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ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF PEPPERMINT (*MENTHA PIPERITA* LINN) AND MENTHOL MINT (*MENTHA ARVENSIS* LINN) ESSENTIAL OILS ON *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* ISOLATED FROM ORODENTAL INFECTIONS.

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ABSTRACT

Introduction: *Aggregatibacter actinomycetemcomitans* is one of the key pathogen associated with periodontal diseases as well as systemic infections. Giving importance to the higher incidence rate of periodontitis and resistance among oral bacteria to antibiotics, there is a need for alternate treatment regimen especially from natural resources. The main objective of the present study was to elucidate antibacterial and antibiofilm potential of the essential oils of *Mentha piperita* Linn and *Mentha arvensis* Linn against *A.actinomycetemcomitans* isolated from patients with orodental infections. **Methodology:** Essential oils of *Mentha piperita* Linn and *Mentha arvensis* Linn leaves were distilled in Clevenger's apparatus by Neo-Clevenger's method. Antimicrobial action was determined by disc diffusion method. Minimum inhibitory concentration (MIC) was determined by microbroth dilution technique. Tissue culture plate method was employed for demonstration of antibiofilm activity. **Results:** 77.9% and 76.5% of the isolates showed antibacterial activity against *Mentha piperita* Linn and *Mentha arvensis* Linn essential oils respectively by disc diffusion methods. The MIC ranged between 3.125µl/ml to 12.5µl/ml. The minimum biofilm inhibition concentration (BIC_{50}) was achieved at 100µl/ml concentration for both the essential oils **Conclusion:** Essential oils of *M.piperita* and *M.arvensis* showed good antibacterial as well as antibiofilm activity at low concentrations and can be considered as an alternate treatment option for the control of periodontitis by *A.actinomycetemcomitans*.

KEYWORDS: *Aggregatibacter actinomycetemcomitans*, *Mentha piperita*, *Mentha arvensis*, Minimum inhibitory concentration (MIC), biofilm inhibition concentration (BIC_{50}).

INTRODUCTION

A.actinomycetemcomitans, a gram negative capnoic, coccobacillus is the primary aetiological agent of aggressive and chronic periodontitis.[1] Dissemination of *A.actinomycetemcomitans* of into extra oral sites can produce life threatening infections like endocarditis, pneumonia and abscess in lung and neck region.[2,3] Along with other obligate anaerobic bacteria, *A.actinomycetemcomitans* is known to act synergistically and produce oral biofilm.[4] Systemic antibiotics along with mechanical treatment of the periodontal pockets are the commonly employed treatment options to eliminate this bacterium.[5] At the same time development of resistance against antibiotics as well as the ability to produce biofilm by the microorganisms placed a new challenge in the treatment of infectious diseases. Studies by various researchers have demonstrated increasing antimicrobial resistance in *A.actinomycetemcomitans*.[5,6] Hence it is of utmost importance to look for newer effective antimicrobial agent from natural sources which has antibacterial as well as antibiofilm potential.

The use of herbal products as oral care agents is an ancient custom and followed in many parts of the world. Plant remedies are increasingly being recognized by scientists as a very important alternative to industrially produced expensive antibiotics and side effects associated with them.

The plant genus *Mentha* contains 25-30 different species and known for its antimicrobial, antiviral and insecticidal activity. Essential oils of mint are extensively used in toiletry, food and pharmaceutical industries because of its aromatic, stimulant and carminative nature.[7] *Mentha piperita*, commonly known as peppermint is a member of this genus and essential oil of peppermint is used as a

flavouring agent in variety of products like toothpastes, mouthwashes, digestive tablets, sweets, ice cream and liquors. [8] The essential oil extracted from this plant was reported to have antibacterial, antifungal and antibiofilm activity.[9] Another species, *Mentha arvensis* commonly known as pudina (menthol mint, corn mint or wild mint) is widely cultivated in Bangladesh, Nepal, India, Srilanka, Thailand and Japan. It is used in folk medicine to control indigestion, peptic ulcer and skin diseases.[10] Antibacterial action of extracts of *M.arvensis* were demonstrated on uropathogens and enteric pathogens by various investigators.[7,10]

In India, trials have been carried out on the safety and antimicrobial activity of medicinal plant extracts and essential oils on various aerobic bacteria and Candida species.[11] However no detailed study on the effects of mint essential oil or their components on periodontal pathogen, particularly *A.actinomycetemcomitans*. Hence the present study investigates the antibacterial and anti biofilm action of *M.piperita* and *M.arvensis* essential oil against this pathogen.

