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ASSESSMENT OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF PIMPINELLA ANISUM SEED EXTRACTS AGAINST STREPTOCOCCUS SANGUINIS AND FUSOBACTERIUM NUCLEATUM -AN IN-VITRO STUDY

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ABSTRACT

Background: Periodontal diseases are conditions due to multifactorial etiology having various treatment approaches**.** Phytochemicals in the periodontal therapy has been evolved in the recent years with their diverse benefits. Pimpinella anisum (anise) seed is an essential seed with many medicinal properties. **AIM:** The aim of this study was to assess the antibacterial, antioxidant activity of Pimpinella anisum methanol seed extract under laboratory condition. **Methods:** In the present study, the methanolic seed extract of Pimpinella anisum was assessed for its antibacterial effects against *Streptococcus sanguinis* and *Fusobacterium nucleatum* using well diffusion technique and MIC (minimum inhibitory concentration) was determined by spectrophotometric method using the dye MTT. The antioxidant effects were assessed by 2, 2-diphenyl-1- picrylhydrazyl radical (DPPH) assay, ABTS 2,2'-azino-bis (3 ethylbenzothiazoline-6-sulfonic acid)) and Ferric reducing antioxidant power (FRAP) assay. This was done in triplicates for each concentration of the extracts. The statistical analysis was done by One-way ANOVA Test followed by Tukey's post hoc test was used to compare the mean ZOI & MIC values between different concentrations of Test extract & Tetracycline group. Kruskal Wallis Test followed by Dunn's post hoc test was used to compare the mean MBC values between different concentrations of Test extract & Tetracycline group. **Results:** The methanolic extract of Pimpinella anisum

seed showed highest mean zone of inhibition of 24.33 at 1000µg/ml, mean MIC and MBC values of 0.4320 and 0.320 respectively at 80µg/ml on *Streptococcus sanguinis* however no inhibitory effect on *F. nucleatum* and showed antioxidant activity by DPPH, ABTS and FRAP assay. **Conclusion:** Pimpinella anisum methanol seed extract had antibacterial effect and antioxidant property only on *Streptococcus sanguinis*. But further studies are required to evaluate antibacterial and antioxidant activity on various other Periodontopathogenic bacteria and under in-vivo conditions.

KEYWORDS: Pimpinella anisum, Anise seed, Minimum inhibitory concentration, S. sanguinis.

INTRODUCTION

The most common form of human periodontal disease includes gingivitis and periodontitis. The etiology of periodontal disease is complex and multifactorial, the fundamental factors being the bacterial species and the immune system.[1] Bacterial species includes gram positive bacteria like *S. sanguinis, S. mitis, S. intermedius, S. oralis, A. viscosus, A. naeslundii, P. micros and gram-negative bacteria like P. gingivalis, Aggregatibacter actinomycetemcomitans, F. nucleatum, P. intermedia, V. parvula,* as well as *Haemophilus, Capnocytophaga,* and *Campylobacter* species.[2] *Streptococcus sanguinis (S. sanguinis)* constitutes the pioneer bacterium on the tooth surface which plays an important role in plaque maturation due to its ability to aggregate with other bacteria resulting in periodontal disease.[3]

Another bacterium implicated in periodontal disease is *F. nucleatum*. It is an anaerobic bacterium belonging to the family Bacteroidaceae and phylum Fusobacteria. It can induce apoptotic cell death and trigger the release of cytokines, elastase and oxygen radicals from leukocytes.[4] As it has been reported to coaggregate with many oral microorganisms, such as *S. sanguinis, S. mutans* and *P. gingivalis.* They are believed to be important bridging organisms between the primary and secondary colonizers during colonization.[5]

The virulence factors produced by the microorganisms will provoke host immune inflammatory response. The tissue destruction caused by the host immune inflammatory response is more when compared to those produced by the bacteria. As a part of the immune responses host cells release pro-inflammatory cytokines (e.g., interleukin-1α, interleukin-1β and tumor necrosis factor-α).[6] These cytokines recruit polymorphonuclear leukocytes

(PMN'S) to the site of infection. Upon stimulation by bacterial antigens (e.g., lipopolysaccharide), PMN'S produce proteolytic enzymes such as elastase and Reactive oxygen species (ROS). ROS (superoxide, hydrogen peroxide and hydroxyl anions) play a central role in generating chronic inflammation and oxidizes DNA, lipids and proteins that contribute to tissue damage in response to periodontal pathogens.[7-8]

The goal of the periodontal treatment is to reduce the inflammation of the tissues, reduce the bacterial load and alter the host response. But the conventional therapy like scaling and root planing (SRP) alone cannot achieve all these goals. Many studies have shown that antibiotics along with conventional therapy like scaling and root planing have a better result.[9] However, overuse of antibiotics has become the major factor of multi-drug resistant strains of several groups of microorganisms. Some other adverse reaction includes allergic/anaphylactic reactions, superinfections of opportunistic bacteria etc.[10] This has generated an urgent need for new antibiotics from medicinal plants. Herbal formulations can provide an option for safe and long-term use. 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 plant extracts or their active components.[11]

