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The effectiveness antimicrobial activity test of citrus peel extract on some periodontal pathogenic bacteria (*In vitro*)

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Abstract
Introduction: Anti-infective periodontal treatment includes mechanical pocket debridement (scaling and root planing) to remove dental calculus and administration of antimicrobial agents. Antimicrobial agents may have side effect such as microbials resistances to antimicrobials. The use of herbal therapy has been considered as alternative of antimicrobial therapy. Citrus (Citrus aurantifolia (chrism.) Swingle) has been shown to have antimicrobial, anti-oxidants and anti-inflammatory effect.
Objective: The aim of this study was to know and to analyze the effectiveness of citrus peel extract to inhibiti the growth of some periodontal pathogens bacteria *in vitro*.
Methods: Citrus peel was extracted by percolation method using ethanol, n-hexane and ethyl acetate solvent to produce a viscous extract citrus peel. The extract was diluted to a concentration of 25%, 12%, 6.25% and 3.125%. Each concentration was tested for its antimicrobial avtivity effectiveness against *A. actinomycetemcomintans, P. gingivalis, F. nucleatum.* The effectiveness of the extract was shown as inhibition zone on BHI broth media.
Results: Citrus peel extract of ethanol at a concentration of 25% has the strongest antibacterial effectiveness against the three bacteria compared to solvents, with a diameter of inhibition zone against *A. actinomycetemcomintans* 16.05 mm, *P. gingivalis* and *F. nucleatum* 12.75 mm and 11.67 mm. There are statistically significant differences among extract concentration of 25%, 12%, 6,25% and 3,125% and citrus peel extract ($p<0.05$).
Conclusion: citrus peel extract has antibacterial effect in inhibiting bacterial growth *A. actinomycetemcomintans, P. gingivalis, F. nucleatum*.

Keywords: Citrus peel extract, anti bacteri, inhibition zone

Introduction
The etiology of the disease is multifactorial and bacterial deposits play an essential role in the pathogenesis. The bacteria that are involved in periodontitis accumulate in the sub-gingival plaque that comprises predominantly of Gram-negative strict anaerobic rods. Among them *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Bacteroides* spp, *Selenomonas* spp. have been associated with chronic or refractory periodontitis [1].
Periodontitis treatment focuses on the effectiveness in killing or reducing the number of bacteria that contaminate the tooth surface and surrounding tissue [2, 3] In most patients, mechanical debridement and anti-infective chemotherapy can readily control the disease.[4] Scaling and root planing may remove subgingival bacteria. Mechanical debridement may fail to remove pathogenic organisms because of their location in subepithelial gingival tissue.[2] Periodontal antibiotic therapy as the evidence for bacterial specificity in periodontitis has accumulated and strengthened [3-5] Systemic antibiotic therapy can also potentially suppress periodontal pathogens. However, inappropriate antibiotic therapy may adversely affect human microbial ecology and give rise to antibiotic resistance [2, 4, 5].
Due to emerging antibiotic resistant infections, considerable attention has been paid to utilize eco-friendly and bio-friendly plant based products for prevention and cure of different human diseases since they are considered as safe and effective. Studies have attempted to shed light on the antibacterial activity of some indigenous medicinal plants. Considering the high costs of the synthetic drugs and their various side effects, the search for alternative products from plants used in traditional system of medicine is justified. In order to promote herbal drugs

there has to be an evaluation of therapeutic potentials of drugs.[5] Resistance is addressed by the use of plant extracts that have antimicrobial properties that can aid the success of periodontitis treatment [3, 5] Medicinal plants are widely used as alternative treatments for health problems in developing countries. Several plants have been studied through actimicrobial activity against some pathogenic bacteria in the oral cavity [3, 5, 6]

Natural ingredients that can be utilized for the treatment of periodontitis are citrus (*Citrus aurantium*) because it has antimicrobial properties. Research on the benefits of citrus for health has been widely practiced [7-9] Research on the ability of citrus peel extract in inhibitioning the growth of Gram positive aerobic bacteria commonly found in the plaques have been many, but there is no clear evidence whether citrus peel extract is effective in inhibitioning the growth of *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* [7, 8] Dhanavade *et al*. who conducted research on orange peel extract showed strong antimicrobial activity when ethanol used as solvent.The antibacterial power of citrus peel has been studied and found to be effective against Gram-positive and Gram-negative bacteria [9]

Methode

The type of this study is laboratory experimental study. This study was conducted at oral biology laboratory, Faculty of Dentistry, Airlangga University Surabaya. Samples of this study were pure culture of *A. actinomycetemcomitans* (ATCC 6154), *P. gingivalis* (ATCC 33 277) and *F. nucleatum* (ATCC 25 586) were cultured with Brain Heart Infusion Broth (BHIB). The number of repetition in this study were 4 times.

