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Dentistry Section

Antimicrobial Efficacy of Various Essential Oils at Varying Concentrations against Periopathogen *Porphyromonas gingivalis*

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ABSTRACT

Introduction: *Porphyromonas gingivalis (P.gingivalis)* is a notorious perio-pathogen with the ability to evade host defense mechanism and invade into the periodontal tissues. Many antimicrobial agents have been tested that curb its growth, although these agents tend to produce side effects such as antibiotic resistance and opportunistic infections. Therefore search for naturally occurring anti-microbials with lesser side effects is the need of the hour.

Aim: The aim of this study was to substantiate the antimicrobial activity of various essential oils; eucalyptus oil, chamomile oil, tea tree oil and turmeric oil against *P. gingivalis*.

Materials and Methods: Pure cultures of *P. gingivalis* were grown on selective blood agar. Antimicrobial efficacy of various concentrations of essential oils (0%, 25%, 50% and 100%) was assessed via disc diffusion test. Zone of inhibition were measured around disc after 48 hours in millimeters.

Results: Zones of inhibition were directly proportional to the concentration of essential oils tested. At 100% concentration all the tested oils possess antimicrobial activity against *P.gingivalis* with eucalyptus oil being most effective followed by tea tree oil, chamomile oil and turmeric oil.

Conclusion: All essential oils tested were effective against *P.gingivalis*. After testing for their clinical safety they could be developed into local agents to prevent and treat periodontitis.

Keywords: Antibacterial, Chamomile, Eucalyptus, Periodontitis, Tea-tree, Turmeric

INTRODUCTION

Periodontitis is an inflammatory disease of the oral cavity of the microbial origin. Host of bacteria have been identified to be associated with it and are termed as 'periopathogens' [1]. Of these, one periopathogen that has grabbed the fancy of researchers is *Porphyromonas gingivalis*. Frequently isolated from active periodontal lesions, it has been positively correlated with progression of periodontal disease [2]. Although a natural member of oral ecology and found in periodontally healthy individuals as well it is highly destructive and attain high numbers in periodontal lesion [3,4]. It has an arsenal of virulence factors which makes it an aggressive 'pathobiont' [5,6].

Over the years the basic treatment of periodontitis has remained constant which is the removal of plaque biofilm and calculus from supra and subgingival surfaces through scaling and root planning [7,8]. This treatment aims at removing whole of the biofilm rather than targeting specific periopathogens. However, it has been observed that all the patients might not respond to mechanical debridement only [9]. One of the reasons for this is that some periopathogens including *P.gingivalis* invade the gingival tissues and are thus spared from mechanical debridement [10]. These hidden pathogens then provide the source for recolonization of periodontal pocket and resurgence of disease. The adjuvant treatment of local drug delivery has been suggested for such tissue invading periopathogens [11].

In local drug delivery, antimicrobial agent is placed within the periodontal pocket in a carrier medium where it is released in to the local area over a period of time [12]. Many anti-microbial agents have been used for this purpose with rather enviable clinical results. Antimicrobial agents that have been used successfully for this purpose include chlorhexidine, tetracycline and metronidazole to name a few [13-15]. But there remains a roadblock in this success march with local delivery of antimicrobials. Previous decades have seen an indiscriminate use of commercial antimicrobials, leading to emergence of multi-drug resistance bacteria [16]. Due to these reasons, natural antimicrobial agents have grabbed attention of researchers. One such group of natural antimicrobial agent that has been used for centuries in naturopathy is the botanically derived essential oils.

Essential oils, also called aromatic plant essences are fragrant volatile substances biosynthesized by plants [17]. The earliest mention of essential oil used for therapeutic purposes is in Ebers papyrus, where more than 800 remedies and treatments have been listed [18]. Since then essential oils have been recognized for their anti-inflammatory, anti-microbial and anti-oxidant properties. The oxygenated terpenoids and some hydrocarbons found in essential oils account for most of their anti-microbial activity [19]. Research suggests that terpenoids diffuse within the cell membrane, irreversibly damaging it and causing bacterial cell death. Being lipophillic in nature, these terpenes causes expansion of membrane, increases its fluidity and inactivates the enzymes embedded within the membrane [20,21].

Although there is a revival of interest in essential oils and their anti microbial properties, the data regarding the efficacy of these oils against oral bacteria especially periopathogens is limited. Here in this study, we assessed the antimicrobial efficacy of above mentioned essential oils at varying concentrations against a major periopathogen *P.gingivalis*.

MATERIALS AND METHODS

This in-vitro microbiological study was carried out in Department of Periodontology, Faculty of Dental Sciences, SGT University in association with Department of Microbiology, Faculty of Medical and Health Sciences, SGT University, Gurgaon, Delhi-NCR. The *P.gingivalis* colonies used in this study were cultivated from the subgingival plaque samples obtained from the Chronic Periodontitis patients reporting to the OPD of the Department of Periodontology. The study commenced in September 2015 and was completed within two weeks of initiation.