One among various herbal plants having active components is Pimpinella anisum (anise) an aromatic annual herb and a grassy plant with white flowers that yields small green to yellow seeds, grows in Turkey, Iran, India, Egypt, and Asia. Anise oil has trans-anethole (85%) as an active ingredient and it is primarily grown for its fruits, commercially called "Anise seeds" that are currently used for flavouring.[12] The essential oil from its fruits is also valuable in perfumery and in medicine which contains eugenol, methylchavicol, anisaklehyde, and estragole. As a medicinal plant, in various disciplines anise has been used as a antiparasitic, antibacterial, antifungal, antipyretic, antiviral, antioxidant, muscle relaxant, analgesic and anticonvulsant activity. It was also reported that anise had several therapeutic effects.[13-14]

There are many studies proving the efficacy of Pimpinella Anisum extracts against pathogens like *A. actinomycetemcomitans, S. aureus, E. coli, Streptococcus pyogenes, Klebsiella pneumoniae*. However, there is paucity of studies establishing the various properties of Pimpinella anisum extracts against *Fusobacterium nucleatum* and *Streptococcus sanguinis*. Hence the present study aimed at assessing the antibacterial and antioxidant ability of Pimpinella anisum seed extracts under laboratory condition.

AIMS AND OBJECTIVES

To assess the antibacterial activity of the Pimpinella anisum methanol seed extract against *Streptococcus sanguinis* and *F. nucleatum* and also to assess the antioxidant activity of Pimpinella anisum methanol seed extract.

MATERIALS AND METHODS

An in-vitro study was carried out to assess the antibacterial, antioxidant activity of Pimpinella anisum methanol seed extract. The Ethical clearance for the study was obtained from the ethical committee and review board of the institution. The seeds were procured from the Department of Medicinal and Aromatic Plants, GKVK. and the laboratory procedures were done at Dextrose technologies Private Limited., Bangalore.

Materials

1. Pimpinella anisum seed
2. Pure Cultures of *Streptococcus sanguinis* ATCC 10556 and *Fusobacterium nucleatum* ATCC 10953.
3. Extraction and Purification: Thimble, Whatman filter paper no.1, Soxhlet, round- bottom flask, heating mantle, water bath and Acetone.
4. Screening of test extracts for antibacterial activity: Vernier caliper.
5. Determination of MIC: Microtitration plates, a spectrophotometric method using the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
6. Determination of MBC: TSA agar
7. DPPH antioxidant assay: 2, 2-diphenyl-1- picrylhydrazyl radical (DPPH), methanol, distilled water, seed extract

   Positive control= ascorbic acid, negative Control is only DPPH solution without seed extract
8. ABTS Radical scavenging Assay: ABTS, potassium persulfate, methanol, quercetin. Positive Control: Quercetin negative control: only ABTS solution without seed extract
9. FRAF Assay: Methanol**,** TPZT, sodium acetate, FeCl3 hexahydrate, Distilled water, FeSO4, Positive Control: FeSO4, negative control is only FRAP solution without seed extract.

Methodology

The invitro study was conducted in following steps:

Step 1: - Collection of Pimpinella anisum seed

Pimpinella anisum seeds were procured. The seeds were then dried in oven at 60 °C for 2–3 days and ground into fine powder. Powdered sample was stored in an air tight bag at room temperature and used for further extraction.

Step 2: - Extraction and Purification of the extracts

- 15 g of the dried seed powder was placed in a thimble made of Whatman Filter paper No1.
- The thimble was kept in the inner tube of soxhlet body in such a manner, that the level of the thimble was lower than the siphoning tube.
- The apparatus was then connected to a round bottom flask which was filled with 200 ml of solvent to which boiling chips was added. The upper portion of the Soxhlet was fitted with condenser.
- The whole apparatus was kept on a heating mantle for 4 hours maintaining the boiling point of the solvent.
- Further the extract was heated on a water bath and was subjected to slow evaporation of the solvent. The solid residue was then washed several times with water and acetone to remove solvent impurities.
- Finally, the pure extract obtained was cooled and stored in vials at room temperature for further usage.

Once the extract is prepared, it was used for the following –

- Thin Layer chromatograph and Phytochemical analysis
- Antimicrobial activity was evaluated by well diffusion technique
- MIC (minimum inhibition concentration) determination against the bacteria by spectrophotometric method using the dye MTT.
- MBC
- Antioxidant activity using DPPH assay, ABTS radical scavenging and FRAP Method.

Step 3: Thin Layer chromatography and Phytochemical analysis

TLC analyses of Ansie seed extract was performed on 20 cm x 10 cm silica gel aluminium plate. Two micro liters of the extract was deposited and developed of hexane: ethyl acetate:

formic acid, 7:10:0.1 (v/v/v), as mobile phase, in a glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried with a hair dryer and then visualized under UV 254 nm, 366 nm and after derivatisation in vanillin-sulphuric acid and anisaldehyde spray reagent. RF value were recorded.

