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Research article

ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL OF *SYZYGIUM AROMATICUM* (L.) MERR.PERRY (CLOVES) AGAINST CLINICAL ISOLATES OF *AGGREGATIBACTER ACTINOMYCETEMCOMITANS.*

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ABSTRACT: Aim: *Aggregatibacter actinomycetemcomitans* implicated in the etiology of aggressive, chronic periodontitis and other systemic infections. The aim of this study was to investigate the antibacterial efficacy of essential oil of a traditional medicinal plant, *Syzygium aromaticum (*L.*) Merr.perry* (cloves) against the clinical isolates of *A.actinomycetemcomitans* obtained from the patients with orodental infections. **Methodology:** Essential oil of clove flower buds was distilled by Neo-Clevenger's method and the antibacterial activity was tested by disc diffusion and agar punch well method. The minimum inhibitory concentration of clove oil was determined by micro broth dilution method. **Results:** 80.8% of the isolates showed sensitivity against clove oil by disc diffusion technique and the minimum inhibitory concentration of clove oil was ranged between 3.125µl/ml to 12.5µl/ml. **Conclusion:** Essential oil of *Syzygium aromaticum* showed good antibacterial activity and can be considered as alternative treatment option for control of periodontitis and other orodental infections caused by *A.actinomycetemcomitans*.

Key words: *Aggregatibacter actinomycetemcomitans, Syzygium aromaticum*, Essential oil of clove, Minimum Inhibitory Concentration.

INTRODUCTION

Aggregatibacter actinomycetemcomitans, a gram negative capnoic cocco bacillus is a principal pathogen implicated in the etiology of aggressive and chronic periodontitis along with other anaerobic organisms. (Henderson B, et al., 2010). It is also associated with non oral infections like endocarditis, abscess in the neck and lung (van Winkelhoff, et al., 1999) and osteomyelitis of the mandible. (Antony B, et al., 2009) Orodental infections like dental caries, gingivitis and periodontitis are considered as the most common chronic infections in humans. In India prevalence of periodontal disease is estimated approximately as 65-100 %. (Saini S, et al., 2003) The major problem in the recent years in the field of health is the increasing incidence of drug resistance among pathogens. Researchers demonstrated the increasing resistance of *A.actinomycetemcomitans* against antibiotics. (Ardila CM, et al., 2010) In this context it makes essential to find a novel and alternate to existing drugs especially from natural resources for the control of orodental infections. Essential oils and other extracts of plants have evoked interest as sources of natural product in the control of microorganism. (Prabuseenivasan S, et al., 2006) The herbs having the known pharmaceutical properties could be the best source of this alternative medicine if the pharmacokinetic and pharmacodynamic actions were fully evaluated. The plant products are safe, cheap, and easily available and are usually devoid of associated risk of chemotherapeutic agents. *Syzygium aromaticum* (*S.aromaticum*) (synonym: *Eugenia cariophylata*) commonly known as clove, is a median size tree (8-12 m) from the Myrtaceae family native from the Maluku islands in east Indonesia. India is one among the largest producers of clove. Clove oil is known for its germicidal, antioxidant, anti-inflammatory and analgesic properties. Clove had been reported as an analgesic for toothache, join pain and antispasmodic since the 13th century. Eugenol was found to be the main compound responsible for this activity. (Cortes Rojas DF, et al.,2014). The mechanism of action of this component was reported as the disruption of cell membrane and denaturation of protein synthesis in wide variety of microorganisms.(Nunez L, et al., 2011).The characteristic smell of clove oil also helps to eliminate halitosis.(Panditha V, et al.,2014). Several studies have demonstrated potent antimicrobial effects of clove

against various organisms including aerobic bacteria and fungi. (Al-mariri A, et al., 2014 & Thosar A, et al.,2013) No detailed reports are available from India regarding the antibacterial effect of essential oil of clove against *A.actinomycetemcomitans* hence this study was undertaken.