Collection of subgingival plaque samples: Five patients with untreated generalized chronic periodontitis were selected for collection of subgingival plaque samples. The patients selected had at least four sites with probing pocket depth of 6 mm or more. To collect the subgingival samples, sterile paper points were used. The area was isolated using cotton rolls and any supragingival plaque and calculus hindering the insertion of paper point into the periodontal pockets was removed using sterile supragingival scalers. Sterile paper points were then inserted slowly with the help of a sterile dental tweezer into the pocket until tissue resistance. Paper point was left in place for around 60 seconds, carefully removed and immediately placed into a sterile container with anaerobic transport media and sent for microbiology department for culture.

***P.gingivalis* culture on selective media:** The composition of selective media per 1000 ml was as follows: Columbia agar (42.5%), Agar (6.5g), 0.1% solution of Hemin (5.0ml), 1% solution of vitamin K1 (1.0 ml), Human blood (50ml), Colistinmethanesulfonate (15.37 mg), Bacitracin (10mg), Nalidixic acid (15 mg) and distilled water. Media was prepared and stored under strict anaerobic conditions to prevent oxidation. Subgingival samples obtained were directly inoculated onto selective media plates for *P.gingivalis*. The inoculated plates were then incubated anaerobically at 37°C for 48 hours. Colonies of *P.gingivalis* developed after 48hours which were round, opaque and convex with 1-2mm diameter. Since whole blood was used the black pigment did not appear at 48 hours since appearance of pigment is slow in non lysed blood.

Confirmation of *P. gingivalis* growth: Since a media selective for *P.gingivalis* was used, only few tests to confirm the presence of desired bacteria were done. The colonies were tested for their ability to fluoresce under ultraviolet light. The colonies were dispersed in 1.0 ml of 96% aqueous ethanol and the suspension was illuminated in a dark room with a 366nm ultra violet light. The colonies failed to produce a red fluorescence which was taken as positive for *P.gingivalis* growth. Further the colonies were tested for their ability to hydrolyse synthetic trypsin substrate N-Benzoyl-DL-Arginine-2-Naphthylamide (BANA). A sterile paper saturated with BANA was taken and colonies were placed over it for 30minutes. This strip was then placed over a filter paper saturated with fast blue BB salt (0.35% w/v in methoxyethanol). The colonies gave a positive reaction with appearance of orange red colour. Both of these test confirmed the colonies to be of *P.gingivalis*.

Preparation of various concentrations of essential oils: Four essential oils; Eucalyptus, Tea-tree, Chamomile and Tur meric oil were tested for their efficacy against *P.gingivalis*. All the essential oils were obtained from Farmaessentials®, Chandigarh, India. All were tested in four concentrations for their antibacterial activity: 0%, 25%, 50% and 100%. The essential oils were diluted in vegetable oil which in itself lacked antimicrobial activity. This was confirmed with the inclusion of 0% group in the study which contained only vegetable oil and no essential oil. Twenty five percent concentrations were prepared by adding 1.25 ml of essential oil in 5ml of vegetable oil. Likewise 50% concentration had 2.5 ml of essential oil in 5 ml of vegetable oil where as 100% concentration had only pure essential oil but no vegetable oil. The dilutions were prepared just before the paper disc diffusion test was initiated.

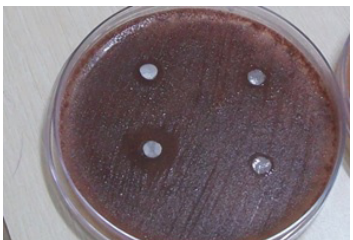
Paper disc diffusion test: Paper disc diffusion was carried out on selective blood agar plates. The *P.gingivalis* culture was aseptically spread on surface of agar plate. Sterilized small filter paper disc with 6 mm diameter were used for the study. The discs impregnated with essential oils were placed on the agar surface. Ten discs of each concentration of each essential oil were taken for the experiment. The agar plates were incubated at 37°C for 48 hours under strict anaerobic conditions. The antibiotic activity of various concentrations of essential oil was assessed by measuring the diameter of the growth inhibition zone in millimeters. All the data was collected and assessed with SPSS 19.0 software for statistical analysis. Both intra group and intergroup comparisons was carried out using student's t-test.