The Anise crude methanolic seed extract was assessed for the presence of alkaloids, flavonoids, glycosides, saponins, sterols, terpenes and tannins by phytochemical screening using standard methods.

Step 4: Screening of pimpinella anisum for antimicrobial activity by well diffusion technique

Test organisms/cultures used were as follows:

i. Streptococcus *sanguinis* ATCC 10556
ii. *Fusobacterium nucleatum* ATCC 10953.

Test compound used was

Pimpinella anisum methanol seed extract

Control used was: Tetracycline

Step 5: Zone of inhibition Test by well diffusion technique

- After, autoclaving the plates were allowed to dry and 6mm wells were punctured on the surface of the agar plate.
- The agar plates were seeded with 100µl of the inoculums and spread evenly over the plate with a sterile glass spreader.
- Each sample (1 - 1000µg/ml) were added to separate wells in the culture plates and incubated at 30℃ for 24hrs.
- After 24 hrs of incubation diameter of the zone of inhibition was measured to nearest millimetre (mm) using a vernier caliper.
- This was done in triplicates for each of the organism.

Step 6: - MIC (minimum inhibition concentration) determination against the bacteria by spectrophotometric method using the dye MTT

Microdilution assay was used to determine the MIC of extract against *S. sangunis and F. nucleatum*

- Series of dilutions of the extract with the concentrations ranging from 1-1000µg/ml were added in the liquid growth medium.
- 100µl microliters of $1x10^5$ CFU of the microorganism culture, 300µl of BHI broth, and 100 µg/ml of serial dilutions of extract were added to each well of a 96-well microplate and mixed well.
- Microplates were incubated for 24 hours at 37 ^{o} C.
- After 30 min of incubation at room temperature and gentle agitation, the Optical density (OD) was measured spectrophotometrically at 540 nm.
- The lowest concentration of the antimicrobial agent that inhibits the growth of the microorganism is considered as the MIC.
- This was done in triplicates for each of the organism.

Step 7: - MBC determination against the bacteria

MBC was determined by subculturing of the wells that displayed no perceivable growth on a sterile agar plate.

- Following the MIC results, the same samples were taken at 100 µl, and was cultured on the TSA agar plates.
- These plates were incubated for 24 hours at 37 $^{o\,C}$.
- Each plate was examined for growth at the end of the incubation period both by the naked eye, and by Colony forming units (CFUs) which was calculated on a grid using a digital colony counter.
- The MBC value was concluded as the lowest concentration that showed no apparent growth on agar subculture.
- This was done in triplicates for each of the organism.[15]

Step 8: - Assessment of antioxidant effect using DPPH assay

- A freshly prepared DPPH solution in 0.5 ml methanol was added to 3 ml of methanolic extract of anise seed to start the antioxidant reaction.
- The decrease in absorbance was measured at different intervals (i.e., 0, 0.5, 1, 3, 5, 10 and 15 min.) at 517 nm.
- The absorbance is correlated with the scavenging action of the test compound. The radical scavenging activities were expressed as percentage of inhibition and calculated according to the following formula equation:
- DPPH radical scavenging activity (%) = [Abs control – Abs sample/Abs control} x100

Where, Abs control is the absorbance of sample at t = 0 min

Abs sample is the absorbance of sample at t = 30 min

Step 9: - Assessment of antioxidant activity using ABTS assay

Radical ABTS+ was prepared through oxidation of ABTS by potassium persulfate. A mixture (1:1; v/v) of ABTS (7Mm) and potassium persulfate (4.95Mm) was prepared and kept in the dark for 16hrs at room temperature. Then, the mixture was diluted with methanol until it reaches absorbance values of 1-1.7 at 734nm.Aliquots of 0.1mL of methanolic extract of each sample (at 4 different concentrations: 20,40,60,80,100 µg/mL; three replicates per sample and concentration) to this 3.9mL of the ABTS+ dilution was added. The absorbance decrease was measured at 734 nm in a UV-30 spectrophotometer. The blank was prepared with ABTS+. The results was expressed in milligram equivalents of quercetin per milligram of dry weight. The calibration line was established using the standard curve.[16]

Step 10: Assessment of antioxidant activity using Ferric Reducing Antioxidant Power (FRAP) Method

Aliquots of 0.2 mL of methanolic extract (at four different concentrations: 20, 40, 60, 80, 100 µg/mL; three replicates per sample and concentration) to this 3.8 mL of FRAP reagent was added. This reagent was prepared formerly by mixing 10 parts of 300 mM sodium acetate buffer solution at pH 3.6, 1 part of 10 mM TPZT, and 1 part of 20 mM FeCl3 hexahydrate. The absorbance increase was measured at 593 nm in a UV-30 spectrophotometer. The blank was prepared by substituting the same amount of diluted extract with methanol. The results was expressed in milligram equivalents of FeSO4 per milligram of dry weight. The calibration line was established using the standard curve.[17]

Statistical analysis

- **Statistical technique used**

Descriptive Statistics

Descriptive analysis includes expression of ZOI, MIC & MBC values in terms of Mean & SD for each concentration & group.

