Formulation and Evaluation of New Periodontal Gel from Pimpinella Anisum L Anise Seed

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Abstract

To determine the antimicrobial effect of anise seed extract on oral microorganisms and to formulate a new periodontal gel from anise seed extract.

Anise seed (Pimpinella anisum) were purchased from the local market. Anise seed were grounded into powder and was added into 70% ethanol which stored in the dark for 48 hours. The extract was filtered and then evaporated by using rotary evaporator. Antimicrobial properties of the anise seed extract were determined using minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) method. The bacteria used were Staphylococcus aureus, Escherichia coli and Aggregatibacter actinomycetemcomitans. Aloevera was added as a base to anise seed extract to form gel.

The anise seed extract was tested against S. aureus, E. coli and A. actinomycetemcomitan. For antimicrobial susceptibility testing (AST), there was zone of inhibition surrounding the disc containing 0.12% chlorhexidine and extracted anise seed which shows potential antimicrobial activity. MIC values of extracted anise seed tested against S. aureus, E. coli and A. actinomycetemcomitan were 3.125 mg/ml, 1.563 mg/ml and 25 mg/ml , respectively. MIC values of chlorhexidine tested against S. aureus, E. coli and A. actinomycetemcomitan were 12.5 mg/ml, 6.25 mg/ml and 6.25 mg/ml, respectively. While for MBC, values of extracted anise seed tested against S. Aureus andE. coli were 6.25 mg/ml and 3.125 mg/ml, respectively. MBC values of chlorhexidine tested against S. Aureus and E. coliwere 25 mg/ml and 12.5 mg/ml, respectively.Conclusion: Anise seed extract were found to have potential antimicrobial effect against S. aureus, E. coli and A. actinomycetemcomitan.

Experimental article (J Int Dent Med Res 2020; 13(3): 928-934)

Keywords: Anise seed, antimicrobial, extract, periodontal gel.

Received date: 23 February 2020 **Accept date:** 06 April 2020

Introduction

Periodontitis, a chronic inflammatory disease, begins with a microbial infection, followed by a host-mediated destruction of soft tissue that causes clinically significant connective tissue and bone destruction.[1] Periodontal pocket formation in the gingiva provides an ideal environment for the growth and proliferation of anaerobic pathogenic bacteria. The oral cavity is colonized by more than 700 species of aerobic and anaerobic microorganisms.

Aggregatibacter actinomycetemcomitans (*A. actinomycetemcomitans*), *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are among the microorganisms implicated in the pathogenesis of periodontitis.[2,3]

A. actinomycetemcomitans is frequently associated with localized aggressive periodontitis.[4,5,6] *A. actinomycetemcomitans* is a non-motile, gram-negative, capnophilic, fermentative coccobacillus which closely resembles several *Haemophilusspecies* but which does not require X or V growth factors.[7] *S. aureus* is a non-motile, gram-positive, cluster-

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forming coccus, non-spore forming facultative anaerobe. *E. coli* is a gram-negative, rod-shaped, coliform facultative anaerobic microorganism. These microorganisms are opportunistic microorganism where patients with periodontal disease represent possible reservoirs for it in the oral cavity. They can also be an infection source to other individuals. [8, 9, 10]

Antibiotics provide the main basis for the therapy of bacterial infections. However, overuse of antibiotics has become the major factor of multi-drug resistant strains of several groups of microorganisms. [11] This has resulted in severe consequences including increased cost of medicines and mortality of patients. Scientists have searched for alternatives to antibiotics. In this view, aromatic plants are becoming more popular due to their antimicrobial effects and stimulating effects on animal digestive systems. [12,13,14] The World Health Organization (WHO) estimated that 80% of the populations rely on tradition medicine for their primary health care needs and most of this therapy involves the use of plants extracts or their active components.[14,15,16] The antimicrobial efficacy attributed to some plants in treating diseases has been beyond belief. It is estimated that local communities have used about 10% of all flowering plants on Earth to treat various infections, although only 1% have gained recognition by modern scientists. [21] Owing to their popular use as remedies for many infectious diseases, searches for plants containing antimicrobial substances are frequent. [22,23,24,25,26,27,28]