RESULTS

The zone of inhibition obtained for varying concentrations of four essential oils included in the study are compiled in [Table/Fig-1]. The zones of inhibition around paper discs infused with these essential oils are depicted in [Table/Fig-2-5]. Since ten discs were used for each concentration, the zones of inhibition were calculated as a mean of all ten values. The zone of inhibition was directly proportional to concentration of essential oil tested. No zone of inhibition was observed around zero percent disc confirming the carrier medium was inert with no antibacterial activity of its own. Turmeric oil had least activity against *P.gingivalis*, whereas eucalyptus oil had maximum. On intragroup comparison [Table/Fig-6] of varying concentration of essential oils, there was significant difference between 25% and 50% concentration of eucalyptus and chamomile essential oils ($p<0.001$). While comparing the difference in antibacterial activity of 50% and 100% concentrations, all the oils tested had highly significant results ($p<0.001$). On intergroup comparison [Table/Fig-7], there was highly significant difference in antibacterial activity of 100% concentration of all essential oils tested against *P.gingivalis*. The antibacterial activity of these oils was in following order: eucalyptus oil, chamomile oil, tea tree oil followed by turmeric oil.

Essential oil	Concentration (%)	Zone of inhibition (mm) ± S.D
Eucalyptus Oil	0	0
	25	1.70 ± 0.258
	50	2.51 ± 0.213
	100	4.5 ± 0.183
Chamomile Oil	0	0
	25	0.52 ± 0.199
	50	1 ± 0.2
	100	1.7 ± 0.183
Turmeric Oil	0	0
	25	0
	50	0.5 ± 0.082
	100	1.12 ± 0.079
Tea tree Oil	0	0
	25	0
	50	0.98 ± 0.092
	100	2.9 ± 0.356

[Table/Fig-1]: Zone of inhibition of various concentrations of essential oils.




[Table/Fig-2]: Zone of inhibition around eucalyptus essential oils discs against *P.gingivalis*. **[Table/Fig-3]:** Zone of inhibition around chamomile essential oils discs against *P.gingivalis*. **[Table/Fig-4]:** Zone of inhibition around tea tree essential oils discs against *P.gingivalis*. **[Table/Fig-5]:** Zone of inhibition around turmeric essential oils discs against *P.gingivalis*.

Essential oil	25% v/s 50%		50% v/s 100%	
	95% CI	p- value	95% CI	p-value
Eucalyptus Oil	1.032-0.588	<0.001	2.176-1.804	<0.001
Chamomile Oil	0.667-0.293	<0.001	0.880-0.520	<0.001
Turmeric Oil	-	-	0.695-0.545	<0.001
Tea tree Oil	-	-	2.164-1.676	<0.001

[Table/Fig-6]: Intragroup comparison of various concentrations of essential oils. p<0.001 is highly significant [data was analysed using t-test]

Essential oil	Chamomile Oil		Turmeric Oil		Tea tree Oil	
	95% CI	p- value	95% CI	p-value	95% CI	p-value
Eucalyptus Oil	2.628-2.972	<0.001	1.334-1.866	<0.001	1.334-1.866	<0.001
Chamomile Oil	-	-	0.448-0.712	<0.001	1.466-0.934	<0.001
Turmeric Oil	-	-	-	-	1.538-2.022	<0.001

[Table/Fig-7]: Intergroup comparison of 100% concentration of essential oil. p<0.001 is highly significant (data was analysed using t-test)

DISCUSSION

Several naturally and synthetically derived agents are being used in dentistry to inhibit the disease causing plaque biofilm these agents include cetylpyridinium chloride and other antimicrobials such as doxycycline, minocycline and metronidazole. These have been used successfully as a mouthwash, topically applied gels or intrapocket local drug delivery agents. These modalities have shown promising results as an adjunct to standard therapy of scaling and root planing [22-24]. The dental community is in search of newer therapeutic agents that apart from having a positive impact on periodontal health will lack the usual side effects of in use antimicrobials. Phytochemicals such as essential oils provide such an alternative.

We tested four essential oils in varying concentrations against the notorious periopathogen *P.gingivalis*. Our data revealed that all four essential oils tested, that is eucalyptus oil, chamomile oil, turmeric oil and tea tree oil had antimicrobial activity against *P.gingivalis* where eucalyptus oil had maximum efficacy and turmeric oil the least. Also, tea tree oil and turmeric oil lacked any antibacterial activity against *P.gingivalis* at 25% concentration. As the concentration of each oil increased, the antibacterial efficacy improved. An interesting observation though is higher antibacterial effect of tea tree oil than chamomile oil at 50% and 100% concentration whereas at 25% concentration chamomile oil was more efficacious. This indicates that certain essential oils such as tea tree oil work better at higher concentrations.