Inferential Statistics

One-way ANOVA Test followed by Tukey's post hoc test was used to compare the mean ZOI & MIC values between different concentrations of Test extract & Tetracycline group.

Kruskal Wallis Test followed by Dunn's post hoc test was used to compare the mean MBC values between different concentrations of Test extract & Tetracycline group.

The level of significance was set at $P<0.05$.

RESULTS

Determination of ANTIMICROBIAL activity

The diameters of zone of inhibition (mm) obtained well diffusion technique are presented in (Table 3, Graph 1a, 1b)

One – way ANOVA test followed by Tukey's post-hoc test was used to compare the mean zone of inhibition between different concentrations of test extract and tetracycline group:

Test group – Methanolic seed extract of Pimpinella anisum

control group – Tetracycline

Comparison of mean ZOI (in mm) b/w different concentration of Pimpinella anisum Seed Extract & Tetracycline for *S. sanguinis* using One-way ANOVA Test

The results of screening test for antimicrobial activity carried out by well diffusion technique revealed that the tetracycline exhibited a mean zone of inhibition with the diameter of 14.67 ± 0.58 and 21 for *S. sanguinis and F. nucleatum* respectively**.** Methanolic seed extract of Pimpinella anisum didn't exhibit any zone of inhibition for F. nucleatum Conc. 1 & 5 µg/ml did not express Zone of Inhibition for S. sanguinis in the present study. The differences in the mean zone of inhibition for S. sanguinis at different concentration of Pimpinella anisum Seed extract was statistically significant at $p<0.001$. Zone of inhibition for S. Sanguinis by Pimpinella Anisum significantly increased with increase in the concentrations & also compared with Tetracycline.

(Table 3, Graph 1a, 1b).

Comparison of mean MIC values at 540 nm b/w different concentration of Pimpinella anisum Seed Extract & Tetracycline for S. *sanguinis* using One-way ANOVA Test

The differences in the mean minimum inhibitory concentration at 540 nm for *S. sanguinis* at different concentration of Pimpinella anisum Seed extract was statistically significant at $p<0.001$. **Minimum inhibitory concentration for S. Sanguinis by P. Anisum showed significant reduction with increase in the concentrations and also compared with tetracycline. (Table 4, Graph 2a, 2b)**

Comparison of mean MBC values (x 102) CFU/mL b/w different concentration of Pimpinella anisum Seed Extract & Tetracycline for *S. sanguinis* using Kruskal Wallis Test. The differences in the mean minimum inhibitory concentration at 540 nm for *S. sanguinis* at different concentration of Pimpinella anisum Seed extract was statistically significant at $p<0.001$. The minimum bactericidal concentration for S. Sanguinis by P. Anisum showed significant reduction with increase in the concentrations from 1 to 40 µg/ml, further which no significant reduction was observed in relation to higher concentrations of P. Anisum and tetracycline. (Table 5, Graph 3a, 3b)

Assessment of antioxidant activity

1. **DPPH assay** The Radical Scavenging Activity [RSA] assessed by DPPH assay of Anise Seed Extract in different concentrations of 20 µg/ml to 100 µg/ml. The half maximal inhibitory concentration (IC50) is a measure of the potency of a substance in inhibiting a specific biological or biochemical function and the same for Anise Seed Extract was 6.66. (Table 6, Graph 4)

2. **FRAF assay** The Radical Scavenging Activity [RSA] assessed by Ferric Reducing Antioxidant Power (FRAP) showed that at the concentration of 20 µg/ml was 17.01 mg, at 40 µg/ml was 28.91mg, at 60 µg/ml was 46.49 mg, at 80 µg/ml was 69.13 mg and at 100 µg/ml was 85.12 mg equivalents of FeSO4 per milligram of dry weight. (Table 7, Graph 5)

3. **ABTS assay** The Radical Scavenging Activity [RSA] assessed by ABT assay of Anise Seed Extract in different concentrations of 20 µg/ml to 100 µg/ml showed that the RSA was varying between 141.48 to 31.68 mg and the maximum activity was observed at 141.48 mg at 20 µg/ml concentration. (Table 8, Graph 6)

DISCUSSION

Periodontal disease has a complicated and multifactorial aetiology in which bacteria are the primary cause.[18] The serial colonisation of more than 600 different bacterial taxa resulted in the development of oral biofilms, one of the most complex and diverse ecosystems. The environment shifts from aerobic to anaerobic as the biofilm moves from supragingival sites to subgingival sites, supporting the growth of primarily Gram-negative obligatory anaerobes while limiting the growth of early Gram-positive facultative aerobes. Supragingival microbes

are mostly associated with gingivitis and root-caries, whereas subgingival species promote the loss of tooth-supporting tissues, resulting in periodontitis.[19]