MATERIALS AND METHODS

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 obtained from the subgingival sites of the patients 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. (Olsen A, et al.,1999) 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. (Couturier MR, et al.,2011)

B. Preparation of clove oil extract.

The flower buds of *Syzygium aromaticum* (Clove buds) obtained from a reputed retail store, were submitted for authentication at National Ayurvedic Dietetics Research Institute, Bangalore [Central Council for research in Ayurveda & Siddha, Department of AYUSH, Ministry of Health & Family Welfare, Government of India], [Authentication no: RRCBI-MUS/108]. The authenticated and certified buds were shade dried and reduced to coarse powder. The essential oil was extracted by Neo - Clevenger's method using Clevenger's apparatus. The powder was taken in a round bottom flask with distilled water (1:10dilution). The flask was then fitted to the Clevenger's apparatus and fixed on the mantle for extraction. The mantle temperature was set to 90^oC and the mixture was boiled for about 8 hours. The distillate was collected in the graduated side arm. When 30-40 ml distillate was collected, the boiling was stopped and the oil was separated and collected in a sterile vial. The essential oil was separated by drying using saturated sodium chloride (NaCl), dichloromethane (CH_2Cl_2) and anhydrous sodium sulphate. (Na_2SO_4). (Ayoola GA, et al., 2008 & Faculty of Science, Chulalongkorn University., 2015).

C. Screening of antimicrobial action of clove oil

The essential oil of clove was tested for antimicrobial action against *A.actinomycetemcomitans* isolates, by agar punch well as well as disc diffusion methods.(J.M Wilkinson,.2006).The preliminary, screening test was done by using undiluted oil.

i) Agar punch well or ditch well method

Antimicrobial susceptibility testing was done by Punch well technique on Cation adjusted Mueller Hinton agar (MHA) plate enriched with 0.6% yeast extract. Two wells of 6mm diameter were bored on the surface of enriched MHA plates with the aid of sterile metallic template and cultures of test isolate (Opacity adjusted to 0.5 Mc Farland opacity Standard) were lawn cultured on them($1.5x10^8$Colony forming Unit/ml). One of the well to which 50 µl of DMSO (Dimethyl sulphoxide) was added and was considered as control. 50µl of undiluted clove oil was added to the other well which served as test. The plates were incubated at 37^oC for 48 hours in candle jar. The zone of inhibition was measured in millimeters after the incubation.

ii) Disc diffusion method

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. One milli litre of undiluted essential oil of clove was added 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 37^0C for 48 hours. A disc incorporated with DMSO (Dimethyl sulphoxide) was included as negative control. The disc diffusion and punch well technique was done in triplicate and the mean value of the zone of inhibition in millimeters was calculated. In addition a tetracycline disc (30µg) the drug of choice for *A.a* 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. (Almeida alves et al, 2011).

iii) Detection of Minimum inhibitory concentration (MIC) & Minimum bactericidal concentration (MBC) of clove oil

The micro broth dilution method was done to determine minimum inhibitory concentration (MIC) according to CLSI guidelines. All tests were performed in 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 0.78 µl/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 concentration of the clove oil was 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 and 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. (Schwalbe R, et al.,2007).

RESULTS

A total of 68 isolates were employed for the demonstration of the antimicrobial action of clove oil. In the initial stage of the study 30 strains were subjected to both punch well and disc diffusion methods. The results of the tests were comparable. As disc diffusion method was easier to perform only this technique was performed for the remaining 38 strains.

Out of the 30 strains tested with agar punch well method, 23 (76.66%) were sensitive and 7 (23.33%) were resistant. Among the 23 sensitive strains 3 (10%) were partially active and 20 (66.7%) were very active to the action of clove oil. In the disc diffusion technique, out of 68 strains tested 55 (80.8%) were sensitive where as 13 (19.2%) were resistant. In this technique 47 (69.1%) were very active, 4 strains (5.8%) were partially active and another 4 were active against the antimicrobial property of clove oil. Table 1 show the results of punch well and disc diffusion assay. The zone of inhibition of clove oil varied from 11 to 29 mm and the mean diameter is found to be 23.12 mm for the sensitive strains. Tetracycline disc (30µg) showed a mean zone of inhibition of 29 mm. The very active isolates showed a zone of inhibition similar to that of tetracycline disc. Out of the 30 strains subjected for the determination of MIC of clove oil, results ranged between 3.125 to 12.5 µl/ml. No visible growth of the organism was noted at 12.5 µl/ml concentration. The minimum bactericidal concentration (MBC) of the tested isolates is found to be 12.5 µl/ml. Table 2 shows the MIC values of clove oil against *A.actinomycetemcomitans* strains.