Eucalyptus, genus of family Myrtaceae has been used to control infectious diseases since the time of ancient Egyptians [25]. Antimicrobial, anti-inflammatory as well as analgesic properties of eucalyptus essential oil have been reported [26,27]. Its predominant component is Citronellal (57%) followed by citronellol (16%) and citronellyl acetate (15%) [28]. However, the antimicrobial bioactivity can be attributed to α- terpineol which showed eight fold higher activity than citronellol against *Staphyllococcu saureus* [29]. Eucalyptus oil inhibited the growth of periodontopathogens including *P.gingivalis* and the minimum inhibitory concentration ranged from 0.25% to 0.5% for various strains [30].

Matricariarecutita, commonly called chamomile belongs to the family Asteraceae. As believed by Anglo-saxons, chamomile is one of the nine sacred herbs given by Lord to humans [31]. Chamomile flowers contain a blue colour volatile oil, of which around 120 constituents have been identified [32]. Major constituent is a terpenoid, α-bisabolol and its oxides which form around 78% of composition. Other constituents which form around 1-15% of composition are azulenes which include chamazulene [33]. The antimicrobial activity of chamomile oil has been confirmed against *S. aureus* and *Candida strains* [34]. There is limited research on the antibacterial effect of chamomile essential oil against common oral pathogens. During the search of literature on chamomile oil we could not find much data on its antimicrobial or antibacterial activity. It is therefore to our knowledge first data on antibacterial effect of chamomile oil against any periopathogen.

Tea tree oil is extracted via steam distillation of leaves and twigs of tree *Melaleucaalternifolia*. It has been used widely for the treatment of cold, cough, sore throats and skin diseases [35]. The anti-microbial activity of this essential oil could be attributed to terpinen-4-ol [36]. Research has demonstrated its ability to inhibit cellular respiration in *Eschercia coli* by disrupting the permeability barrier of bacterial cell membrane [37]. Similar to our study, it has been found to have significant antibacterial activity against four common intra canal oral pathogens [38]. Tea tree oil also shows significant adhesion-inhibiting activity against *P. gingivalis* [30].

Curcuma longa, commonly termed turmeric is a botanical relative of family Zingiberaceae [39]. It's a commonly used spice along with vast history of use as a therapeutic agent. The extract of turmeric is an oleoresin which has two fractions: a yellow brown heavy fraction and a light volatile oil fraction. The extract as a whole consists of many curcuminoids, monoterpenoids and sesquiterpenoids [40]. Curcumin is the principle curcuminoid responsible for antioxidant, anti-inflammatory and antimicrobial activity of turmeric [41]. The major component of turmeric essential oil is aromatic tumerone (20-30%) [42]. Although used as a mosquito repellent and in treatment of respiratory diseases, its antibacterial and antifungal activity has also been identified [43-46]. Research on its effect against periopathogens is lacking but still it has been used clinically as a mouthwash, local drug delivery agent as well as subgingival irrigant [47-49].

There are increasing numbers of research paper on the anti-microbial efficacy of various essential oils for their prospective use as surface disinfectants, food preservatives and alternative medicinal therapy. However articles on their antibacterial efficacy against oral pathogens especially periopathogens are limited. Takarada et al., assessed the antimicrobial effect of essential oils on cariogenic and periodontopathic bacteria including *P.gingivalis* [30]. They found that periodontopathic bacteria were killed completely by exposure for 30 seconds to 0.2% manuka oil, tea tree oil and eucalyptus oil. Tea tree and manuka oil showed significant adhesion inhibiting activity against *P.gingivalis* [27]. These results are in accordance with our study. Among all the oils tested, manuka and tea tree oil had particular strong antibacterial activity against periodontopathic bacteria. In our study, we found eucylyptus oil to be more effective than tea tree oil against *P.gingivalis*.

Since periodontitis is highly prevalent disease, undesirable effects of several antimicroial agents presently being used in treatment of oral disease and augmented resistance of oral bacteria to antibiotics are a cause of concern. Alternate products that are safe but equally effective are required for the treatment as well as the prevention of disease. Essential oils such as those tested in this study could provide a good alternative to usual commercial antibiotics.

LIMITATION

There are certain limitations of the study that we carried out. First, only one periopathogen *P.gingivalis* was tested for antibacterial efficacy of these essential oils. The reason is fastidious growth condition required for growth of these anaerobic periopathogens. Another drawback is that minimum inhibitory concentrations of these essential oils were not determined. The results of our study indicate their potential use in oral cavity especially intrapocket placement which is the ecological niche of *P.gingivalis*. Our follow-up research will be on their safety and cell toxicity at various concentrations. Establishing the safety of these oils intraorally will pave the path for their full scale use as a local drug delivery agent for the treatment of periodontitis.

CONCLUSION

To conclude both eucalyptus and tea tree oil possess a significant antibacterial activity against *P.gingivalis* followed by chamomile and turmeric oil. Also as the concentration of essential oils increases so does the antibacterial efficacy.

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