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Comparative Antimicrobial Efficacy of Tulsi Leaf (Ocimum Sanctum) Extract and Doxycycline on Periodontal Pathogens: An In Vitro Study

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Abstract

Objective:

To assess the in vitro antimicrobial activity of Tulsi leaf extract on periodontal pathogens and to compare its efficacy with doxycycline, a known adjunct to periodontal therapy in individuals with periodontitis.

Materials And Methods:

Ethanolic extract of Tulsi leaves was prepared and four different concentrations (2%, 5%, 10% and 15%) were obtained. Dental plaque and gingival crevicular fluid were collected from the subjects. Doxycycline was used as positive control and dimethyl formamide as a negative control. Both the extracts and controls were subjected to microbiological investigation against Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis. Agar well diffusion method was employed to test the antimicrobial efficacy.

Results:

TUKEY Post HOC test was used to find the means that are significant. Results are considered statistically significant when $P \leq 0.05$. At highest concentration (15%), Tulsi extract demonstrated the widest zone of inhibition (18mm for Porphyromonas gingivalis and 20mm for Aggregatibacter actinomycetemcomitans). The widest zone of inhibition by doxycycline (positive control) was 23mm and by dimethyl formamide (negative control) was 3mm.

With increasing concentration, the zones of inhibition by Tulsi leaf extract increase. Tulsi leaf extract had greater antibacterial efficacy against Aggregatibacter actinomycetemcomitans than Porphyromonas gingivalis.

Conclusion:

In comparison to doxycycline which was used as the positive control in the study, Tulsi leaf extract exhibited an almost similar antimicrobial efficacy against Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis species. Natural phytochemicals isolated from traditional medicinal plants like Tulsi might serve as potential adjunct in the management of periodontitis to overcome the side effects of synthetic drugs.

Keywords: Tulsi leaf extract, Ocimum sanctum, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Doxycycline, Antimicrobial efficacy.

Introduction

Chronic periodontitis is an inflammatory disease that affects the supporting structures of the teeth. This disease is widely prevalent in all age groups and is most commonly seen in adults. The severity of this disease increases with age and has a higher predilection for males.[1]

One of the main causative factors of periodontitis is plaque or biofilm. Bacteria present in the biofilm accumulate around the teeth and initiate the disease. A change in the ecological niche of the bacteria from a healthy microflora to a dysbiotic microflora leads to destruction of the tissues and progression of the disease. During this microbial shift, some of the key periodontal pathogens such as red complex bacteria, Aggregatibacter species and others colonise the biofilm and lead to connective tissue breakdown.

The treatment strategies for effective plaque control can either be mechanical or chemical. Mechanical plaque control is the mainstay, while chemical plaque control is an adjunctive therapy. Chemical plaque control generally includes antiseptics and antibiotics in various forms. Antimicrobial agents have been playing a prominent role in managing periodontal diseases and conditions. Apart from their beneficial effects, they also carry certain undesirable side effects. The emergence of drug resistance in human pathogenic bacteria and several other unwanted side effects with antibiotics has triggered immense interest in the search for new antimicrobial alternatives of plant origin.[2]

Herbal extracts have not received due attention in the management of periodontal diseases. World Health Organization stated that herbal extracts are the best source to obtain a variety of drugs. It is estimated that 25% of modern medicine of the 21st century have plant derivatives as their active ingredients or principle agents. Studies have shown the beneficial effects of Tulsi, Aloe vera and Neem in the treatment of periodontitis. Among these herbs, Tulsi (Ocimum sanctum) stands out as a time-tested pioneer medicinal herb. Tulsi has long been recognized as an antioxidant and as a Cyclo oxygenase (COX)-2 inhibitor.[3] The medicinal properties of Tulsi have been described in the Indian medicinal text "Ayurveda", which is 5000 years old. Various parts of the Tulsi plant have antimicrobial, immunomodulatory, anti-diabetic and anti-inflammatory properties.[4]

The active phytochemicals of Ocimum sanctum are responsible for the various medicinal actions. Currently, the research related to Ocimum sanctum is gaining momentum worldwide to analyse and evaluate its effects, side effects and therapeutic uses in various acute and chronic pathological diseases and conditions, including periodontitis.[5]

This study seeks to assess the antimicrobial efficacy of Tulsi leaf extract on periodontal pathogens and compare its effectiveness with that of doxycycline.

Materials And Methods

Source Of The Study

The patients for this study were chosen from the outpatient department, GITAM Dental College and Hospital, Visakhapatnam.