Nowadays, there are many periodontal gels readily available in the market. However, certain periodontal gel contains chemical agents that can cause unwanted side effects. This study was conducted to explore the antimicrobial effect from one of the natural herbs, which is anise (*Pimpinella anisum L*.) instead of antibiotics. As an aromatic plant, anise is an annual herb and a grassy plant with white flowers and small green to yellow seeds, which grows in Turkey, Iran, India, Egypt, and Asia. [29] Anise oil has anothole (85%) as an active ingredient and it has contained eugenol, methylchavicol, anisaklehyde, and estragole. As a medicinal plant, anise has been used as a stimulating effect of digestion and antiparasitic, antibacterial, antifungal and antipyretic.[30, 31, 32] It was also reported that anise had several therapeutic effects. [33] Anise is primarily grown for its fruits, commercially called “seeds” that are currently used for flavouring. The essential oil from its fruits is also valuable in perfumery and in medicine. [34,35]

Another one of the ancient medicinal plant is aloe vera. Aloe vera is a succulent plant with thick, fleshy, serrated, lanceolate-shaped leaves of green-greyish color. Aloe vera inner gel is obtained from the lower leaves of the plant by slicing the leaf open. The gel is clear, odorless, and tasteless and should be free of leaf skin or yellow parts.[36] As for the mechanism of action, it causes stimulation of macrophage and fibroblast activity, increased collagen and proteoglycan synthesis as well as anti-inflammatory effect. [28] The antimicrobial effect of mouthwash containing aloe vera has been demonstrated in an in vitro study, in which this phytotherapic agent inhibited the growth of diverse oral microorganisms, such as *S. mutans, S. sanguis, A. viscosus* and *C. albicans.*[37] Aloe vera contains six antimicrobial agents such as lupeol, salicylic acid, urea, nitrogen, cinnamonic acid, phenols and sulfur which have inhibitory action on fungi, bacteria and viruses.[38]

Materials and methods

Extraction of *Pimpinella anisum L* anise seed

Anise seed purchased from local market had been used in this research.100 grams of anise seed was measured using weighing balance and grinded using grinder. The powder was then added into 70% of 2000 ml ethanol in a ratio of 1:20 and stored in the dark for 48 hours. Then, the solution was filtered through Whatman No.1 filter paper and evaporated using Eyela rotary evaporator to remove the ethanol. After filtration, the anise seed extract was kept in a universal bottle. Figure 1 shows extraction of anise seed.

Assessment of antibacterial activity

Gram-positive *S. aureus* (ATCC 13709), gram-negative *E. coli* (ATCC25922) and *A. Actinomycetemcomitan* (ATCC 43718) were purchased from Aldrich, USA and used in this study to represent oral microorganisms.The microorganisms were sub-cultured on the blood agar and inoculated into the brain heart infusion (BHI) broth as stock culture, used throughout the experiment. Figure 2 shows the preparation of bacteria culture.

Preparation of McFarland standard

McFarland Standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland Standard. A fresh, pure culture of *S. aureus*, *E. coli* and *A. actinomycetemcomitan*were inoculated in the blood agar.[19] This procedure was prepared in aseptic technique, 3ml of normal saline was added into test tube by using volumetric micropippette. The bacteria was streaked out using sterilized loop and mixed it in the normal saline. The solution was stirred until the solution appears homogenous and free of clumps. The turbidity (0.5) was measured using densitometer.[39]

Revival of Strains

A loopful of each microorganisms from the test tubeswere transferred onto the BHI agar plate by using sterile loop respectively. *S. Aureus* and *E. coli*cultures were incubated in 37°C with 5% carbon dioxide incubator for 24 hours, whereas A. actinomycetemcomitan culture were incubated for more than 48 hours due to it slow growth. Figure 4 shows the growth of *S. aureus*, *E. coli* and *A. actinomycetemcomitan* on blood agar.

