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ANTIMICROBIAL EFFECTS OF *CITRUS SINENSIS* PEEL EXTRACTS AGAINST PERIODONTOPATHIC BACTERIA: AN *IN VITRO* STUDY

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ABSTRACT

Background. Use of plant extracts and phytochemicals with known antimicrobial properties may have great significance in therapeutic treatments.

Objective. To assess the *in vitro* antimicrobial potential and also determine the minimum inhibitory concentration (MIC) of *Citrus sinensis peel* extracts with a view of searching a novel extract as a remedy for periodontal pathogens.

Materials and Methods. Aqueous and ethanol (cold and hot) extracts prepared from peel of *Citrus sinensis* were screened for in vitro antimicrobial activity against *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* and *Prevotella intermedia*, 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. Confidence level and level of significance were set at 95% and 5% respectively.

Results. *Prevotella intermedia* and *Porphyromonas gingivalis* were resistant to aqueous extracts while *Aggregatibacter actinomycetemcomitans* was inhibited at very high cncentrations. Hot ethanolic extracts showed significantly higher zone of inhibition than cold ethanolic extract.

Minimum inhibitory concentration of hot and cold ethanolic extracts of *Citrus sinensis* peel ranged between 12-15 mg/ml against all three periodontal pathogens.

Conclusions. Both extracts were found sensitive and contain compounds with therapeutic potential. Nevertheless, clinical trials on the effect of these plants are essential before advocating large-scale therapy.

Key words*: agar well diffusion, antimicrobial activity, periodontal disease, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Porphyromonas gingivalis*

INTRODUCTION

The practice of ethnomedicine is a complex multi--disciplinary system constituting the use of plants, spirituality and the natural environment and has been the source of healing for people for millennia [26]. The use of plants for medicinal purposes dates back to Vedic period. However, up to few decades back the herbal medicines were replaced by synthetic medicines due to their quick effect. Side effects of allopathic medicines is reverting the global trend towards green medicine.

Finding healing powers in plants is an ancient idea. People on all continents have long applied poultices and imbibed infusions of hundreds, if not thousands, of indigenous plants, dating back to prehistory. According to the World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs, therefore such plants should be investigated to understand their properties, safety and efficacy for a search of new potent antimicrobial compounds. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of world [22].

Orange, the tasty, juicy fruit, belonging to the family *Rutaceae* is botanically known as *citrus sinensis*. *Citrus sinensis* is one of the most important and widely grown fruit crop, with total global production reported

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to be around 120 million tons. Orange trees are widely cultivated in tropical and subtropical climates for its tasty juice and medicinal value.

Citrus senensis peel has many medicinal properties and is widely used against various ailments, such as colic, upset stomach, cancer, diuretic, cormunative, immuno – enhancing, stomachic, tonic to digestive system, immune system and skin. It is also used to treat and prevent vitamin deficiencies, colds, flu, and scurvy and helping to fight viral and bacterial infections. Antibacterial effects of orange peel have been demonstrated in the literature [11]. *Dubey* et al. showed potent antibacterial activity (against *Pseudomonas aeruginosa*, *Staphylococcus aureus, Staphylococcus epidermidis*, *Shigella flexineri*, *Bacillus subtilis* and *Escherichia coli*) of extract from fruit of Orange peels using disk diffusion method [5]. *Chabuck* et al. observed orange peel extract to be effective against *Klebsiella pneumonia* [4].

Periodontal disease is one of the most common dental health problems in the human communities. Several oral microorganisms have been studied as periodontopathogens. *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia*, are particularly implicated because of the wide scope of their virulence factors and because of their interactions with a large variety of Gram-negative and Gram--positive bacteria in the development of periodontal diseases. These microorganisms are often recovered in higher numbers from periodontal pockets with active attachment loss and may establish complex ecological relationships among several oral bacteria and modulate host immune system and influence the outcome of the treatment [8, 24].

Thorough literature search did not reveal any studies investigating the effect of orange peel extract on oral disease pathogens.

Hence, the present study was undertaken with the following objectives:

1. To assess and compare the in vitro antibacterial properties of different extracts of *Citrus sinensis* against common periodontal pathogens.
2. To determine the minimum inhibitory concentration (MIC) of each extract of both the plants against each pathogen with a view of searching a novel extract as a remedy for periodontal diseases.

MATERIALS AND METHODS

The study protocol was reviewed and approved by the Institutional Ethical Committee of Jordan University of Science and Technology, Jordan.