Table 1: Results of punch well and disc diffusion assay.

Methods employed	No of strains tested	Total No of sensitive isolates & percentage	Grading * Sensitive isolates			Total No of resistant isolates & percentage
			Partially active	Active	Very active	
Agar Punch well	30	23 (76.7%)	03 (10%)	00	20 (66.7%)	07 (23.3%)
Disc diffusion	68	55 (80.8%)	04 (5.8%)	04 (5.8%)	47 (69.1%)	13 (19.2%)

*< 9 mm- Resistant; 9-12 mm – Partially active; 13-18 mm - Active; >18mm – Very active.

Table 2: MIC results of 30 strains of *A.actinomycetemcomitans* tested against essential oil of Clove by micro broth dilution.

Various concentration of clove 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
No & Percentage of strains survived in each concentration after the incubation ➡	(-)	(-)	(-)	(-)	(+) 6 (20%)	(+) 9 (30%)	(+) 30 (100%)	(+) 30 (100%)
A.actinomycetemcomitans ATCC 29522	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)

(+) Growth present; (-) No growth.

DISCUSSION

Clove oil possessed many medicinal properties and was used as an analgesic in dental practice from time immemorial. The present study was performed to elucidate the antibacterial activity of essential oil of clove against clinical strains of *A.actinomycetemcomitans* a principal pathogen in aggressive periodontitis. Clove oil and its major components like eugenol and β-caryophyllene were found to have good antibacterial activity against various cariogenic organisms and anaerobic periodontal pathogens as shown in the literature.(Cai L et al.,1996 & Moon S, et al.,2011) Reports demonstrating antibacterial action of clove oil on *A. actinomycetemcomitans* are lacking from India, except a study which showed good antimicrobial activity of aqueous extract of clove against single clinical isolate of *A.actinomycetemcomitans* by disc diffusion assay. (Pathak A, et al.,2011).Hence this study comprising 68 clinical isolates of A. *actinomycetemcomitans* attempts to fulfill the lacunae. Among the 68 strains used in the study 55 isolates showed high antibacterial action.

The antibacterial activity of various herbal products, other than clove oil was tested against *A.actinomycetemcomitans* using agar punch well or disc diffusion method by few investigators. (Ocheng F, et al., 2014 & Satthanakul, et al., 2015). In the present study we compared the efficacy of both the methods in the screening of the antimicrobial property of the essential oil of clove. Disc diffusion was found to be superior to punch well technique, as it is comparatively easy to perform, less cumbersome and consumed less quantity of extract. The results of the study could be compared with Kirby Bauer's disc diffusion technique employed in routine antimicrobial susceptibility testing.

MIC determination was considered more accurate than the disc or agar well diffusion methods, due to the poor diffusion of the extract into the surrounding agar medium might occur in the later two techniques. As the bacterial cells are in direct contact with extract in broth dilution and the MIC values indicate the definite nature of the antibacterial activity of the extract as seen in the literature. (J M Wilkinson, 2006).

In the present study clove oil showed good antibacterial activity in low concentrations and the MIC values ranged between 3.125µl/ml to 12.5µl/ml. Moon et al reported a MIC of 0.8mg/ml against ATCC A.*actinomycetemcomitans* 43717. (Moon S, et al, 2011)

Antibacterial efficacy of clove oil reflected in our study has provided the justification for utilizing therapeutic potential of clove oil incorporated in the oral care products especially for the control of periodontitis caused by *A.actinomycetemcomitans.* The practice of using such herbal formulations as supplements in oral care products or alternative medicine in developing countries like India will reduce the cost of the treatment as well as adverse side effects by the chemo therapeutic agents used for treatment.

CONCLUSION

This study shows that essential oil of Clove has bactericidal effect on *A.actinomycetemcomitans*. This is a promising finding for developing an alternate treatment regimen for the control of orodental infections caused by *A.actinomycetemcomitans*.

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