Antimicrobial Susceptibility Testing (AST)

Antimicrobial Susceptibility Testing (AST) was done as a screening for antimicrobial effect. Three mueller hinton (MH) agar plates were prepared and the plates were labelled as *S. aureus*, *E. coli* and *A. actinomycetemcomitan*. A loopful of culture from the plate were taken and put into a sterilized test tube containing 3ml of normalsaline. The tube was mixed throughly. Mc Farland standard which turbidity of 0.5 were prepared for each bacteria. A sterile cotton swab was took and dipped into the test tube. While taking out the swab form the tube the soaked swab was rotated firmly against the upper inside wall of the tube to get rid of excess fluid. The cotton swab containing the culture was swabed evenly onto the MH agar plate labelled as gram positive*S. aureus*. Previous steps were repeated for another MH agar plates of gram negative E. coli followed by*A. actinomycetemcomitan*. 0.12% Chlorhexidine as positive control, sterile distilled water, blank disc and 70% ethanol as negative control, extracted aniseseed were prepared. MH agar plates were divided into five portion. One disc was prepared for each compound. The disc was dipped into 20 µl chlorhexidine and placed it onto a filter paper to remove the excessive compound. Then, the disc was lifted by using tweezer and placed at chlorhexidine portion on the MH agar plate labelled as *S. Aureus,E. coli followed by A. actinomycetemcomitan.*The step was repeated for other compounds. The MH plates labelled with *S. Aureus* and *E. coli* plates were incubated in 37°C with 5% carbon dioxide incubator for 24 hours, whereas*A. actinomycetemcomitan* plate were incubated for more than 48 hours due to it slow growth.

Minimum Inhibitory Concentration (MIC) test and Minimum Bactericidal Concentration (MBC) test.

Minimum inhibitory concentration (MIC) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentration (MBC) as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media (40).Preparation of *S. aureus*, *E. coli* and *A. actinomycetemcomitan*with McFarland standard of 0.5 was done by diluted to 10^8 CFU/ml with 0.9% of normal saline.[41]

S. Aureus and *E. coli*well were incubated in 37°C with 5% carbon dioxide incubator for 24 hours, whereas A. actinomycetemcomitan well were incubated for more than 48 hours due to it slow growth. The MIC value was determined by looking at the changes of turbidity. The tubes were examined for visible growth and was recorded growth as (+) and no growth as (-).

The MBC of the sample was determined by subculturing all the content of the well onto a MH agar plate. The concentration of the sample that showed no visible growth of bacteria was the MBC.[42] Figure 5 shows the 96-empty well and preparation of MBC and MIC tested of bacterias.

Results

Antimicrobial susceptibility testing (AST)

AST was done to test the antimicrobial activity of the extracted anise. There was zone of inhibition surrounding the well containing clorhexidine and extracted anise against *S. aureus*, *E. coli* and *A. actinomycetemcomitan*.The zone of inhibiton of tested bacterias was shown in Table 1.

Compound	Zone of inhibition (mm)		
	S. aureus	*E. coli*	*A. actinomycetemcomitan*
Anise	9	6	11
Chlorhexidine	20	17	11
Distilled water	0	0	0

Table 1. Shows the zone of inhibiton for *S. aureus*, *E. coli* and *A. Actinomycetemcomitan*with different compound.

Minimum Inhibitory Concentration (MIC) test and Minimum Bactericidal Concentration (MBC) test.

Below are the results of the growth of bacteria after MIC test. The MIC value was determined by visual based on the turbidity seen on the well.For MBC test, all the mixture of each well in the MIC test were cultured into a MH agar. The growth of the bacteria after overnight incubation was examined.

Well	1	2	3	4	5	6	7	8	9
Concentration (mg/ml)	50	25	12.5	6.25	3.125	1.563	0.782	0.391	0.196

Table 2. Shows the dilution method of concentration for the solution.

Tables 3 and 4 below shows the result of the MIC test.

Well	1	2	3	4	5	6	7	8	9
Growth of S. aureus	-	-	-	-	+	+	+	+	+
Growth of E. coli	-	-	-	-	-	+	+	+	+
Growth of A. actinomycetemcomitan	-	+	+	+	+	+	+	+	+
Conc. (mg/ml)	50	25	12.5	6.25	3.125	1.563	0.782	0.391	0.196

Table 3. Extracted anise against *S. aureus,E. coliand A. actinomycetemcomitan.*

MIC values of extracted anise seed tested against *S. aureus*, *E. coli* and *A. actinomycetemcomitan* were 3.125 mg/ml, 1.563 mg/ml and 25 mg/ml , respectively. While for MBC, values of extracted anise seed tested against *S. aureus* and *E. coli* were 6.25 mg/ml and 3.125 mg/ml, respectively. However, MBC values for *A. actinomycetemcomitan*cannot be determinedue to the difficulty of culturing bacteriaon agar plates.

