

Comparative Evaluation of citrus sinensis extract on Porphyromonas Gingivalis, Aggregatibacter Actinomycetemcomitans and Prevotella Intermedia with Chlorhexidine: an In-Vitro Study

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Abstract:

Background: Periodontal pathogens like Aggregatibacter Actinomycetemcomitans, Porphyromonas Gingivalis, and Prevotella Intermedia etc. are considered to be the primary etiologic factors for the periodontal diseases. Chlorhexidine is an antimicrobial agent considered to be gold standard which has a broad antibacterial activity and is normally used for chemical plaque control. But chlorhexidine is known to cause staining when used for a longer time. Hence other agents with herbal contents are being researched that can be used on regular basis.

Materials and Methods: Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC) and Zone of Inhibition (ZOI) were used to assess the antibacterial effect of citrus sinensis extract against periodontal pathogens and it was compared to chlorhexidine using the micro dilution process and the culture method.

Results: For Citrus Sinensis extract, MIC and MBC value of P. Gingivalis was 50ug/ml and of A. Actinomycetemcomitans & P. Intermedia was 100ug/ml. ZOI of P. Gingivalis was 15mm at 50ug/ml, and A. actinomycetemcomitans and P. Intermedia was 12 mm and 11 mm at 100ug/ml respectively. For Chlorhexidine, MIC and MBC value of P. Gingivalis was 0.2 ug/ml and 1.6 ug/ml respectively. And for both A. Actinomycetemcomitans & P. Intermedia, MIC and MBC value was 12.5 ug/ml. ZOI of P. Gingivalis was 13mm at 1.6 ug/ml, while ZOI of A. Actinomycetemcomitans & P. Intermedia was 12 mm at 12.5 ug/ml.

Conclusion: Citrus sinensis extract has antibacterial action against the microorganisms P. Gingivalis, A. Actinomycetemcomitans, and P. Intermedia. It is demonstrated that it may be utilised as a natural supplement to chlorhexidine for the treatment of chemical plaque.

Key Word: Citrus sinensis extract, chlorhexidine, Antibacterial, Minimum inhibitory concentrations, Minimum Bactericidal concentrations, Zone of Inhibition.

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I. Introduction

Periodontal infections are multifactorial and are elicited by a complex of bacterial species interacting with host tissues cells that cause the release of a broad array of inflammatory cytokines, chemokines and mediators which lead to the periodontal destruction. The complex microbial biofilms colonizing the sulcular region trigger the initiation of the disease. The microbial complexes considered as periodontal pathogens like Aggregatibacter Actinomycetemcomitans, Porphyromonas gingivalis and Prevotella Intermedia etc. They are the portion of the climax community in the biofilms at sites expressing progressing periodontitis and are a part of the 'red complex'¹.

Chlorhexidine, an antimicrobial agent, is a cationic bisbiguanide with broad antibacterial activity, a low mammalian toxicity and a strong affinity for binding to skin and mucous membranes. It has a wide spectrum of activity that encompasses the gram-positive and gram-negative bacteria, yeasts, dermatophytes and few lipophilic viruses. It has a membrane-active type of antimicrobial activity and damages the inner cytoplasmic membrane. Chlorhexidine has been used by the dental profession for more than two decades as it is recognized as the primary agent for chemical plaque control².

Citrus sinensis is the botanical name for orange, a tasty, juicy fruit that belongs to the Rutaceae family. Orange trees are widely cultivated in tropical and subtropical climates which are most commonly grown and valued fruit crop, with an estimated global output of 120 million tons.

Citrus sinensis peel has many medicinal properties and is widely used against various ailments, such as colic, upset stomach, cancer, diuretic, carminative, 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 literature³. This in-vitro study was planned to evaluate antibacterial efficacy of various dilutions of citrus sinensis extract and to compare that with 0.2 % chlorhexidine on periodontal pathogens such as P. Gingivalis, Aggregatibacter Actinomycetemcomitans and Prevotella Intermedia.

II. Material And Methods

An in vitro study was carried out after approval from Institutional Ethics Committee, to evaluate MIC, MBC and ZOI of citrus sinensis extract on P. Gingivalis, A. Actinomycetemcomitans and P. Intermedia and to compare the results with chlorhexidine.