The citrus Peel was dried in the drying cupboard for 10 days and were blended into simplicia powder, and inserted into a closed bottle, to be soaked with n-hexane solvent for 3 hours. The mixtrure is inserted into the percolator, and the solvent of n-hexane is poured sufficiently over the simplicia powder. The mixtrure in percolator was left for 24 hours and opened after 24 hours, the liquid was collected into a clear colored bottle. Percolation was stopped when liquid was evaporated without any residue. Liquid is concentrated by a rotary evaporator at temperature of < 400 ^{0}C until a viscous extract is obtained which has been dried with dryer. The simplicia powder is then dried again in the drying cupboard for 24 hours, then immersed with ethyl acetate solvent for 3 hours.The same process repeated with ethanol and n-hexane solvent. The extraction results are poured into the bottle.

Instruments and medium were sterilized in the autoclave at 121 °C. for 15 minutes. The extract of citrus peel weighed using electronic balance. The mass was adjusted to the desired concentration by dissolving it with the Brain Heart Infusion Broth (BHIB) medium. Eight container was prepared, was filled with 10 ml extract of citrus peel to obtain extract of citrus peel with 100% concentration. Five other container are then filled with 5 ml of BHIB. 5 ml of 100% citrus extract added to 0.02 ml of DMSO (0.2% total volume) and transfered to second tube with 50% citrus peel extract (double dilution), until the concentration reaches 25%, 12.5%, 6.25% and 3.125%. The tubes are then labeled according to their concentration. Thirtyseven gram of Brain Heart Infusion Broth (BHIB) medium was prepared, and dissolved into 1000 ml of aquadest for 40 petri dish (20 ml / petri), then heated on a magnetic heating oven to boil. Then the media was sterilized in the autoclave for 15 minutes with 2 ATM and 121 °C. Once sterilized, the media is stored in a refrigerator. If it will be used again, the medium is reheated to boil and poured into petri dish to cool down.

Specimen breeding activities are held out in an anaerobic atmosphere with CO_2 incubators. *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* stem-cell specimen bacterium has been cultured purely on Brain Heart Infusion (BHI) media which has been prepared in previous procedures in an anaerobic incubator. A total of 1-2 ose from pure cultured test bacteria which have been cultured and fertilized with suspended 0.9% NaCl solution to obtain a standardized 0.6 Mc Farland or equivalent to a bacterial count of 1 x 106 CFU / ml Brain Heart Infusion. The sterilized broth is then keep inside refrigerator with removed and left indoor. Ose is heated with bunsen for sterlisation.

A. actinomycetemcomitans, *P. gingivalis* and *F. nucleatum* bacteria are taken from the Brain Heart Infusion Broth and at the time of opening should be close to bunsen for the bacteria *A. actinomycetemcomitan*, *P. gingivalis* and *F. nucleatum* to be not contaminated.

BHI Broth medium which has contained bacteria were placed on disc paper, then paper discs was dripped with 10 µl of peel extract Citrus liquid for each concentration.

Set for 15-20 minutes until it dries and incubated in a CO_2 incubator with a temperature of 37 ^{0}C for 18-24 hours. The diameter the inhibition zone was measured by observing the clear zone present around the disc paper with g a caliper. This experiment was repeated four times. Data have been analyzed using the statistical tests of Anova and Mann-Whitney.

Result

The effectiveness of citrus peel extract on *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* by using three solvents ie ethanol, n-hexane and ethyl acetate with concentrations of 25%, 12.5%, 6.25% and 3.125% can be known by measured the diameter of the tested bacterial inhibition zone. Inhibition zone is a clear zone in the area around a circular test material showing no bacterial growth. Inhibition zone increases when the diameter of the clearer area in this circle is wider. The inhibition zone diameter of citrus peel extract with different solvents and concentrations can be seen in Figures 1, 2 and 3.