Lipopolysaccharides (LPS) and DNA from these bacteria enable the gingival fibroblast to activate the nuclear factor-k and activating protein-1 pathways, resulting in the generation of inflammatory cytokines, MMP (matrix metalloproteinases) which results in overproduction of lipid peroxides, inflammatory mediators, and oxidised proteins. In a nutshell, we can state that tissue damage and ROS production are mutually exclusive in the presence of periodontal infections.[20]

According to World Health Organization data, 80% of the world's population relies mostly on traditional remedies that entail the use of plants. Over half of all modern therapeutic medications are derived from natural products. Essential oils from medicinal plants stand out as potent sources of resistance-modifying chemicals. Secondary metabolites present in essential oils include tannins, terpenoids, alkaloids, and flavonoids, all of which have antibacterial activities in vitro.[21-25]

Pimpinella anisum is one such medicinal plant which is produced largely for its fruits (aniseeds), which are gathered between August and September. Trans-anethole (80-95%) (Fujita and Nagasawa, 1960), estragole (Zargari, 1989), eugenole (Monod and Dortan 1950), pseudoisoeugenol (Reichling et al., 1995), methylchavicol and anisaldehyde (Wagner et al., 1984), terpene hydrocarbons (Kartnig et al) The phenol ester 4-methoxy- 2-(1-propene-yl)-phenol-2-methyl-butyrate, which is characteristic of anise (5%), is an uncommon chemical. The essential oil is used as an expectorant, carminative, and in cough mixes, particularly in paediatrics, and the major phenyl-propane constituents, such as trans-anethole and estragole, have a stabilising effect on the autonomic nervous system (Hänsel et al., 1999). A search in the literature showed that there are various studies done on Pimpinella anisum and its medicinal value.[26-27]

A study done by Bektas Tepe et al in the year 2005 evaluated possible antioxidant and antimicrobial activities of Pimpinella anisetum and Pimpinella flabellifolia oils wherein P. anisetum oil exerted greater antioxidant activity than that of P. flabellifolia also showed moderate antimicrobial activity against all microorganisms tested. hence suggesting its antioxidant and antibacterial action.[28]

In this in-vitro study, the organisms used were Pure Cultures of *Streptococcus sanguinis* ATCC 10556 and *Fusobacterium nucleatum* ATCC 10953. The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, *hot continuous extraction (Soxhlet),* aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents).[29] In this study we used The Soxhlet extraction method as it is automatic continuous extraction method with high extraction efficiency was used. The various solvents that are used in the extraction procedures are Water, acetone, alcohol, choloroform, ether etc. Alcohols (EtOH and MeOH) are universal solvents in solvent extraction for phytochemical investigation.[30]

Also previous study by Al-Bayati FA (2008) has reported methanol extract of Pimpinella anisum to be exhibiting antibacterial activity against most tested pathogens in his study.[31] Hence in the present study we used methanol extraction method for obtaining the active ingredient of Pimpinella anisum. Phytochemical Analysis was done, which showed the presence of all 6 organic chemicals except sterols. (Table 1)

In this study we have assessed the antimicrobial property by using agar well diffusion method in which tetracycline was used as a control, as it has proven to be possessing "broad spectrum" of bacteriostatic activity against both Gram-positive and Gram-negative species.[32]

In the present study antioxidant activity of Pimpinella anisum seed extract was evaluated by DPPH antioxidant assay, ABTS Radical scavenging Assay and FRAF Assay. According to the Scopus citation rates, the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical cation-based assays and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-based assays are two of the most common assays for measuring antioxidant capacity.[33]

The FRAP (Ferric Reducing Ability of Plasma/Ferric Reducing Antioxidant Power) test is an unique method for determining the antioxidant capacity of different antioxidants. The FRAP assay analyses an antioxidant's ability to convert ferric tripyridyltriazine complex (Fe +3 - TPTZ) to ferrous complex (Fe +2-TPTZ) at low pH.[34]

According to the results obtained from this study, the methanolic extract of Pimpinella anisum seed had no inhibitory effects on growth of Fusobacterium nucleatum. *Fusobacterium nucleatum* being gram negative bacteria unlike gram positive bacteria, a lipopolysaccharide

layer is present in the cell wall of gram-negative bacteria which doesn't allow passage of materials like antibiotic through their cell wall. This could be the reason for having no antibacterial effects of methanol extract of Pimpinella anisum seed on *Fusobacterium nucleatum* in our study and concentration 1 and 5 µg / ml didn't express zone of inhibition for S.sanguinis.[35]

Mean Zone of inhibition of tetracycline at conc 30 µg/ml was 14.67 which was equal to Pimpinella anisum seed extract at 20 µg/ml for S.sanguinis. This is in contrast with the study done by Ahmed M, Amer (2019) where they reported, tetracycline to have strong antimicrobial activities against tested bacteria**.** However they used ethanolic and aqueous extract we used methanolic seed extract of Pimpinella anisum. Also mean zone of inhibition for *S.sangunis* by Pimpinella anisum seed extract significantly increased with increase in the concentration with highest Mean Zone of inhibition of Pimpinella anisum seed extract for *S.sanguinis* was found to be 24.33 at conc 1000 µg/ml. After screening for the zone of inhibition, the minimum inhibitory concentration and minimum bactericidal concentration of Pimpinella anisum seed extract was assessed and compared with that of control tetracycline.[36]