Procurement of plant material

Oranges (*Citrus sinensis*) were purchased from local market and orange peels were obtained.

Extraction

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 rotaevaporator. 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 [16].

Qualitative analysis on phytochemical constituents [19]

Test for tannins

0.5 g of powdered sample of each plant was boiled in 20 mL of distilled water in a test tube and then filtered. The filtration method used here was the normal method, which includes a conical flask and filter paper. 0.1% $FeCl_3$ is added to the filtered samples and observed for brownish green or a blue black colouration, which shows the presence of tannins.

Test for saponins

2 g of powdered samples of each plant was boiled separately with 20 mL of distilled water in a water bath and filtered. 10 mL of the filtered sample was mixed with 5 mL of distilled water in a test tube and shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and observed for the formation of emulsion, which indicated the presence of saponins.

Test for flavonoids

A few drops of 1% NH_3 solution was added to the aqueous extract of each plant sample in a test tube. A yellow coloration confirms the presence of flavonoid compounds.

Test for terpenoids

5 mL of aqueous extract of each plant sample was mixed with 2 mL of $CHCl_3$ in a test tube. 3 mL of concentrated H_2SO_4 was carefully added to the mixture to form a layer. An interface forms with a reddish brown coloration if terpenoids constituent is present.

Test for cardiac glycosides

1 mL of concentrated H_2SO_4 was prepared in a test tube. 5 mL of aqueous extract from each plant sample is mixed with 2 mL of glacial CH_3CO_2H containing 1 drop of $FeCl_3$. This mixture was carefully added over to 1ml of concentrated H_2SO_4 already present in the test tube.

Test for alkaloids

200 mg plant material in 10 mL methanol, filtered; a 2 mL filtrate + 1% HCl + steam, 1mL filtrate + 6 drops of *Dragendroff* reagent, orange precipitate indicated the presence of respective alkaloids.

Test for carbohydrates

Molisch's test was used to detect the presence of carbohydrates. One drop of concentrated sulphuric acid was added to about 1g of the herbal extract, and then three drops of 1% α-napthol in 80% ethanol were added to the mixture, without mixing to form an upper phase. Formation of brown or purple ring at the interphase indicated the presence of carbohydrates.

Test of phenol

To 2-3 mL of aqueous or alcoholic extract few drops of 5% $FeCl_3$ solution was added. Formation of deep blue-black colour indicated the presence of phenols.

Test microorganisms

Three periodontal disease causing bacteria, *P. gingivalis* ATCC 3327, *A. actinomycetem comitans* ATCC 33384 and *P. Intermedia* ATCC 2564 were procured from Microbial Type Culture Collection, IMTECH, Chandigarh. These microorganisms were subcultured on the specific media, brain heart infusion agar (*P. gingivalis*) and MRS agar (*A. actinomycetem comitans* and *P. intermedia*) that were attained from HiMedia Laboratory Pvt. Ltd., Bombay, India and incubated aerobically at 37°C. Identification of all strains was confirmed by standard biochemical and staining methods [3].

Screening for Antimicrobial Activity

The hot and cold aqueous and ethanol extracts of the *citrus sinensis* were used for the antimicrobial screening using the agar well diffusion method. The media was punched with 7 mm diameter wells and were filled with various concentrations of the extracts 5 mg/ml, 10 mg/ml, 15 mg/mL, 20 mg/ mL and 25 mg/mL. The plates were then incubated at 37ºC for 24 hours. After incubation, zone of growth inhibition for each extract was measured in millimeters by using a special scale designed for their purpose by Himedia Laboratories pvt Ltd, measuring diameter between the edges of the lawn. Each extract was tested five times.

Determination of minimum inhibitory concentration (MIC)

MIC is defined as the lowest concentration of a compound/extract/drug that completely inhibits the growth of the microorganism in 24 h [23]. For MIC, 9 dilutions of each extract were done with brain heart infusion (BHI) broth microdilution assay. In the initial tube, 20 microliter of extract was added into the 380 microliter of BHI broth. For dilutions, 200 microliters of BHI broth was added into the next 9 tubes separately. Then from the initial tube, 200 microliter was transferred to the first tube containing 200 microliter of BHI broth. This was considered as 10^{-1} dilution. From 10^{-1} diluted tube 200 microliter was transferred to second tube to make 10^{-2} dilution. The serial dilution was repeated up to 10^{-9} dilution for each extract. From the maintained stock cultures of required organisms, 5 microliter was taken and added into 2 mL of BHI (brain heart infusion) broth. In each serially diluted tube 200 microliter of above culture suspension was added. The tubes were incubated at 37°C for 24 hours and observed for turbidity [20].