Place Of The Study

1. Department of Periodontics and Oral Implantology, GITAM Dental College and Hospital.
2. Department of Pharmacology, GITAM Institute of Medical Science and Research.
3. Microbial world testing centre, Nalgonda.

Inclusion Criteria

a) Patients with good systemic health.

b) Patients diagnosed with probing pocket depth ≥ 5mm, in more than 30% teeth present.

Exclusion Criteria

1. Smokers
2. Medically compromised patients
3. Patients on antibiotic therapy
4. Pregnant and lactating women

The patients were given a description of the study protocol and a written informed consent was obtained for the same. The institutional ethical committee has given its approval to the study protocol.

Plaque Samples collection

The premolar and molar buccal surfaces of the maxillary and mandibular teeth were chosen. To decrease the soft debris contaminating the plaque, the

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individuals were instructed to swish with plain water. Cotton gauge was used to isolate the area of plaque collection. A sterile Gracey curette was inserted into the pocket to collect the subgingival plaque sample, and a sterile stainless steel Jacquette scaler was used to collect the supragingival plaque. To collect gingival crevicular fluid (GCF), pre-sterilized paper points were inserted into pockets and held there for 30 seconds.

Transportation of samples of plaque and gingival crevicular fluid

Transportation of plaque samples and paper points was done using the Brain Heart Infusion Broth (BHI) medium, which was employed as a transport medium and for the initial growth of microorganisms.

Figure 1: Brain heart infusion broth (BHI) and Paper points in Brain heart infusion broth

Preparation of cold extract

In the city of Visakhapatnam, Tulsi (Ocimum sanctum) leaves were purchased from local markets and courtyards. After being detached from the stem, the leaves were thoroughly cleaned and then dried for seven days. Then, dried leaves were ground up individually in an electric grinder until a uniform powder was produced. From the blended powder, using the "cold extraction procedure," ethanol extract was prepared.

250 g of finely ground Tulsi were macerated in 100 % ethanol for three days. To obtain a clean filtrate, the alcoholic decoction was filtered through Whatman #1 filter paper. In order to get a solid residue of Tulsi extract, the filtrate so produced was reduced at a low temperature of 60°C. It took 250 grams of Tulsi powder to dissolve in 1 litre of ethanol, and the result was about 18 grams of solid residual extract.

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Figure 2: Preparation of ethanolic extract of Tulsi leaves

Figure 3: Dried Tulsi leaves

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Figure 4: Fine powder grinded from Tulsi leaves

Figure 5: Ethanolic extract of Tulsi leaves

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Figure 6: Filtration of ethanolic extract of Tulsi leaves using Whatman filter paper.

Figure 7: Filtered ethanolic extract of Tulsi leaves

Preparation of four different concentrations of Tulsi leaf extract

To get an extract with a 10% concentration, one gram of this extract was diluted in 10 ml of dimethyl formamide. Similar to this, Tulsi extract concentrations of 2%, 5%, and 15% were prepared by diluting with the proper proportions of solvent. As a positive control, doxycycline was used. Dimethyl formamide, the solvent used to prepare the extract, was used as a negative control.

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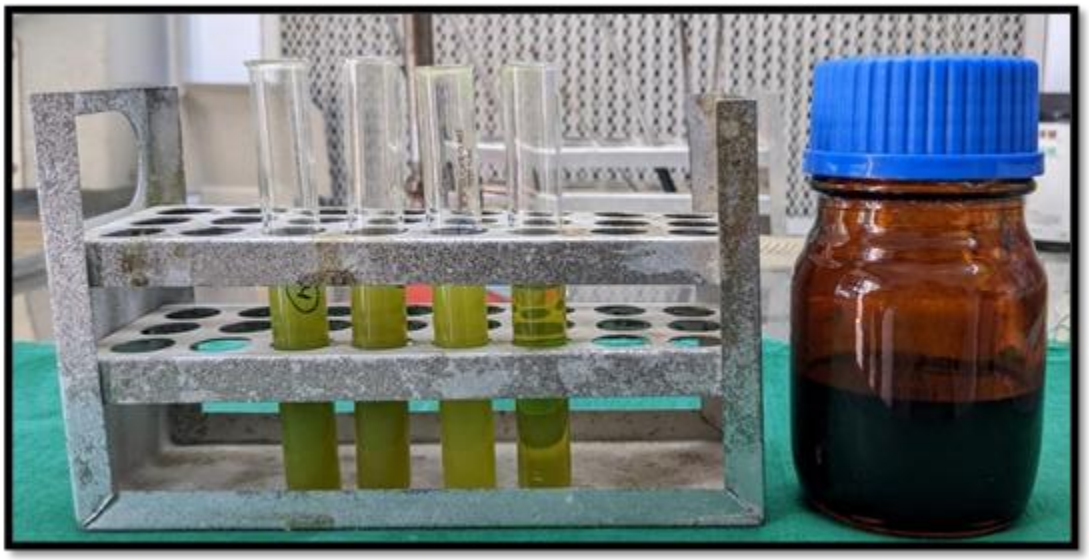
Figure 8: Four different concentrations (2, 5, 10 and 15%) of ethanolic extract of Tulsi.



