaves, juice and roots of *C. sinensis*, which include the following: Flavonoids, steroids hydroxy amides, alkanes and fatty acids, coumarins, peptides, carbohydrates, carbamates and alkylamines, carotenoids, volatile compounds and nutritional elements such as potassium, magnesium, calcium and sodium. [5,6]

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Antibacterial effects of orange peel have also been demonstrated in the literature. Mehmood et al. showed potent antibacterial activity (against enteric pathogens) of extract from orange peels. Orange peel extract was also found to be effective against *Klebsiella pneumonia* by Akdemir. [7-9] Previously our team has a rich experience in working on various research projects across multiple disciplines. [10-15]

Natural products have been and will be important sources of new pharmaceutical compounds. Recently, there has been a renewed interest in natural product research due to the failure of alternative drug discovery methods to deliver many lead compounds in key therapeutic areas. [16,17] In this sense, considering the health benefits of *C. sinensis*, it presents excellent options for treating or helping in a disease due to its bioactive compounds. Thus this study aims to evaluate the antibacterial efficacy of *Citrus sinensis* against *E. faecalis* biofilm.

Methodology

Preparation of *Citrus sinensis* aqueous extract

Sweet Oranges (*Citrus sinensis*) were purchased from the local market and the orange peels were obtained. The peels were carefully washed under running tap water followed by sterile distilled water. These were air dried at room temperature (30°C) for two days, pulverized to a fine powder using a sterilized mixer grinder and stored in air-tight bottles. Two different solvents namely ethanol (hot and cold) and water (hot and cold) were used for extraction to obtain a total of 4 extracts. For the purpose of extraction, a 10 g amount of the pulverized peel was separately soaked in 100 ml of ethanol (96%) and cold sterile distilled water for 24 h. Also the same amount (*i.e.* 10 g) of pulverized peel was immersed in 100 ml of hot sterile distilled water (100°C) and allowed to stand for 30 min on a water bath with occasional shaking and kept undisturbed for 24 h. Each preparation was filtered through a sterilized Whatman No.1 filter paper and the filtered extract was concentrated under vacuum below 40°C using Heidolph, VE-11 rota evaporator. The dried extract thus obtained was exposed to UV rays for 24 h and checked for sterility on nutrient agar plates and stored in labeled sterile bottles in a freezer at 4°C until further use.

MIC and MBC of *Citrus sinensis* extract in comparison with CHX and saline

Bacterial strain: The bacterial strain of Enterococcus faecalis used in this study was obtained from the American Type Culture Collection (ATCC 14506). The E. faecalis strain was inoculated containing 3 ml Brain Heart Infusion (BHI) broth, incubated in a B.O.D. incubator for 24 h in a 5% CO_2 atmosphere at 37°C. After this period, the microorganisms were subcultured and used for further study.

Experimental groups

Group 1: Saline (0.9%)

Group 2: Chlorhexidine (0.2%)

Group 3: *Citrus sinensis* aqueous extract

Minimum inhibitory concentration using microdilution broth technique

For this procedure, 9 dilutions were done with Brain Heart Infusion (BHI) broth microdilution assay. In the initial tube 20 µl of the test sample was added into 380 µl of BHI broth. For dilutions 100 µl of BHI broth was added into the next 9 tubes separately. Then from the initial tube, 100 µl was transferred to the first tube containing 100 µl of BHI broth. This was considered as 10^{-1} dilution.

From 10^{-1} diluted tube 100 µl was transferred to the second tube to make 10^{-2} dilution. The serial dilution was repeated upto 10^{-9} dilution for each drug. From the maintained stock cultures of required organisms, 5 µl was taken and added into 2 ml of BHI broth. In each serially diluted tube, 100 µl of above culture suspension was added.

From the maintained stock cultures of required organisms, 5 µl was taken and added into 2 ml of BHI broth. In each serially diluted tube, 100 µl of above culture suspension was added. Test was done on Saline, CHX, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml dilutions of *Citrus sinensis* extract. The tubes were incubated at 37°C for 48-72 h in an anaerobic jar and observed for turbidity.