MATERIALS AND METHOD

A.COLLECTION AND IDENTIFICATION OF STRAINS

The study was conducted at a tertiary care hospital at Mangaluru, coastal Karnataka, South India. The study was approved by the Institutional Ethics Committee (Ref. No FMMC /IEC /395 /2010).

A.actinomycetemcomitans was isolated from different patient populations with orodental infections attending the dental colleges in and around Mangaluru. The paper point specimens collected from the sub gingival sites of the patients, after taking informed consent were transported in reduced transport fluid and sub cultured on Dentaid -1 media. The plates were incubated at 37^0C in a candle jar for 48 hours. Pin point (1mm diameter), glistening colonies with central 4-6 pointed star like configuration were presumptively identified as *A.actinomycetemcomitans* and were confirmed by Grams stain, positive catalase test and biochemical reactions.[12] These strains were further subjected to Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDITOF–MS), an advanced method of identification based on protein profile of the organism using Bruker MALDI biotyper.[13]

B.PREPARATION OF ESSENTIAL OILS.

The leaves of peppermint and wild mint were obtained from a reputed retail store and submitted for authentication at National Ayurvedic Dietetics Research Institute, Bengaluru [Central Council for research in Ayurveda & Siddha, Department of AYUSH, Ministry of Health & Family Welfare, Government of India]. Details were given in table I. The authenticated and certified leaves were shade dried and reduced to coarse powder. The essential oil was extracted by Neo-Clevenger's method using Clevenger's apparatus.

Table I: Details of authentication.

Common name	Scientific name	Family	Extracts used	Authentication number
Peppermint	*Mentha piperita* Linn	Lamiaceae	Essential Oil from leaf	RRCBI-MUS/112
Pudina (Menthol mint, Wild mint, Corn mint.)	*Mentha arvensis* Linn	Lamiaceae	Essential Oil from leaf	RRCBI-MUS/111

C. SCREENING OF ANTIMICROBIAL ACTION OF ESSENTIAL OILS

The essential oils of both herbs were tested for antimicrobial action against Sixty eight (68) *A.actinomycetemcomitans* isolates, by disc diffusion method. The preliminary, screening test was done by using undiluted oil. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentrations (MBC) were determined by Microbroth dilution methods.

i) Disc diffusion method.[14]

Disc diffusion technique was done on Cation adjusted Mueller-Hinton agar (MHA) plate enriched with 0.6% yeast extract. A 48 hour culture of *A.actinomycetemcomitans* grown on enriched brain heart infusion (BHI) broth, opacity was compared with Mc Farland 0.5 standard ($1.5x10^8$ Colony forming Unit/ml) were swabbed over the agar plate. Separate sets of the discs with essential oils were prepared by adding One milli litre of undiluted essential oil of *M.piperita* and *M.arvensis* to 100 sterile discs of 6mm diameter. The discs were then placed over the enriched MHA and the zone of inhibition was measured in millimeters after incubation under candle jar (5-10% CO_2) at 37^0 C for 48 hours. A disc incorporated with DMSO (Dimethyl sulphoxide) used as a diluents was included as negative control.

The disc diffusion technique was done in triplicate and the mean value of the zone of inhibition in millimeters was calculated. In addition a doxycycline disc (30µg) the drug of choice for *A.actinomycetemcomitans* was also used as a reference antimicrobial compound along with the test. *A.actinomycetemcomitans* ATCC 29522 was also tested in parallel along with the clinical strains.