Microorganism preparation

a. Porphyromonas gingivalis

For this in vitro study, a gram-negative, anaerobic rod-shaped periodontal pathogen called Porphyromonas gingivalis was chosen. Five patients with chronic periodontitis (with periodontal probing depth ≥ 5 mm in more than 30% of the teeth) were selected, and subgingival plaque samples along with gingival crevicular fluid were collected.

After carefully drying the sites and isolating them with sterile cotton rolls, GCF samples were collected by gently inserting small, sterile paper points into the pockets for 10 to 15 seconds. plaque samples obtained through scalers and curettes and paper points were then transferred to the lab in transport vials filled with sterile Brain Heart Infusion (BHI) broth.

Figure 9: Brucella Blood Agar media (BBA)

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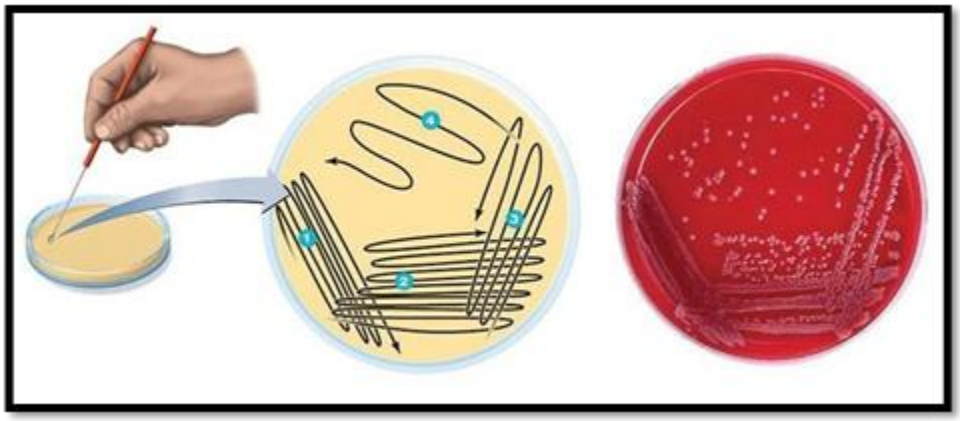
The transported vials were incubated at 37oC under anaerobic conditions for 48 to 72 hours in McIntosh Filde's jar. Following incubation, the bacteria were cultured and maintained on blood agar plates in an anaerobic atmosphere in the McIntosh fields jar.

Figure 10: Streak plate method



The following tests were performed to confirm the growth of Porphyromonas gingivalis.

Microscopic examination of colony morphology

1. Motility
2. Gram staining
3. Biochemical tests: Catalase test, Triple sugar iron test, Urease test, Citrate test

Aggregatibacter actinomycetemcomitans (AAC)

An exogenous bacterium called Aggregatibacter actinomycetemcomitans (AAC) belongs to the Pasteurellaceae family and Actinobacillus genus. They are chemoorganotrophic, facultatively anaerobic, and gram-negative. Using tryptic soy serum bacitracin vancomycin agar (TSBV agar), Aggrgatibacter actinomycetemcomitans was isolated, identified, and characterised from dental plaque samples. Five separate clinical samples of dental plaque were used to inoculate TSBV agar media in sterile petri dishes. The plates were incubated for 24 hours at 37 °C.

Microscopic examination of colony morphology

4. Motility
5. Gram staining
6. Biochemical tests: Catalase test, Triple sugar iron test, Urease test, Citrate test

Figure 11: Tryptone Soy Bacitracin and Vancomycin agar plate (TSBV)

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Microbiological assay

Agar-containing hot media was transferred into petri dishes, where it was left to cool for 20 minutes. A total of three wells—one for the Ocimum sanctum extract, one for the positive control, and one for the negative control were punched into the plate at intervals of 25 millimeters (mm). A micropipette was used to load 50 µl of the specific concentration of Ocimum sanctum extract into the well designated for the test sample. Ocimum sanctum extract in four different concentrations was loaded onto four of these plates. Similarly, 50 µl each of the positive control (doxycycline) and the negative control (dimethyl formamide) was poured into the wells designated for each.