There were 3 groups of organisms tested on 10 different concentrations of the citrus sinensis extract for Minimum Inhibitory Concentration (MIC). The concentrations that resulted positive of MIC were then checked for Minimum concentration, Bactericidal Concentration (MBC). And, the positive concentrations in MBC were checked for the Zone of Inhibition (ZOI).

Procedure methodology

MINIMUM INHIBITORY CONCENTRATION (MIC) PROCEDURE:

Stock solution of the antimicrobial agent prepared by adding 100 µg of orange peel extract to 1 ml of thioglycolate (TG) broth medium (µg/1ml). For MIC, 9 dilutions of the drug prepared with 380 µl of TG broth medium using microdilution method (Schwalbe et al.). 20 µl of drug from the stock solution added into the initial tube. For dilutions, 200 µl of TG broth was added into the next nine tubes separately. 200 µl was transferred to the first tube containing 200 µl of TG broth. (considered as 10-1 dilution.) From 10-1 diluted tube, 200 ml was transferred to the second tube to make 10-2 dilution. The serial dilution was repeated up to 10-9 dilution for each drug. From the maintained stock cultures of required organisms (P.G, A.A and P.I), 10 µl was added to 2 ml of TG broth. In each serially diluted tube, 200 µl of above culture suspension was added and tubes were sealed air tightly and incubated for >48 h in an anaerobic jar/chamber and observed for turbidity. The minimum concentration of the drug in the tube which does not show any turbidity is considered as the MIC of the drug.

MINIMUM BACTERICIDAL CONCENTRATION (MBC) PROCEDURE:

A pure culture of a specified microorganism grown overnight, then diluted in growth-supporting broth (typically Mueller Hinton Broth) to a concentration between 1×10^5 and 1×10^6 cfu/ml. A stock dilution of the antimicrobial test substance is made at approximately 100 times the expected MIC (if known). Further 1:1 dilutions are made in test tubes. All dilutions of the test product(s) are inoculated with equal volumes of the specified microorganism. A positive and negative control tube or well is included for every test microorganism to demonstrate adequate microbial growth over the course of the incubation period and media sterility, respectively. The tubes were incubated at the appropriate temperature and duration. Turbidity indicates growth of the microorganism and the MIC is the lowest concentration where no growth is visually observed. The MBC is the lowest concentration that demonstrates a pre-determined reduction (such as 99.9%) in CFU/ml when compared to the MIC dilution.

ZONE OF INHIBITION (ZOI) PROCEDURE:

Nutrient agar plates were inoculated by rubbing sterile cotton swabs dipped into bacterial suspensions of P. Gingivalis, A. Actinomycetemcomitans and P. Intermedia (overnight cultures grown at 37°C on nutrient agar) over the entire surface of the plate. Six such sets were prepared. After inoculation, 10 mm diameter five wells were cut into the surface of the each agar plate using sterile cork borer. The positive concentrations of orange peel extract from minimum bactericidal concentration were added into wells in different plates containing above mentioned 2 different bacteria's and one for one set of bacteria distilled water. Plates will be incubated at 37°C for 24 hours. The diameter of zones of inhibition will be measured using a Digital Vernier Caliper of all the plates. The mean score of zones of inhibition was calculated and statistical analysed. All these concentrations of orange peel extract were prepared and compared with chlorhexidine mouthwash.

III. Result

In MIC, P. Gingivalis showed sensitivity at 100 µg/ml and 50 µg/ml, whereas A. Actinomycetemcomitans and P. Intermedia showed sensitivity at 100 µg/ml for citrus sinensis extract (Table 2).

For Chlorhexidine, P. Gingivalis showed sensitivity from 0.2 to 100 µg/ml, whereas A. Actinomycetemcomitans and P. Intermedia showed sensitivity from 12.5 to 100 µg/ml (Table 2).

In MBC, P. Gingivalis showed bacterial activity at 0.2, 0.4 and 0.8 ug/ml for Chlorhexidine(Image 2)
 For P.Gingivalis ZOI for CS showed 20mm at 100% and 15mm at 50%(Table 1)(Image 3) .The measurements for ZOI for CHX showed 28mm at 100%, 25mm at 50%, 23mm at 25%, 20mm at 12.5%, 18mm at 6.25%, 15mm at 3.12 and 13mm at 1.6%(Table 1)(Image 4).