The results were expressed in terms of the diameter of zone of the inhibition as: < 9 mm resistant. 9-12 mm - partially active; 13-18 mm - active; >18mm - very active.[15]

ii) Detection of Minimum inhibitory concentration (MIC) & Minimum bactericidal concentration (MBC) of essential oils.[16]

The micro broth dilution method was done to determine minimum inhibitory concentration (MIC) according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Thirty (30) strains of *A.actinomycetemcomitans* were used for the study. All tests were performed on cation adjusted Mueller-Hinton broth enriched with 2.5% sterile sheep blood. Briefly, serial doubling dilutions of the extract were prepared in a 96-well microtiter plate ranging from 100µl/ml to 0.78µl/ml. Finally from a 48 hour culture, diluted 100 µl (10^6cfu/ml) of bacterial suspension was added to each well except broth control. The final concentrations of the essential oil were doubled in each well after addition of equal amount of the test strains. Each well had a final volume of 200µl.The plates were covered loosely with plastic film to ensure that the bacteria did not get dehydrated during incubation. The test was done in triplicates and they were incubated at 37°C for 24-48 hours in candle jar. The highest dilution which showed lack of visual turbidity was considered as MIC. The MBC was defined as the lowest concentration of the extract at which the incubated microorganism was completely killed. This is observed by plating out the test bacteria into enriched BHI agar from the MIC wells which showed no visible growth. The procedure was done in triplicates.

D.DETERMINATION OF ANTIBIOFILM ACTIVITY

Antibiofilm potential of these essential oils was tested on 30 strong biofilm producing strains of *A.actinomycetemcomitans* detected by microdilution, similar to the MIC assay by tissue culture plate method.[17]The essential oils were added to the growth medium at the time of inoculation and the cells were allowed to form biofilm. Serial dilutions of the herbal extracts were prepared in 96 polystyrene tissue culture plate from 200µl/ml to 3.125 µl/ml. Medium without essential oil was used as a non treated well and served as growth control. 100µl of the *A.actinomycetemcomitans* culture grown on tryptic soy broth with 1% glucose was added to each well and incubated at 37^0C in candle jar for 48 hours and the contents of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml phosphate buffer saline and fixed with 2% sodium acetate followed by staining with 0.1% crystal violet. Excess of the stain was rinsed thoroughly with deionised water and biofilm formation was quantified by measuring the absorbance (OD - Optical density) at 570 nm using a microplate reader.[BIORAD, USA] The test was done in triplicates.

The percentage of biofilm inhibition was calculated using the following formula. The biofilm inhibition concentration (BIC_{50}) was defined as the lowest concentration of extract that showed 50% inhibition on the biofilm formation. [18]]

$$BIC_{50} = \frac{[\text{OD growth control} - \text{OD sample}]^*}{[\text{OD growth control}]} \times 100$$

*Growth control: Medium without essential oil
Sample : Medium with essential oil.

E.STATISTICAL ANALYSIS

The results were analysed by mean, standard deviation and t-test using SPSS-23 statistical software. In all cases p value <0.05 was considered significant.

RESULTS

A total of 68 strains were used for the antimicrobial study using disc diffusion method. Out of 68 strains tested against *M.piperita* essential oil 53 (77.9%) were sensitive and 15 (22.1%) were resistant. Among 53 sensitive strains 45 were very active, 7 were active and 1 was partially active. In the case of *M.arvensis* oil out of 68 tested strains 52 (76.5%) were sensitive and 16 (23.5%) were resistant. Among 52 sensitive strains 38 were very active, 10 were active and 4 were partially active. Table 2 shows the result of disc diffusion assay. Mean zone of inhibition for *Mentha piperita* was 24.3mm (Range 18–29 mm) and for *Mentha arvensis* was 22.67mm. (Range 19-29 mm). Doxycycline (30µg) disc showed a mean zone of inhibition of 29 mm. Significant difference was not observed between the antibacterial actions of essential oils. (p value - 1.000, > 0.005, Not significant) Out of 30 strains tested, MIC ranged between 3.125µl/ml to 12.5µl/ml and the MBC was found at 12.5µl/ml for both the essential oils. Table 3 shows the value of MIC assay. The biofilm inhibition concentration (BIC_{50}) was achieved at 100 µl/ml for both essential oils.

Table 2: Results of disc diffusion assay.