Each bacterium was inoculated onto four agar plates for four different concentrations of the Tulsi extract (2%, 5%, 10%, and 15%). In order to examine both bacteria, a total of 8 plates were inoculated. To get the average or mean of the results, each test was run five times.

Figure 12: Brain heart infusion broth (BHI), Tryptone Soy Bacitracin and Vancomycin agar (TSBV), Brucella Blood Agar (BBA) and paper points

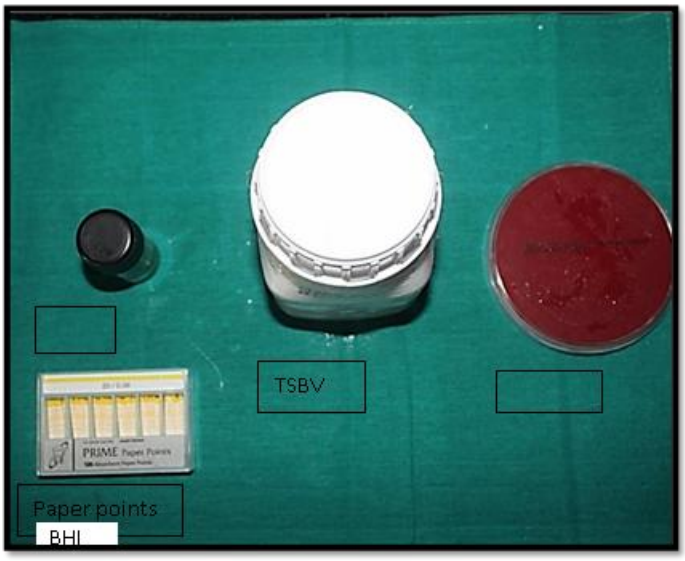

Figure 13: Incubator

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Figure 14: Collection of sub-gingival plaque sample using paper points

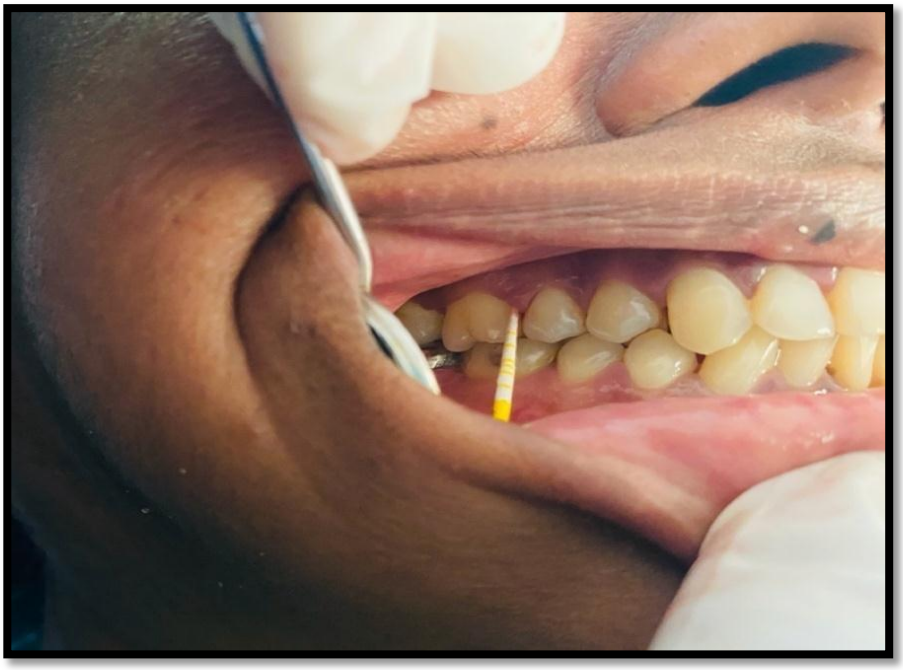

Figure 15: After sample collection paper points submerged into the brain heart infusion broth