Fig 1: Inhibition zone diameter test the against *A. actinomycetemcomitans*

Fig 2: Inhibition zone diameter test the against *P. gingivalis*

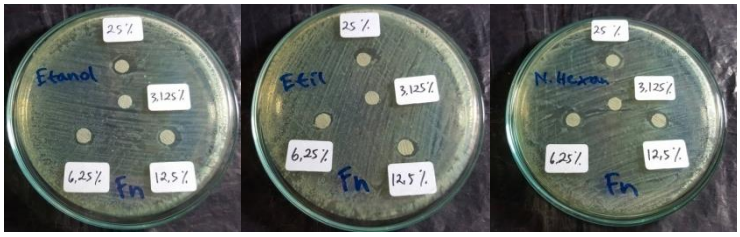

Fig 3: Inhibition zone diameter test the against *F. nucleatum*

Figures 1, 2 and 3 show that citrus peel extracts of various concentrations and various solvents form a clear zone around the wells so it appears that they are effective in inhibited the growth of *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* bacteria.

Table 1: Comparison of inhibitory zone diameter of A. actinomycetemcomitans, P. gingivalis and F. nucleatum bacteria from each citrus peel extract concentration by ethanol solvent

citrus peel extract	bakteria	Mean	Median ± Interquartile range	p value		
				25%	12,5%	6,25%
Concentration 25%	Aa	16,05	16.05±0,25		0,02*	0,02*
	Pg	13,07	13,05±0,17		0,02*	0,02*
	Fn	12,22	12,25±0,18		0,02*	0,02*
Concentration 12,5%	Aa	13,07	13,05±0,32			0,02*
	Pg	11,15	11,15±0,25			0,02*
	Fn	11,07	11,05±0,17			0,02*
Concentration 6,25%	Aa	11,35	11,35±0,25			
	Pg	9,62	9,65±0,33			
	Fn	9,40	9,40±0,35			
Concentration 3,25%	Aa	0	0	0,01*	0,01*	0,01*
	Pg	0	0	0,01*	0,01*	0,01*
	Fn	0	0	0,01*	0,01*	0,01*

Mann-Whitney Test
(*) Significant $p<0.05$

Table 1 shows the largest inhibitory zone diameter against *A. actinomycetemcomitans* bacteria present in citrus peel extract with ethanol solvent at 25% concentration, the smallest being at 6.25% concentration. The largest inhibitory zone diameter against *P. gingivalis* bacteria at 25% concentration while the smallest at concentration of 6.25%. The largest inhibitory zone diameter against bacteria *F. nucleatum* at 25% concentration while the smallest is at concentration 6.25%. Inhibitory zones were not seen at of concentration 3.125%. There was a significant difference of clear zone between each concentration ($p<0.05$).

Table 2: Comparison of diameter of the inhibitory zone of each concentration of citrus peel extract with ethyl acetate solvent to *A. actinomycetemcom*itans, *P.gingivalis* and *F.nucleatum* bacteria

Citrus peel extract	bakteria	Mean	Median ± Interquartile range	p value		
				25%	12, 5%	6, 25%
Consentration 25%	Aa	11,67	11,65±0,32		0,02*	0,02*
	Pg	9,62	9,60±0,42		0,02*	0,02*
	Fn	9,55	9,55±0,25		0,02*	0,02*
Consentration 12,5%	Aa	10,65	10,65±0,25			0,02*
	Pg	9,12	9,15±0,17			0,02*
	Fn	8,75	8,75±0,25			0,02*
Consentration 6,25%	Aa	9,72	9,75±0,32			
	Pg	8,10	8,10±0,22			
	Fn	8,15	8,15±0,10			
Concentration 3,25%	Aa	0	0	0,01*	0,01*	0,01*
	Pg	0	0	0,01*	0,01*	0,01*
	Fn	0	0	0,01*	0,01*	0,01*

Mann-Whitney Test
(*) Significant $p<0.05$

Table 2 shows the largest inhibitory zone diameter of *A. actinomycetemcomitans* bacteria present in citrus peel extract with ethyl acetate solvent at 25% concentration while the smallest is at 6.25% concentration. The largest inhibitory zone diameter against *P. gingivalis* bacteria was in citrus peel extract with ethyl acetate solvent at 25% concentration while the smallest at concentration of 6.25%. The largest inhibitory zone diameter against *F. nucleatum* bacteria was in citrus peel extract with ethyl acetate solvent. at 25% concentration while the smallest was at concentration at 6.25%. Inhibitory zones were not seen at of concentration 3.125%. There was a significant difference of inhibition zone between each concentration (p<0, 05).