Minimum bactericidal concentration by agar well diffusion method

The minimum bactericidal concentration was determined by taking 10 µl aliquot of the bacterial suspensions at concentrations where no visual growth of the microorganisms was observed. The 10 µl of CSE (75 and 100 µg/ml), CHX and saline were used for the MBC testing. In the media plate around 6 mm dm wells were made and filled with the test solutions. After incubation at 37°C for 24 h, the zone of inhibition of each test sample was measured in mm.

Statistical analysis

Statistical evaluation of the data was performed using SPSS version 21.0. The statistical test used was ANOVA for multiple comparisons, followed by Duncan's test. Values of $p<0.05$ were considered statistically significant and are indicated by an asterisk. All tests were performed in three independent experiments.

Results

E. faecalis were inhibited most by extracts of *Citrus sinensis* peel. MIC (Minimum Inhibitory Concentration) of *Citrus sinensis* peel was 37.5 µg/ml and MBC (Minimum Bactericidal Concentration) was 59.42 µg/ml [Table 1, Table 2], [Figure 1, Figure 2].

Moreover, minimum inhibitory concentrations of the aqueous extracts were much higher than the ethanolic extracts. Cowan reported that the potency of *Citrus* fruit peel is enhanced by the type of solvent used indicating that there are some active ingredients in orange peel which have high antimicrobial effect but which would not be released except when orange fruit peel is used in conjunction with a particular solvent. [31-36]

Cowan mentioned that most of the antibiotic compounds already identified in plants are reportedly aromatic or saturated organic molecules which can easily be solubilized in organic solvents.
[37-40]

In a study conducted by Jeyaseelan et al. in which leaf extracts of *Ricinus communis* L were investigated against *Staphylococcus aureus* and *Escherichia coli* and hot ethanolic extract showed better effectiveness.

Jeyaseelan et al. explained that the better activity of hot extracts may be due to the chemical changes caused by the hot treatment, and the resulting molecules may be more active than the biomolecules found in the cold extracts. [41-46]

The antimicrobial potency of plants is believed to be due to tannins, saponins, phenolic compounds, essential oils and flavonoids.

These compounds are known to be biologically active and therefore aid the antimicrobial activities of the plants. These secondary metabolites exert antimicrobial activity through different mechanisms.

Tannin as observed in *Citrus sinensis* peel extract have been found to form irreversible complexes with proline rich protein resulting in the inhibition of cell protein synthesis.

Another secondary metabolite compound observed in the ethanolic extract was alkaloid. One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms.

These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines. [47-49] Just et al. revealed the inhibitory effect of saponins on inflamed cells and is found to be present in the extracts of *Citrus sinensis* peel.

Flavonoids, another constituent of both the plants exhibited a wide range of biological activities like antimicrobial, anti-inflammatory, anti-angiogenic, analgesic, anti-allergic, cytostatic and antioxidant properties. Terpenoids observed in ethanolic extracts is speculated to be involved in membrane disruption by the lipophilic compounds. [50-54]

Hence, strength of the study noted were the peels of fruits of *Citrus sinensis* which are generally treated as wastes can serve as an effective and economical antimicrobial agent as they are available for no cost, and have no side effects.

In future, *in vivo* clinical studies should be conducted to confirm *in vitro* results and for the assessment of safety and efficacy by incorporating these plant extracts into dental products such as mouth rinses and toothpastes.

Conclusion

Citrus sinensis peels extract demonstrated antimicrobial activity against *E. faecalis in vitro* warranting further *in vivo* clinical studies to determine the exact dosages and its effectiveness in practical situations. Toxicity studies should also be done to determine safety. Need of the hour is to execute more and more screening of natural products or plant parts to set a primary platform for further phytochemical, pharmacological and *in vivo* studies that may open the possibilities of finding new clinically effective antibacterial compounds against bacterial resistant pathogens.

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