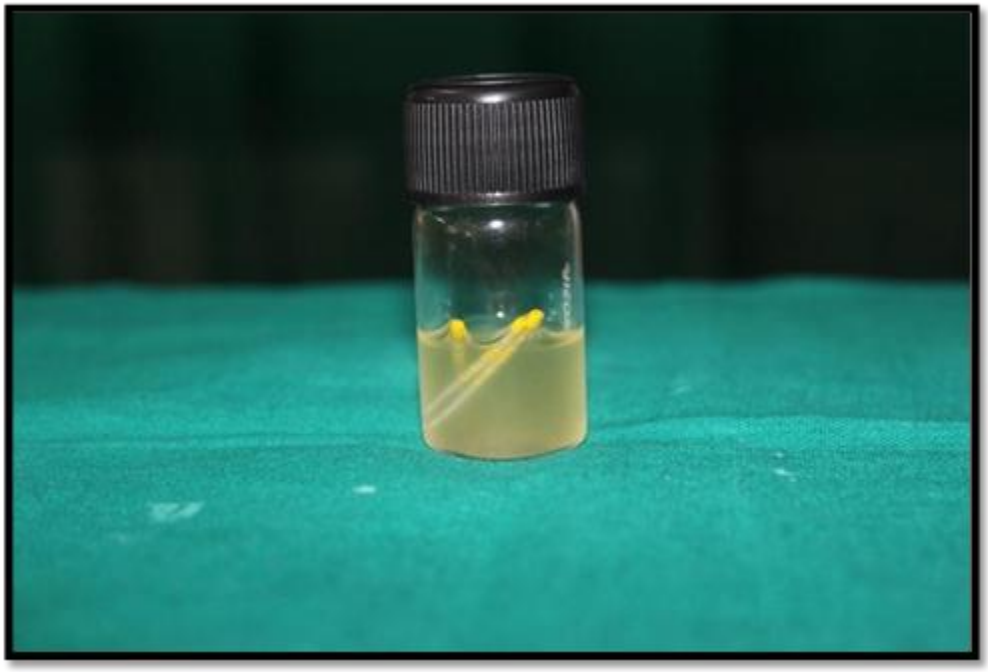

Figure 16: Streaking of the collected sample onto the specific medium plates Tryptone soy bacitracin and vancomycin (TSBV)- Aggregatibacter actinomycetemcomitans; Brucella blood agar plates (BBA) - Porphyromonas gingivalis

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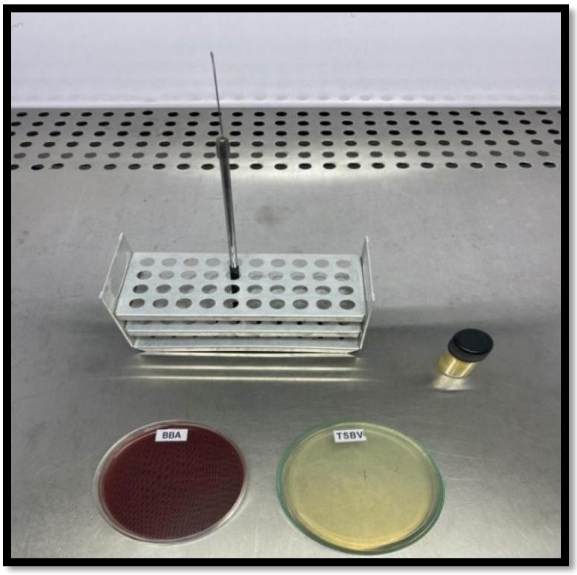

Figure 17: Growth of cultured organisms after 24-48 hrs incubation period

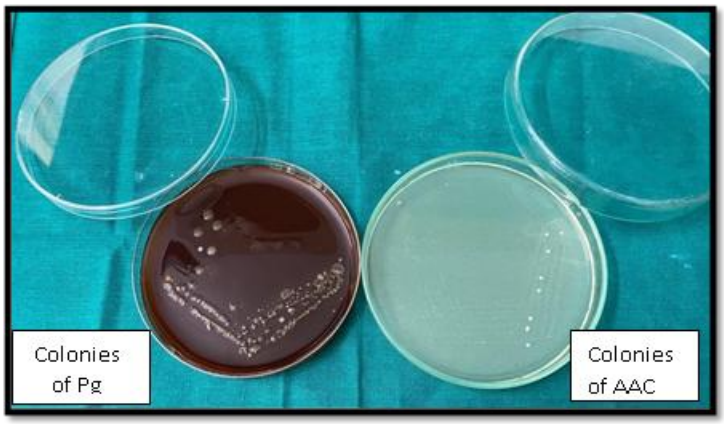

Figure 18: Agar well diffusion method

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Figure 19: Four different concentrations of ethanolic extract of Tulsi (2, 5, 10 and 15%) on agar plates along with Doxycycline (positive) and Dimethyl formamide (negative) controls.

Observations And Results

Statistical Analysis

Data entry was done in MS Excel and analysed using SPSS V22 software. Normality was checked by using the Kolmogorov-Smirnov test. Descriptive statistics was represented as Mean with Standard deviation. TUKEY Post HOC test was used to find the means that are significant. With respect to the nature of the distribution, all the tests were applied. Statistically significant was only considered when P<0.05.