In our study mean minimum inhibitory concentration and mean minimum bactericidal concentration for Pimpinella anisum against *S. sangunis* was seen at concentration 80µg/ml which were 0.4320 and 0.320 respectively whereas a study done by Fatemeh lavaee et al mean minimum inhibitory concentration and mean minimum bactericidal concentration (microdilution method) was 33.571 and 65.714 respectively.[37] Different values can be related to diversities of plant extraction concentration, various methodologies and differences in cultivated geographic area of plants. (Fatemeh Lavaee et al) On further increasing the concentration of the extract there is no significant differences in both MIC and MBC.

When MIC of Pimpinella anisum methanol seed extract at 80µg/ml was compared with control tetracycline there was statistically significant difference with tetracycline having better inhibiting capacity. When MBC of Pimpinella anisum methanol seed extract at 80µg/ml was compared with control tetracycline showed no statistically significant difference with tetracycline, hence we can conclude that extract is comparable to the drug used and extract has similar MBC as the tetracycline.

In our study DPPH radical scavenging activity of Pimpinella seed extract may be due to the presence of total flavonoids, tannin similar with study done by shobha RI and Andralla (2018). It was observed that as the concentration of extract increased, the antioxidant activity also increased. Aniseed extract reducing capacity of ABTS have been confirmed in other study done by shobha RI (2018).[38]

According to Hagerman et al., the highest level of radical scavenging demonstrated by the methanol extract points to the presence of high molecular weight phenolics, particularly tannins, which have the ability to quench free radicals (ABTS). This ability is dependent more on the molecular weight, number of aromatic rings, and nature of hydroxyl group substitution than the specific functional groups (1998).[39]

Our antibacterial and antimicrobial activity results are in agreement with those of Hassim et al. Pensec et al. who suggested a positive relationship between the total phenolic content and the biological capabilities (antioxidant and antimicrobial activities) of the plant extracts.[40-41]

Table 1: Qualitative phytochemical analysis of methanol extract of Pimpinella anisum.

Sl. No.	Photochemical constitutents	Observations
		Methanol
1	Terpenoid	+ve
2	Flavonoid	+ve
3	Saponin	+ve
4	Alkaloid	+ve
5	Glycoside	+ve
6	Tannin	+ve
7	Sterol	-ve

Table 2: Quantitative screening of phytoconstituents.

Sl. No.	Photochemical tested	RF value
		Sample Pimpinella Anisum seed
1	Terpenoid	0.21
2	Flavonoid	0.83
3	Saponin	0.82
4	Alkaloid	0.92
5	Glycoside	0.90
6	Tannin	0.91

Table 3: Comparison of mean ZOI (in mm) between different concentration of Pimpinella anisum Seed extract & Tetracycline for *S. Sanguinis* using One-way ANOVA Test.

Groups	N	Mean	SD	Min	Max	p-value
Conc. 10 µg/ml	3	11.00	1.00	10	12	<0.001*
Conc. 20 µg/ml	3	14.67	0.58	14	15	
Conc. 40 µg/ml	3	19.67	0.58	19	20	
Conc. 80 µg/ml	3	15.00	1.00	14	16	
Conc. 160 µg/ml	3	21.00	2.65	19	24	
Conc. 320 µg/ml	3	21.33	0.58	21	22	
Conc. 640 µg/ml	3	21.33	0.58	21	22	
Conc. 1000 µg/ml	3	24.33	1.16	23	25	
Tetracycline	3	14.67	0.58	14	15	

Table 4: Comparison of mean MIC values at 540 nm between different concentration of Pimpinella anisum Seed extract & Tetracycline for *S. sanguinis* using One-way ANOVA Test.

Groups	N	Mean	SD	Min	Max	p-value
Conc. 1 µg/ml	3	1.2533	0.3467	0.853	1.455	<0.001*
Conc. 5 µg/ml	3	0.7663	0.0015	0.765	0.768	
Conc. 10 µg/ml	3	0.6530	0.0010	0.652	0.654	
Conc. 20 µg/ml	3	0.6100	0.0010	0.609	0.611	
Conc. 40 µg/ml	3	0.5960	0.0010	0.595	0.597	
Conc. 80 µg/ml	3	0.4320	0.0010	0.431	0.433	
Conc. 160 µg/ml	3	0.3540	0.0010	0.353	0.355	
Conc. 320 µg/ml	3	0.2960	0.0010	0.295	0.297	
Conc. 640 µg/ml	3	0.1977	0.0006	0.197	0.198	
Conc. 1000 µg/ml	3	0.0977	0.0006	0.097	0.098	
Tetracycline	3	0.0520	0.0020	0.050	0.054	

Table 5: Comparison of mean MBC values (x 102) CFU/mL between different concentration of Pimpinella anisum Seed extract & Tetracycline for *S. sanguinis* using Kruskal Wallis Test.