Essential oil	No of strains tested	Grading * Sensitive isolates			Total No of sensitive isolates & percentage	Total No of resistant isolates & percentage	Mean zone of inhibition produced	Standard deviation
		Partially active	Active	Very active				
M.piperita	68	1	7	45	53 (77.9%)	15 (22.1%)	24.3	5.268
M.arvensis	68	4	10	38	52 (76.5%)	16 (23.5%)	22.67	5.96

* < 9 mm- Resistant; 9-12 mm – Partially active; 13-18 mm - Active; >18mm – Very active.
(p value= 1.000, > 0.05, Not significant.)

Table 3: **MIC of 30 strains of *A.actinomycetemcomitans* tested against Mentha essential oils.**

Various concentrations of essential oil tested ➡	100 µl/ml	50 µl/ml	25 µl/ml	12.5 µl/ml	6.25 µl/ml	3.125 µl/ml	1.56 µl/ml	0.78 µl/ml
Number and percentage of strains survived in each concentration after the incubation (*Mentha piperita*) ➡	(-)	(-)	(-)	(-)	(+) 03 (10%)	(+) 04 (13.3%)	(+) 30 (100%)	(+) 30 (100%)
Number and percentage of strains survived in each concentration after the incubation (*Mentha arvensis*) ➡	(-)	(-)	(-)	(-)	(+) 02 (6.6%)	(+) 09 (30%)	(+) 30 (100%)	(+) 30 (100%)
A.actinomycetemcomitans ATCC 29522	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)

(+) ➡ Growth present, (-) ➡ Growth absent.

DISCUSSION

A.actinomycetemcomitans is a principal pathogen in aggressive periodontitis with rapid loss of attachment and bone destruction in the periodontal tissues. It is very difficult to eradicate this bacterium from the sub gingival biofilm by conventional periodontal therapy alone. In India >90% of the populations are suffering with periodontal diseases. [19] Not many reports are available from India regarding the pathogenic potential of *A.actinomycetecomitans* and antimicrobial action by herbal extracts.

Essential oil of peppermint was employed in dental care in the ancient times as an analgesic agent for treating toothache. Therapeutic preparations from peppermint leaf were used as mouth rinse to reduce gingival inflammation after periodontal treatment and also to control periodontal pathogens.[20] *Mentha arvensis* or Pudina leaves were extensively used in preparations of food throughout the world. Johnson et al [7] demonstrated the antibacterial property of nodal callus extract of *Mentha arvensis* against gram positive and gram negative bacteria. Menthol, d-carvone,d-sylvesterene and citronellol were found to be responsible for its antimicrobial property. Few investigators had reported the antimicrobial activity of essential oils of medicinal plants other than pudina and peppermint against *A.actinomycetemcomitans.* [21,22]

Out of 68 strains of *A.actinomycetemcomitans* used to demonstrate antibacterial property by disc diffusion method, 53 (77.9%) and 52 (76.5%) were sensitive against *M.piperita* and *M.arvensis* oil respectively. In disc diffusion assay the zone of inhibition produced by very active isolates were showed similar results with standard reference drug, doxycycline (30µg).

The essential oils showed antibacterial activity at low concentration and the MIC ranged between 3.125µl/ml to 12.5µl/ml for both the oils tested. A study by Shapiro et al [24]on antibacterial activity of peppermint oil showed an MIC of 0.1-0.3% (w/v) against reference strain of *A.actinomycetemcomitans*. According to the established parameters reported by Durate etal[25] and Freires et al[26] based on MIC concentration range as a parameter to determine the intensity of antibacterial activity, an essential oil with MIC of ≤100µg/ml is considered to have very strong antibacterial activity with a score of '++++'. Thus the present study proved that the essential oils of mentha have very strong antibacterial activity against *A.actinomycetemcomitans*.

Ability to form biofilm is one of the virulence factors in bacteria. Biofilm formation plays a role in development of drug resistance and facilitate occurrence of chronic bacterial infections.[27] In the present study antibiofilm activity was observed at a concentration of 100µl/ml for both the essential oils. To the best of our knowledge no reports were available regarding the antibiofilm activity of *A.actinomycetemcomitans* by essential oils of mentha and this is the first study of this kind from the Indian subcontinent.

The results of our study provided the justification for the use of these essential oil in oral care products. Mouth washes or rinses contain herbal agents with antibiofilm activity will be helpful in the prevention of biofilm formation which in turn will prevent the orodental colonisation, adherence and infection by *A.actinomycetemcomitans*.

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