Well	1	2	3	4	5	6	7	8	9
Growth of *S. aureus*	-	-	+	+	+	+	+	+	+
Growth of *E. coli*	-	-	-	+	+	+	+	+	+
Growth of *A. actinomycetemcomitan*	-	-	-	+	+	+	+	+	+
Concentration (mg/ml)	50	25	12.5	6.25	3.125	1.563	0.782	0.391	0.196

Table 4. Chlorhexidine against *S. aureus, E. Coli and A. actinomycetemcomitan.*

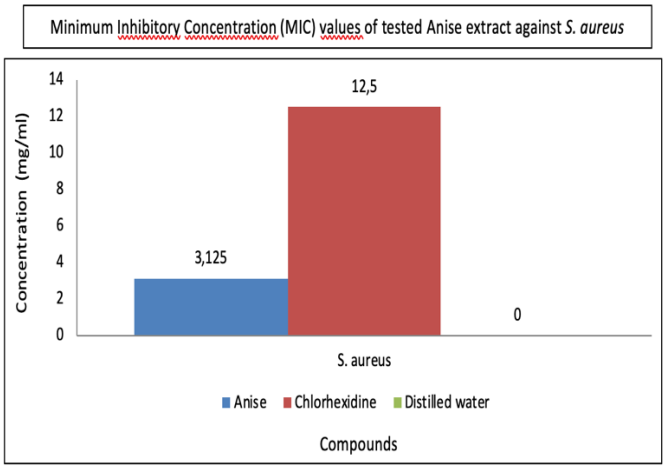

Chart 1. Shows comparison MIC value of anise extract, positive control chlorhexidine and negative control distilled water against *S. Aureus*.

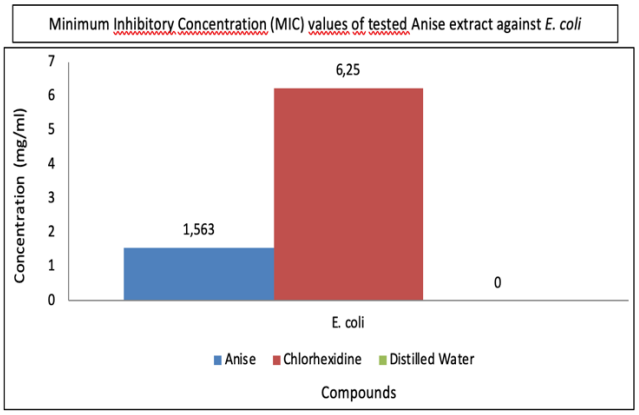

Chart 2. Shows comparison MIC value of anise extract, positive control chlorhexidine and negative control distilled water against *E.coli*.

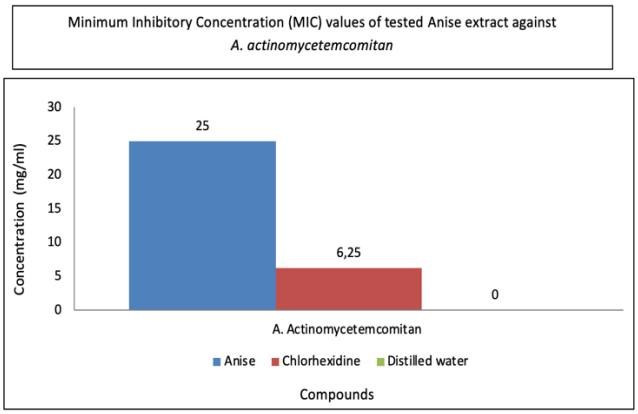

Chart 3. Shows comparison MIC value of anise extract, positive control chlorhexidine and negative control distilled water against*A. actinomycetemcomitan*.

MIC values of chlorhexidine tested against *S. aureus*, *E. coli* and *A. actinomycetemcomitan* were 12.5 mg/ml, 6.25 mg/ml and 6.25 mg/ml, respectively. MBC values of chlorhexidine tested against *S. Aureus* and *E. coli* were 25 mg/ml and 12.5 mg/ml, respectively. However, MBC values for *A. actinomycetemcomitan* cannot be determinedue to the difficulty of culturing bacteria on agar plates.