For A. Actinomycetemcomitans, The measurements for ZOI for citrus sinensis showed 12mm at 100%(Table 1)(Image 3). The measurements for ZOI for CHX showed 21mm at 100%, 18mm at 50%, 13mm at 25% and 12mm at 12.5%(Table 1)(Image 4).

For P. Intermedia the measurements for ZOI for CS showed 11 mm at 100%(Table 1)(Image 3) .The measurements for zone of inhibition for CHX showed 20 mm at 100%, 17mm at 50%, 13mm at 25% and 12mm at 12.5%(Table 1)(Image 4)

Table no 1 : Shows Zone Of Inhibition of different bacteria

		100 ug/ml	50 ug/ml	25 ug/ml	12.5 ug/ml	6.25 ug/ml	3.12 ug/ml	1.6 ug/ml
Citrus sinensis extract	P.G	20mm	15 mm	-	-	-	-	-
	A.A	12 mm	-	-	-	-	-	-
	P. I	11 mm	-	-	-	-	-	-
Chlorhexidine	P.G	28 mm	25 mm	23 mm	20 mm	18 mm	15 mm	13 mm
	A.A	21 mm	18 mm	13 mm	12 mm	-	-	-
	P. I	20 mm	17 mm	13 mm	12 mm	-	-	-

P.G: P. Gingivalis; A.A: A. Actinomycetemcomitans; P.I: P. Intermedia

Table No 2: Shows graphical comparison of Zone Of Inhibition of different bacteria

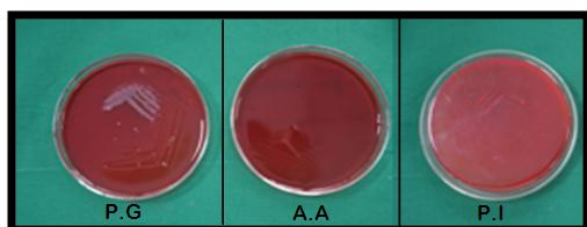
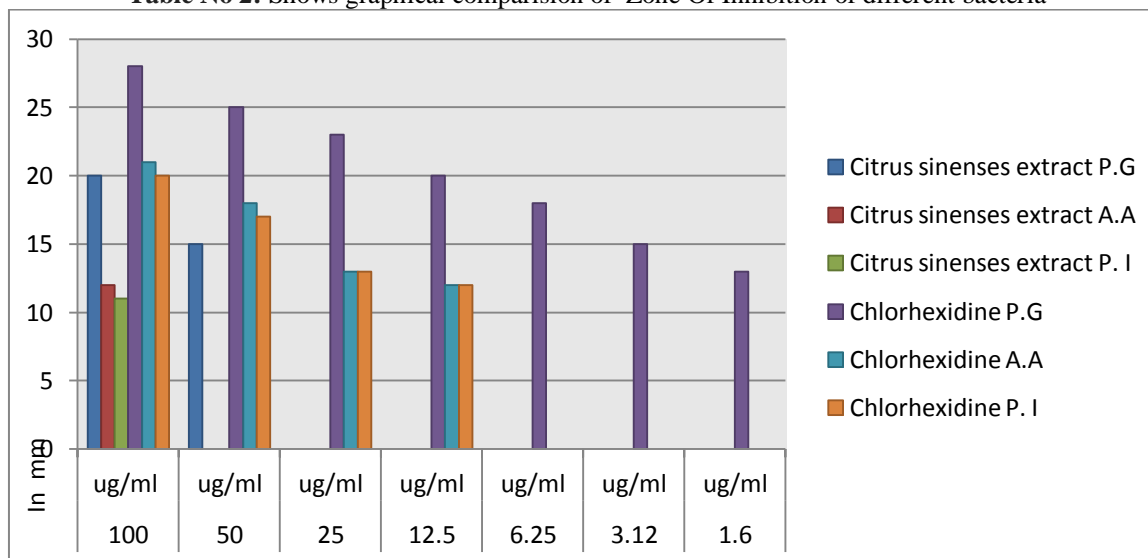