Observation

The plates were incubated for 24 hours at 37oC. The zone of inhibition was measured using a Vernier caliper and an unaided eye after the incubation period of 3 days. The diameter of the disc was taken into account while measuring the size of zones to the nearest millimeter.

Results

Zones of inhibition against Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans displayed by Tulsi extract (at various doses), Doxycycline, and Dimethyl Formamide are reported. For both the bacteria, there was a significant difference in the zone of inhibition ($p < 0.05$). Dimethyl Formamide had the least zones of inhibition, and Doxycycline showed the widest zones of inhibition against both species (Table 1 and 2).

With increasing concentration, the zones of inhibition by Tulsi leaf extract increased. According to the findings, at concentrations of 5, 10, and 15%, Tulsi leaf extract had greater antibacterial effectiveness against Aggregatibacter actinomycetemcomitans than Porphyromonas gingivalis. Post-hoc tests revealed that all pairwise comparisons were statistically significant

Figure 20: Zones of inhibition for Porphyromonas gingivalis by ethanolic extract of Tulsi, Doxycycline and Dimethyl formamide.

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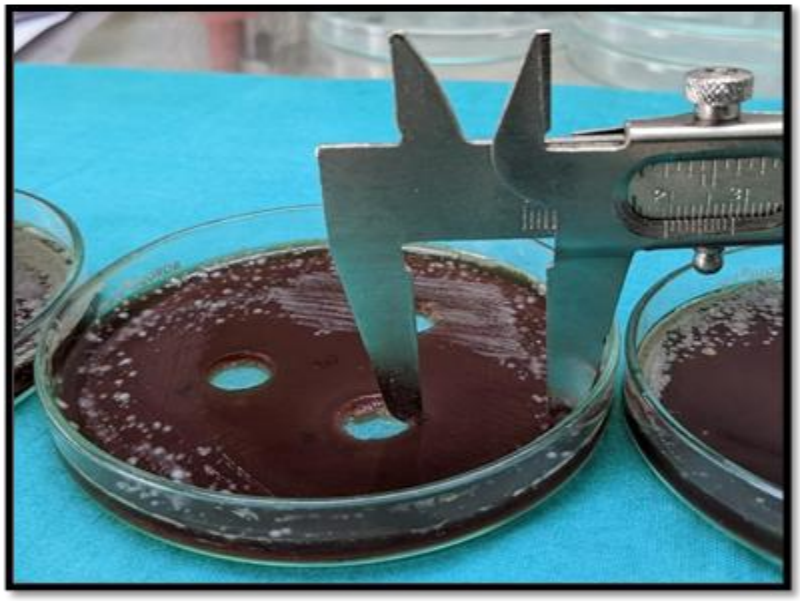
Figure 21: Zones of inhibition for Aggregatibacter actinomycetemcomitans by ethanolic extract of Tulsi, Doxycycline and Dimethyl formamide.

Figure 22: Measuring zones of inhibition using vernier callipers.



Tables And Graphs

Table 1: Analysis of mean zones of inhibition for each concentration of Tulsi leaf extract, Doxycycline and Dimethyl formamide on Porphyromonas gingivalis.

Type of Bacteria	Concentration	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		P Value	Post hoc analysis using Tukey's test
					Minimum	Maximum		
	Tulsi 2%	.8800	.08367	.03742	.7761	.9839		
	Tulsi 5%	1.4200	.08367	.03742	1.3161	1.5239		

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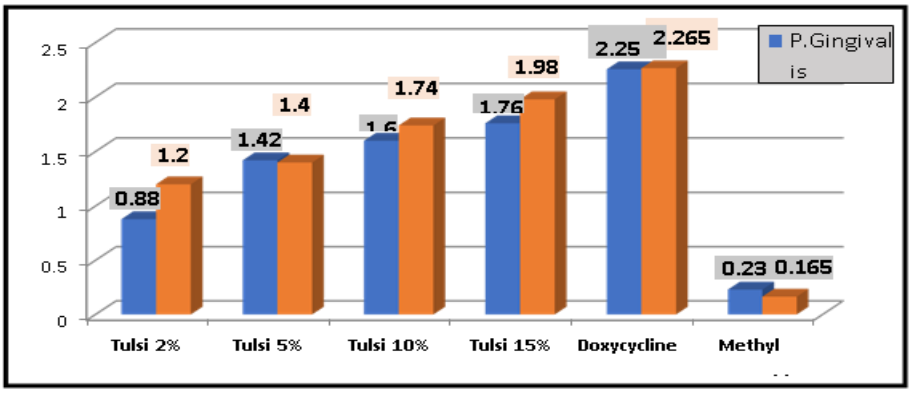
P. gingivalis	Tulsi 10%	1.6000	.12247	.05477	1.4479	1.7521	0.000*	D>T15>T10>T5>T2>MF
	Tulsi 15%	1.7600	.05477	.02449	1.6920	1.8280		
	Doxycycline	2.2550	.06048	.01352	2.2267	2.2833		
	Dimethyl Formamide	.2300	.04702	.01051	.2080	.2520		