Discussion

Periodontal disease is a chronic infectious disease resulting from a response towards a complex dental biofilm containing various types of pathogenic bacteria.[43,44] Periodontal disease caused destruction of tooth-supported tissue such as gingiva, periodontal ligament, alveolar bone and cementum.[45,46,47] Failure of getting treatment for periodontal disease may lead to tooth loss.48[,49,50,51] Most of the prophylactic and therapeutic interventions are done to reduce the number of bacteria in such a way that optimum oral health can be obtained and maintained. Antimicrobial agents are useful to support this effort by effectively inhibit the formation of dental biofilm and removing established dental biofilm.[52,53,54,55]

Anise seed has been used since ancient time due to its antimicrobial, anti-inflammatory, and antioxidant properties, as a remedy for the treatment of many diseases.[20, 30] Among the reported pharmacological effects, anise seed extracts were also active as anti-ulcer, antispasmodic, antibiotic and performance enhancement (immunomodulation).[38]

Pharmacodynamically, the major routes of elimination of anise seed are via urination and exhalation.[39] To obtain all these properties from the active ingredients in the anise seed, extraction with ethanol was done. The ratio of anise seed to organic solvents can be 1:10 or 1:20. It does not affect the extraction of active compound in anise seed.[29] We used the ratio of 1:20 for this research. Ethanol 70% was used as the organic solvent to make the extracted anise seed soluble in water for dilution and more active compound of the anise seed being extracted.

The first objective of this research was to determine the antimicrobial effect of anise seed extract on oral microorganisms. There are a lot of study found that anise seed has antimicrobial properties.[18, 22, 29] Furthermore, anise seed was found also to have antifungal effects where the essential oil of anise completely inhibited the fungal tested.[39] In this research we tested the anise seed extraction with gram-positive *(S.aureus)* and gram-negative (*E. coli and A. actinomycetemcomitans)*bacteria species. Extracted anise seed showed to have a potential antibacterial activity against *S.aureus*, *E. coli and A. actinomycetemcomitans* species. However, there was weaker antibacterial activity against *A. actinomycetemcomitans* bacteria. The MIC values of extracted anise seed tested against *S. aureus*, *E. coli* and *A. actinomycetemcomitans* were 3.125 mg/ml, 1.563 mg/ml and 25 mg/ml, respectively. These results are in agreement with the study done by Oktay et al. 2003, they found that anise seed extract sample showed high antibacterial activity against gram-positive bacteria and weak activity against gram-negative bacteria.[29]

The second objective of this research was to formulate a new periodontal gel from anise seed extract. Aloe vera have been utilized in dentistry since 1982.It plays a pivotal role to combat periodontal diseases and enhances the defense mechanism.[42] The pharmacologic actions of aloe vera have been mentioned in many studies including its anti-inflammatory, anti-bacterial, anti-viral and anti-fungal properties. Due to its anti-bacterial properties it is effective in preventing halitosis, gingivitis, stomatitis and periodontitis.[43] Reports of successful changes in root sensitivity containing aloe vera toothpastes have been confirmed. Research demonstrated

that aloe vera is not toxic when applied topically aloe.[41] Aloe vera gel might enhance the ability of hydrocortisone to reduce swelling if applied topically.[41] In this research project, we use aloe vera which act as a base and carrier to mix with anise seed extract to produce anise seed extract gel. The combination of high amount of herbal remedies like aloe vera and anise seed may double up antimicrobial effect towards oral microorganisms. The use of aloe vera gel in topical applications has widely been confirmed in the clinical studies as safe.[41] Aloe vera in tooth gel is used to cleanse teeth and gingiva as effectively as toothpaste. It is a great alternative for people with sensitive teeth or gums. The aloe vera tooth gel used in the study had no added fluoride content but still exerted almost an equal amount of antimicrobial activity.[44] Due to the double antimicrobial pharmacological effect, clinical trials are recommended to evaluate the beneficial effects of anise seed extract gel in human models.

Conclusions

Anise seed extract was found to have a potential antimicrobial activity against *S. Aureus*,*E. coli* and *A. Actinomycetemcomitan* so that it could beformulated into a gel and maybe used in treating patient with periodontal disease.

Acknowledgements

We would like to extend our deepest appreciation to the laboratory staff Faculty of Dentistry and Institute for Medical Molecular Biotechnology, Universiti Teknologi Mara, Sungai buloh for guiding and providing us workplace, materials, and apparatus to be used for this research.

Declaration of Interest

The authors report no conflict of interest.

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