Image 1: MBC-CITRUS SINENSIS

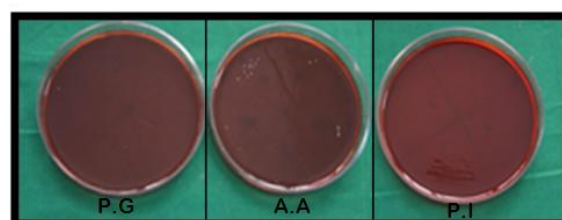


Image 2: MBC-CHLORHEXIDINE

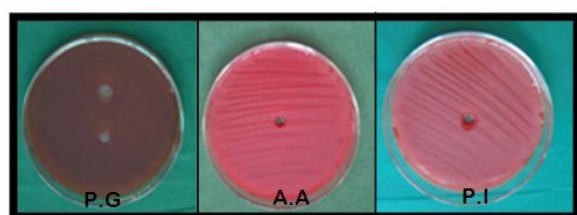


Image 3: ZOI-CITRUS SINENSIS

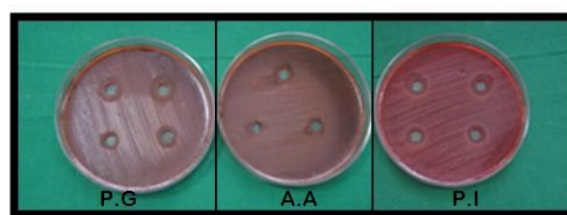


Image 4: ZOI-CHLORHEXIDINE

IV. Discussion

Anti-plaque agents which are based on the use of broad-spectrum antimicrobial agents like chlorhexidine, quaternary ammonium compounds and antibiotics have shown development of resistance to antimicrobials, considerable side effects and also the emergence of uncommon infections due to their improper use. Sufficient scientific evidence is available suggesting that the nature of orange fruit influences its antimicrobial efficacy⁴.

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. *Prevotella Intermedia* and *Porphyromonas gingivalis* showed antibacterial activity with citrus sinensis extracts. Moreover, minimum inhibitory concentrations of the extracts were lesser. Same observations have been reported earlier^{5,6}. Nisha et al.⁷ and Nair et al.⁸ 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 solvent⁷.

The antimicrobial potency of plants is believed to be due to tannins, saponins, phenolic compounds, essential oils and flavonoids⁹. 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 protein¹⁰ resulting in the inhibition of cell protein synthesis. Parekh and Chanda¹¹ 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. Dubey et al. showed potent antibacterial activity (against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Shigella flexneri*, *Bacillus subtilis* and *Escherichia coli*) of extract from fruit of Orange peels using disk diffusion method¹². Chabuck et al. observed orange peel extract to be effective against *Klebsiella pneumonia*¹³.

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 lines¹⁴. Just et al.¹⁵ 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 properties¹⁶. Terpenoids observed in ethanolic extracts is speculated to be involved in membrane disruption by the lipophilic compounds¹⁷.

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. Chlorhexidine, which is an antibacterial agent, acts against the Gram-ve and +ve microbes. It has been the gold standard for plaque control. However, it demonstrates a few side effects such as discolorations of fillings, teeth and tongue, taste interference, mucosal lesions, aphthous stomatitis, burning sensation and desquamation of soft tissue etc. These problems merit further consideration¹⁸. The current study has used a pure citrus sinensis extract in comparison to the gold standard Chlorhexidine. All the varied concentrations, right from 0.2% to the purest form i.e. 100% were tested for MIC, MBC and ZOI against the periodontal pathogens. The results show that the purest form of the extract i.e. 100% has shown to be effective against both the periodontal pathogens *P. Gingivalis*, *A. Actinomycetemcomitans* and *P. Intermedia* whereas chlorhexidine still being the gold standard.

As the current study is in-vitro, clinical application cannot be done. Further studies need to be carried out in-vivo for the clinical application of results.

V. Conclusion

Based on the present study we can conclude that citrus sinensis extract possesses antimicrobial activity against periodontal pathogens *P. Gingivalis*, *A. Actinomycetemcomitans* and *P. Intermedia* in its purest form. Clinical efficacy should be studied especially against the periodontal disease initiation and progression.

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