Groups	N	Mean	SD	Min	Max	p-value
Conc. 1 µg/ml	3	18.200	0.100	18.10	18.30	<0.001*
Conc. 5 µg/ml	3	6.300	0.100	6.20	6.40	
Conc. 10 µg/ml	3	5.200	0.100	5.10	5.30	
Conc. 20 µg/ml	3	4.200	0.100	4.10	4.30	
Conc. 40 µg/ml	3	4.067	0.153	3.90	4.20	
Conc. 80 µg/ml	3	0.320	0.010	0.31	0.33	
Conc. 160 µg/ml	3	0.290	0.010	0.28	0.30	
Conc. 320 µg/ml	3	0.403	0.508	0.10	0.99	
Conc. 640 µg/ml	3	0.000	0.000	0.00	0.00	
Conc. 1000	3	0.000	0.000	0.00	0.00	

µg/ml						
Tetracycline	3	0.000	0.000	0.00	0.00	

Table 6: Radical scavenging activitiy % (DPPH) of Anise seeds.

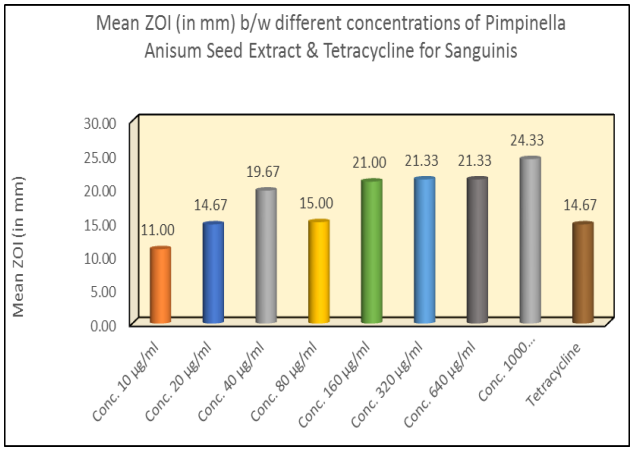
Concentration in µg/ml	Control	Sample	%RSA	IC50
20	0.52	0.42	19.2308	1.33336
40	0.52	0.38	26.9231	2.66678
60	0.52	0.31	40.3846	4.0002
80	0.52	0.2	61.5385	5.33362
100	0.52	0.12	76.9231	6.66704

Table 7: Calculation of scavenging activity by FRAP assay.

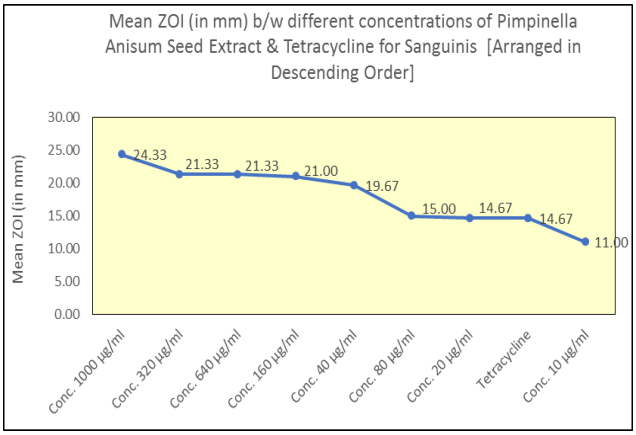
Seed Extract Concentration in µg/ml	Sample	mg/FeSO4 mg
20	0.152	17.01
40	0.399	28.91
60	0.762	46.49
80	1.234	69.13
100	1.566	85.12

Table 8: Calculation of scavenging activity by ABTS assay.

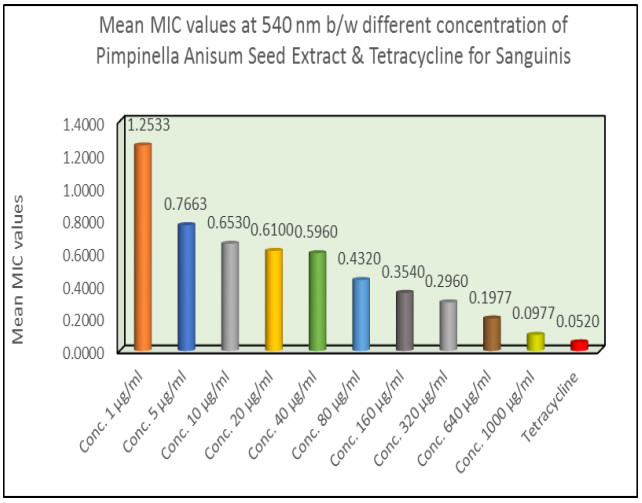
Seed Extract Concentration in µg/ml	Sample	mg/QCT mg
20	1.552	141.48
40	1.389	127.59
60	0.861	82.62
80	0.556	56.64
100	0.263	31.68