Table 2: Analysis of mean zones of inhibition for each concentration of Tulsi leaf extract, Doxycycline and Dimethyl formamide on Aggregatibacter actinomycetemcomitans.

Type of Bacteria	Concentration	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		P Value	Post hoc analysis using Tukey's test
					Minimum	Maximum		
A. actinomycetemcomitans	Tulsi 2%	1.2000	.07071	.03162	1.1122	1.2878	0.000*	D>T15>T10>T5>T2>MF
	Tulsi 5%	1.4000	.07071	.03162	1.3122	1.4878		
	Tulsi 10%	1.7400	.05477	.02449	1.6720	1.8080		
	Tulsi 15%	1.9800	.04472	.02000	1.9245	2.0355		
	Doxycycline	2.2650	.04894	.01094	2.2421	2.2879		
	Dimethyl Formamide	.1650	.05871	.01313	.1375	.1925		

Chart 1: Mean zones of inhibition for each concentration of Tulsi leaf extract, Doxycycline and Dimethyl formamide on Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans.



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Discussion

Periodontitis is mainly caused by dysbiotic oral microbiota. In this polymicrobial community, every bacterium has unique and synergistic activities that lead to the disease process. The dysbiotic polymicrobial communities, along with its complex pathogenic mechanism, aids in a severe destructive process.[6]

Porphyromonas gingivalis (Pg) is thought to be one of the most common keystone pathogens in periodontal infections. Its virulence factors disrupt the host–microbial homeostasis, leading to inflammation and alveolar bone loss. Aggregatibacter actinomycetemcomitans is another putative periodontal pathogen that is usually found associated with aggressive and chronic periodontitis conditions.

The treatment strategies for effective plaque control can either be mechanical or chemical. Mechanical plaque control is the mainstay, while chemical plaque control is an adjunctive therapy. Chemical plaque control generally includes antiseptics and antibiotics in various forms. The planktonic bacteria that were left behind following mechanical debridement as well as bacteria from other oral habitats colonizes the pocket again.

Antimicrobial agents have been playing a prominent role in managing periodontal diseases and conditions. These antimicrobials can either be administered systemically or locally. These agents are used exclusively or in conjunction with non-surgical or surgical periodontal treatment and management.

One of the main adverse effects of synthetic antimicrobial medicines is the development of antibiotic resistance. Doxycycline, a medication most frequently used to treat aggressive periodontitis, also has certain unwanted side effects. The goal of this study was to determine whether Ocimum sanctum could be a viable alternative to these antibiotics in the treatment and management of periodontal diseases and conditions.[7]

Herbal plants are potential sources of medicine. Ocimum sanctum, often known as Tulsi, is one of the many different medicinal plants that stands out as a tried-and-true top remedy. The antibacterial properties of Ocimum sanctum leaves can be imparted to essential oils like eugenol, caryophyllene, germarene-A, clemene, and caryophylline oxide. Additionally, rosmarinic acid, ursolic acid, and oleanolic acid are among the several phytochemicals found in Tulsi leaves. According to Mondal et al. (2007), Ocimum sanctum's essential oils and biologically active components exhibit antibacterial characteristics.[8]

The phytochemicals and essential oils in Ocimum sanctum, which are effective against bacteria that cause systemic diseases, may also act against periodontal pathogens like Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans in human dental plaque.