Statistical analysis

The data obtained were analyzed using SPSS (Statistical Package for Social Sciences) version 11.5 (SPSS Inc., Chicago, Illinois, USA). Descriptive statistics (Mean value and SD) along with comparison in mean zone of inhibition between the extracts and at different concentrations of both plants were performed using One way analysis of variance (ANOVA) with bonferroni *post hoc*. Confidence level and level of significance were set at 95% and 5% respectively.

RESULTS

Hot and cold aqueous extracts (up to 25 mg/mL conc.) of *citrus sinensis* peel showed no antimicrobial activity against any of the three periodontal pathogens. Zone of inhibition of the extracts (in mm) significantly increased ($p \leq 0.05$) as the concentration of the extracts increased. Mean zone of inhibitions were significantly higher with hot ethanolic extract than cold ethanolic extract against all three periodontal pathogens (Table 1, 2 and 3).

Prevotella intermedia and *Porphyromonas gingivalis* were found to be resistant against hot water and cold water extracts of Citrus sinensis. Minimum inhibitory

Table 1. Mean zone of inhibition (mm) of all extracts of *Citrus sinensis* on *Aggregatibacter actinomycetemcomitans*

Extracts	Concentrations (mean±SD)					P value
	25 mg/mL	20 mg/mL	15 mg/mL	10 mg/mL	5 mg/mL	
Cold ethanol	10.87±0.44[a]	9.12±0.22[c]	8.20±0.31[e]	R	R	0.01*
Cold water	R	R	R	R	R	-
Hot ethanol	11.00±0.41[b]	9.54±0.21[d]	8.77±0.12[f]	7.65±0.55[g]	R	0.024*
Hot water	R	R	R	R	R	-
P value	0.02*	0.004*	0.034*	0.013*	-	

Test applied – One way ANOVA with bonferroni *post hoc*, *P≤0.05 (statistically significant)
Post hoc: Values with same letter superscripted do not vary significantly.

Table 2. Mean zone of inhibition (mm) of all extracts of *Citrus sinensis* on Prevotella intermedia

Extracts	Concentrations (mean±SD)					P value
	25 mg/mL	20 mg/mL	15 mg/mL	10 mg/mL	5 mg/mL	
Cold ethanol	10.60±0.82[a]	8.60±0.73[c]	8.40±0.91[e]	R	R	0.041*
Cold water	R	R	R	R	R	-
Hot ethanol	11.00±0.07[b]	10.80±0.80[d]	9.40±0.76[f]	R	R	0.033*
Hot water	R	R	R	R	R	-
P value	0.002*	0.034*	0.018*	0.014*	-	

Test applied – one way ANOVA with bonferroni post hoc, *P≤0.05 (statistically significant)
Post hoc: Values with same letter superscripted do not vary significantly

Table 3. Mean zone of inhibition (mm) of all extracts of *Citrus sinensis* on *Porphyromonas gingivalis*

Extracts	Concentrations (mean±SD)					P value
	25 mg/mL	20 mg/mL	15 mg/mL	10 mg/mL	5 mg/mL	
Cold ethanol	10.41±0.48[a]	9.33±0.47[c]	8.25±0.94[e]	R	R	0.015*
Cold water	R	R	R	R	R	-
Hot ethanol	11.00±0.07[b]	10.80±0.88[d]	8.40±0.70[f]	R	R	0.023*
Hot water	R	R	R	R	R	-
P value	0.011*	0.034*	0.028*	0.011*	-	

Test applied: One way ANOVA with bonferroni *post hoc,* *P≤0.05 (statistically significant)
Post hoc: Values with same letter superscripted do not vary significantly

concentration of hot and cold water extracts against *A. actinomycetemcomitans* were found to be very high, viz, 28.9 and 30 mg/mL, respectively. Minimum inhibitory concentration of hot and cold ethanolic extracts of *Citrus sinensis* peel ranged between 12-15 mg/mL against all three periodontal pathogens (Table 4).

Table 4. Minimum inhibitory concentration (MIC) of *Citrus sinensis* peel extracts against periodontal pathogens on specific media for microorganism

Extract	*Aggregatibacter actinomycetemcomitans* (mg/mL)	*Prevotella intermedia* (mg/mL)	*Porphyromonas gingivalis* (mg/mL)
Cold ethanol	13.2	13	12.8
Cold water	30	-	-
Hot ethanol	12.2	12.1	12.5
Hot water	28.9	-	-

Preliminary phytochemical analysis (Table 5), revealed the presence of alkaloids and tannins in *Citrus sinensis* peel. Carbohydrates and glycosides and saponins were only found in aqueous extract. Triterpenoides were only revealed in ethanolic extracts.