Table 3: Comparison of diameter of the inhibitory zone of each concentration citrus peel extract with n-hexane solvent against *A. actinomycetemcomitans*, *P.gingivalis* and *F.nucleatum* bacteria

Citrus peel extract	bakteria	Mean	Median ± Interquartile range	p value		
				25%	12,5%	6,25%
Consentration 25%	Aa	11,67	11,65±0,32		0,02*	0,02*
	Pg	9,62	9,60±0,42		0,02*	0,02*
	Fn	9,55	9,55±0,25		0,02*	0,02*
Consentration 12,5%	Aa	10,65	10,65±0,25			0,02*
	Pg	9,12	9,15±0,17			0,02*
	Fn	8,75	8,75±0,25			0,02*
Consentration 6,25%	Aa	9,72	9,75±0,32			
	Pg	8,10	8,10±0,22			
	Fn	8,15	8,15±0,10			
Concentration 3,25%	Aa	0	0	0,01*	0,01*	0,01*
	Pg	0	0	0,01*	0,01*	0,01*
	Fn	0	0	0,01*	0,01*	0,01*

Mann-Whitney Test
(*) Significant p<0.05

Table 3 shows the largest inhibitory zone diameter against *A. actinomycetemcomitans* bacteria was present Extract of citrus peel with n-hexane solvent at 25% concentration while the smallest is at concentration at 6.25%. The largest inhibitory zone diameter against *P. gingivalis* bacteria was in citrus peel extract with n-hexane solvent at 25% concentration while the smallest was at 6.25% concentration. The largest inhibitory zone diameter against *F. nucleatum* bacteria was in citrus peel extract with n-hexane solvent at 25% concentration while the smallest was at 6.25% concentration. Inhibitory zones were not seen at of concentration 3.125%. There was a significant difference of clear zone between each concentration (p<0.05).

Discussion

Taiwo *et al*. (2007) who is investigate the antibacterial effects of two plant extracts, *Citrus aurantifolia* linn (citrus) and *Tithonia diversifolia* poaceae (sunflower) using disk diffusion susceptibility testing on 53 fresh human bacterial pathogens isolated showed the diameter of inhibitions zone of *Citrus aurantifolia* linn extracts are 10, 12, 11, 17 and 16 mm for *Staphylococcus* sp., *E. coli, Klebsiella* sp., *Proteus* sp. and *Pseudomonas* sp., respectively. Fourty four percent of the Gram positive and 69% (18 of 26) of the Gram negative pathogens showed the diameter of of inhibition zone = 10 mm to *Citrus aurantifolia* linn [9]. Test results inhibitory zone of citrus peel extract *against bacteria A. actinomycetemcomitans, P. gingivalis* and *F. nucleatum* showed a clear zone on the media BHI Broth circular around the wells [10, 11]. Clear zone indicates the antibacterial effectiveness of each medicinal nconcentration. Mean of inhibition zone diameter each concentration about 10-19 mm. The results showed citrus peel extract has a strong inhibitory zone antibacterial effectiveness *in vitro* against bacteria *A. actinomycetemcomitans, P. gingivalis* and *F.nucleatum* which is a periodontal bacterial pathogen. Citrus peel extract with ethanol, n-hexane, and ethyl acetate may inhibition the growth of bacteria *A. actinomycetemcomitans, P. gingivalis* and *F. nucleatum*. Ethanol solvent is the most effective compared to the n-hexane and ethyl acetate solvents [12]. Antibacterial effectiveness obtained from an ethanol extract of the strongest antibacterial effectiveness in inhibiting the growth of bacteria *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*. This is because ethanol with a lower polarity can dissolve the compound alkaloids, diglikosida, phenolic, flavonoid, tannin and a little essential oil [12, 13]. The inhibition zone Meansuring less than 5 mm were formed on the agar diffusion test showed weak inhibition categorized whereas inhibition zone Meansuring 5-10 mm categorized as moderate, strong categorized 10-19 mm and 20 mm or more are categorized very strong [10-12] This research shown, citrus extract with concentration of 12.5% and 25% with ethanol solvent and 25% concentration with n-hexane solvent having strong inhibition power.

Conclusions

This study concluded that citrus peel extract as effective in inhibiting the growth of some bacteria *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum in vitro*. Citrus peel extract with ethanol solvent has the strongest antibacterial effectiveness against all three periodontal pathogenic bacteria compared with n-hexane and ethyl acetate solvents, this is characterized by the largest inhibition diameter zone of 16.05 mm. Minimum Inhibitory Concentration (MIC) of citrus peel extract with ethanol,n-hexane and ethyl acetate solvent to bacteria *A. actinomycetemcomitans, P. gingivalis* and *F. nucleatum* were at concentrations of 6.25%.

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