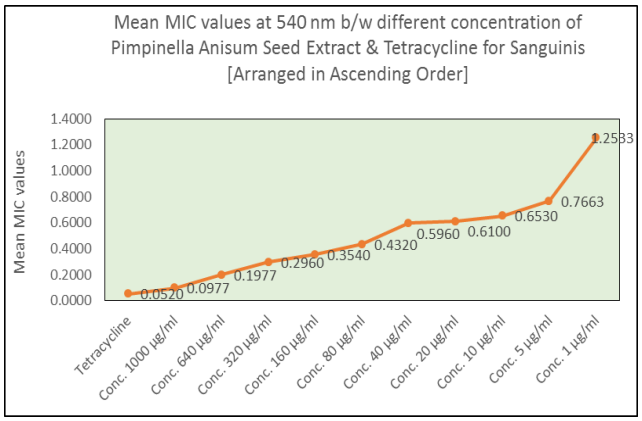
Graph 1a: Mean ZOI (in mm) b/w different concentrations of Pimpinella Anisum Seed Extract & Tetracycline for Sanguinis.



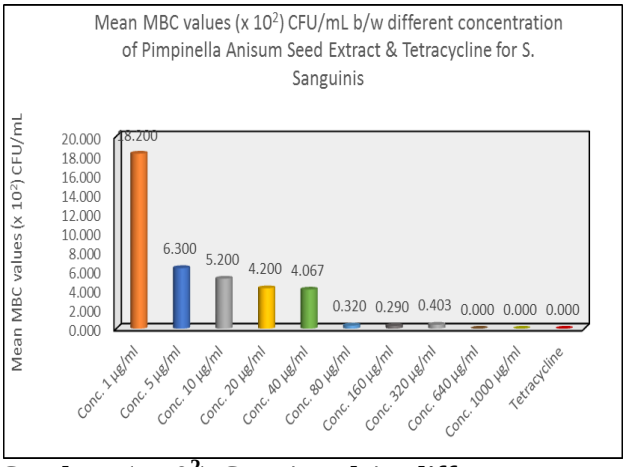
Graph 1b: Mean ZOI (in mm) b/w different concentrations of Pimpinella Anisum Seed Extract & Tetracycline for Sanguinis. [Arranged in Descending Order]



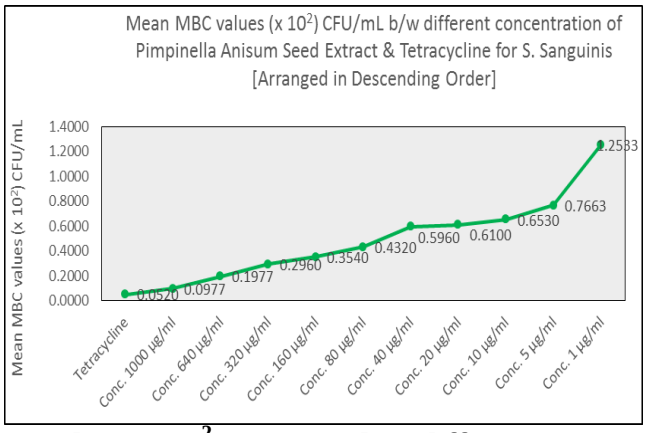
Graph 2a: Mean MIC values at 540 nm b/w different concentration of Pimpinella Anisum Seed Extract & Tetracycline for Sanguinis.



Graph 2b: Mean MIC values at 540 nm b/w different concentration of Pimpinella Anisum Seed Extract & Tetracycline for Sanguinis. [Arranged in Ascending Order]



Graph 3a: Mean MBC values (x 10^2) CFU/mL b/w different concentration of Pimpinella Anisum Seed Extract & Tetracycline for S. Sanguinis



Graph 3b: Mean MBC values (x 10^2) CFU/mL b/w different concentration of Pimpinella Anisum Seed Extract & Tetracycline for S. Sanguinis. [Arranged in Descending Order]

CONCLUSION

1. Methanol extract of Pimpinella anisum seed showed significant mean zone of inhibition against *Streptococcus sanguinis* when compared to control tetracycline but had no antibacterial effects on *Fusobacterium nucleatum*.
2. Methanol extract of Pimpinella anisum seed showed significant mean inhibitory and mean bactericidal effect on *Streptococcus sanguinis*. However, tetracycline showed better MIC values when compared with the seed extract and MBC values of the extract were comparable to that of tetracycline.
3. In the present study Methanol extract of Pimpinella anisum seed showed strong antioxidant activity by DPPH, ABTS and FRAP assay when compared with different standards (Ascorbic acid, ferrous sulphate and quercetin respectively).

Limitations of the present study

It is an in-vitro study conducted to study the antimicrobial and antioxidant property of methanol extract of Pimpinella anisum seed. The outcome of the study cannot be correlated to the in vivo scenario. The other limitation of the study was that, other periodontal pathogens like *P.gingivalis*, *T.forsythia*, *T.denticola* were not evaluated.

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