Table 5. Phytochemical analysis of orange peel extract

Chemical constituent	*Citrus sinensis*	
	Aqueous	Ethanol
Alkaloids	+	+
Anthraquinones	-	-
Carbohydrates and glycosides	+	_
Tannin and phenolic	+	+
Protein	-	-
Triterpenoides	-	+
Saponins	+	_
Flavonoids	+	+

DISCUSSION

A number of medicinal plants described in Ayurveda still need to testify according to the modern parameters to ensure their activity and efficacy. Drugs used in Ayurveda are mostly prepared by extraction with water, as in ancient time people do not usually had the access to more lipophilic solvents. This is of concern, as mostly healers do not extract all the active compound(s) that are present in the plant and consequently the prepared drug might not contain all the pharmacologically active

compounds. In this study, the ethanol extracts of all plants showed greater antibacterial activity as compared to their water extracts. *Prevotella intermedia* and *Porphyromonas gingivalis* were found to be resistant with aqueous extracts but showed antibacterial activity with hot and cold ethanolic extracts. Moreover, minimum inhibitory concentrations of the ethanolic extracts were lesser than the aqueous extracts. Same observations have been reported earlier [6,13]. *Nisha* et al. [15] and *Nair* et al. [14] also reported better antibacterial activity with orange peel extract prepared in organic solvent. *Nisha* et al. 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 [15].

In the present study, better antibacterial activity was shown by hot ethanolic extract as compared to cold ethanolic extract against all the three periodontal pathogens. Similar results were obtained in a study conducted by *Jeyaseelan* and *Jashothan* [9] in which leaf extracts of *Ricinus communis L* were investigated against *Staphylococcus aureus* and *Escherichia coli* and hot ethanolic extract showed better effectiveness. *Mehta* et al. also reported greater zone of inhibition exhibited by hot methanolic extracts of leaves of *Epipremnum aureum* against several gram positive and gram negative bacterias [12]. *Jayaseelan* and *Jashothan [9]* explained that the better activity of hot extracts may be due to the chemical changes caused by the hot treatment, and the resulting biomolecules may be more active than the biomolecules found in the cold extracts.

The zone of inhibition of all extracts against periodontal pathogens in the present study increased significantly with increase in concentration which is in agreement with previous studies [11, 25]. The minimum inhibitory concentrations of *citrus sinensis* peel ethanolic extracts against periodontal pathogens in the present study ranged between 12.1-14.7 mg/mL. The MIC values obtained are much lower than that obtained by *Lawal* et al. [11] against *Salmonella typhi, Salmonella paratyphi* and *Aeromonas hydrophila*. The difference in findings may be due to the difference in bacteria studied. To the best of our knowledge, the present study is a pioneer study demonstrating the effect of orange peel extract on periodontal pathogens.

The antimicrobial potency of plants is believed to be due to tannins, saponins, phenolic compounds, essential oils and flavonoids [1]. 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 cinensis* peel extract have been found to form irreversible complexes with proline rich protein [22] resulting in the inhibition of cell protein synthesis. *Parekh* and *Chanda* [18] reported that tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues.

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 [17]. *Just* et al. [10] revealed the inhibitory effect of saponins on inflamed cells and is found to be present in the extracts of *citrus sinenesis* peel. Flavonoids, another constituent of both the plants, exhibited a wide range of biological activities like antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties [7]. Terpenoids observed in ethanolic extracts is speculated to be involved in membrane disruption by the lipophilic compounds [2].

Ethnomedicine have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. Recycling of fruit waste is one of the most important ways of utilizing it in a number of novel products which essentially required for human, animal and plant nutrition as well as in the pharmaceutical industry. The results of the present study give substantial evidence that the extracts are active against periodontal pathogens. In future, *in vivo* clinical studies should be conducted to conform *in vitro* results and for the assessment of safety and efficacy by incorporating these plant extracts into dental products such as mouth rinse.

CONCLUSIONS

In vitro investigations in the present study confirmed the antimicrobial potential of *citrus sinensis* peel against periodontal pathogens. Further *in vivo* studies are warranted to determine the exact dosages and its effectiveness in practical situations. Toxicity studies should also be done to determine safety.

Conflict of interest.
The authors declare no conflict of interest.

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