AAC and Pg, the potent pathogenic microorganisms in patients with chronic periodontitis, were cultured. AAC, a gram-negative anaerobe, nonmotile, small, short, straight or curved rod with rounded ends is strongly linked to periodontal destruction. AAC has five serotypes and several biotypes based on different polysaccharide composition. This pathogen possesses many virulence factors like collagenase, protease, endotoxin, Fc binding protein termed as Omp34, leukotoxin Super antigen producing T cell apoptosis and factors inducing bone resorption.[9]

Porphyromonas gingivalis (Pg), a nonmotile gram negative organism, an asaccharolytic rod-like obligate anaerobe associated with periodontal tissue destruction. Pg possesses vesicles, a capsule which protects it against phagocytosis by leukocytes, fimbriae for adhesion. Pg also has several virulence factors like collagenase, proteases, hemolysin, endotoxin, fatty acids, Hydrogen sulphide and Ammonia. Pg when removed from the periodontal biofilm reverses the dysbiosis suggesting that dysbiosis could be treated by particularly targeting the keystone pathogen.[10]

Bale BF, Doneen AL, Vigerust DJ (2017) in their study demonstrated that the microorganisms prevalent in periodontal disease include Pg, AAC, Tf, Td and Fn. These pathogens when enter into systemic circulation, release endotoxins. These endotoxins induce pro-inflammatory cytokine release, upregulate endothelial adhesion molecules and enhance the risk of atherothrombotic events. Pg, Tf and Td are the organisms which act synergistically in the subgingival environment and are considered as the principal causative microorganisms of periodontitis.

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Zones of inhibition against Pg and AAC that were shown by Tulsi extract (at 2%, 5%, 10%, and 15%), Doxycycline, and Dimethyl formamide were quantified and assessed. For both bacteria, there was a significant difference in the zone of inhibition caused by several antimicrobial drugs ($p \leq 0.05$). Dimethyl Formamide had the smallest zones of inhibition, whereas doxycycline showed the widest zones of inhibition against both species.

With increasing concentration, the zones of inhibition for tulsi leaf extract increased. The antibacterial efficacy of Tulsi leaf extract was considerably higher for AAC than for Pg. Post-hoc tests revealed that all pairwise comparisons were statistically significant.

Compared to Tulsi extract in the current investigation, doxycycline was found to be more efficacious against AAC and Pg. However, the long-term use of doxycycline may be constrained by its well-known adverse effects, which include altered taste perception, oral thrush, gastrointestinal discomfort, and the development of resistant organisms.

Similar findings were found in a study by Pranati Eswar and colleagues (2016). The aim of the study was to investigate the anti-microbial efficacy of Tulsi leaf extract against AAC in human dental plaque. The authors came to the conclusion that a 6% concentration of Tulsi leaf extract had the strongest antibacterial efficacy against AAC.

Traditional herbal medicines like Tulsi are also widely accessible, affordable, simple to use, and culturally acceptable. Tulsi leaf extract, in contrast to synthetic antibiotics, has a higher safety margin, minimal toxicity, and also there is no evidence of any drug interactions in humans. Hence, it might be suggested for long-term use.

It is clear from the literature search that relatively few studies have examined the antibacterial activity of Ocimum sanctum on oral disease-causing pathogens. According to an in vitro investigation, Tulsi was most effective at 3 mg concentration against Streptococcus mutans, and chlorhexidine 0.12% was most effective at 3 mg concentration against Streptococcus aureus.[11]

Several writers have previously postulated various modes of action for Tulsi extract. According to Vishwabhan et al., the essential oil component of tulsi imparts its antibacterial properties. According to Singhal et al., the antibacterial activity of Tulsi leaf extract can be linked to its potential to convert silver ions into silver nanoparticles, which have antibacterial properties and are effective against Gram-positive and Gram- negative bacteria.

The antimicrobial properties of Tulsi against a variety of organisms have been demonstrated in numerous earlier studies by Agarwal et al., Rathod Shah et al., and Prasanna Balaji. Moreover, there is evidence suggesting that plant products are also being used as an effective therapy against periodontal diseases and conditions.

In summary, Tulsi leaf extract has the potential to function as an antibacterial adjuvant and could serve as an alternative to a number of synthetic medications in the treatment of periodontal illnesses and disorders. The present investigation used an in vitro study design to investigate the antibacterial efficacy. Therefore, it is necessary to validate these laboratory results in clinical and real-life conditions. Additionally, the study solely examined the anti-microbial effects of Tulsi against AAC and Pg. It is advised to test its anti-microbial effectiveness against other prevalent periodontal pathogens.

Summary And Conclusion

Tulsi has the potential to become the "drug of choice" in the management of periodontitis. In summary, Tulsi leaf extract has the potential to function as an antibacterial adjuvant and could replace synthetic medications in the treatment of periodontal diseases and conditions. In order to establish clear indications and implications of Tulsi in periodontal therapy, researchers are encouraged to conduct additional studies and clinical trials. These studies will give an insight into the activity of Tulsi extract against periodontal pathogens and to evaluate the long-term efficacy of Ocimum sanctum, additional research is necessary.

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