Original Research Article

In vitro antimicrobial action of plant products of herbal origin on *Aggregatibacter actinomycetemcomitans*

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Abstract

Introduction: Orodental infections and other systemic infections caused by *Aggregatibacter actinomycetemcomitans* is well documented. Increasing resistance by these bacteria against antibiotics is a great concern. Phytochemical agents obtained from herbal plants are an alternative method for the control of infections produced by these drug resistant bacteria. An attempt is done to study the antibacterial efficacy of various plant preparations on *A.actinomycetemcomitans*.
Materials and methods: Alcoholic extracts of betel leaves, mango leaves, aloe vera and boiled extract of arecanut were tested against clinical and standard strains of *A.actinomycetemcomitans* by disc diffusion, MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) detection.
Results: Out of the 68 strains tested by disc diffusion method, the alcoholic extracts *P.betle* showed maximum sensitivity (73.5%) followed by *M.indica* (70.6%) and *Aloevera* (19.1%). Boiled areca nut extract showed no action on tested strains of *A.actinomycetemcomitans*. MIC ranged between 31.25 to 62.5mg/ml and the MBC was 62.5mg/ml for alcoholic extracts of betel leaves, mango leaves and aloe vera.
Conclusion: The present study revealed the antibacterial potential of the herbal plant products tested and can be used as an alternative treatment option for control of periodontitis and other orodental infections caused by *A.actinomycetemcomitans*.

Key words

A.actinomycetemcomitans, Orodental infections, Herbal plant extracts, Disk diffusion, MIC and MBC.

Introduction

Aggregatibacter actinomycetemcomitans a gram negative coccobacilli is implicated in the etiology of aggressive and adult periodontitis and various other systemic infections like endocarditis, abscesses in brain, neck, lungs etc. [1-3]. Considering the increased incidence of orodental infections like periodontitis and the increased resistance to antimicrobials by oral bacteria [4], there is a need of alternative products, such as natural agents. 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 present study is planned to asess the antimicrobial potential of various herbal plant products used in traditional medicines on *A.actinomycetemcomitans*.

Materials and methods

The herbal extracts which are used in traditional medicine were prepared and tested for its antibacterial potential by standard procedures against *A.actinomycetemcomitans.*

Procurement and authentication

The medicinal plants and their products were purchased from reputed retail store as well as from local farmers.

All the medicinal plants and plant products were authenticated by scientists at National Ayurvedic Dietetics Research Institute, Bangalore. Under the control of Central Council for research in Ayurveda and Siddha, Department of Ayush, Ministry of Health and Family Welfare, Government of India and provided with authentication number for each herb. Details of the herb tested are given in **Table - 1**.

Table - 1: Details of herb tested for antibacterial property.

Common name	Scientific name	Extracts used	Authentication No
Aloevera leaf	*Aloe vera* (L.) N.Burman	Alcoholic extracts (Leaves)	RRCBI-AP/178
Betel leaf	*Piper betle* Linn	Alcoholic extracts (Leaves)	RRCBI-AP/6151
Mango leaf	*Mangifera indica*	Alcoholic extracts (Leaves)	RRCBI-MUS/110
Areca nut	*Areca catechu* Linn	Aqueous extract (Fruit)	RRCBI-MUS/114

Preparation of alcoholic extract of *Aloe vera*

Procedure

The alcoholic extract of *Aloevera* was prepared by simple maceration procedure. The *Aloe vera* leaves were washed with detergent, rinsed with water and dried with sterile cotton wool. They were aseptically sliced and 100 grams of the pulp was placed in a 500 ml conical flask stoppered with aluminum foil. 500 ml of 70% ethyl alcohol was added and allowed to stand at room temperature for a period of 24 hours with frequent agitation on a mechanical shaker until the soluble matter had dissolved. The mixture was then filtered, the filtrate was collected in a pre-weighed porcelain dish and the solvent was evaporated at 60^0C by using water bath to yield residue. The filtrate was tested for the sterility on blood agar and the plates were incubated at 37^oC for 24 hours.

Preparation of alcoholic extract of Betel and mango leaves

The leaves of Mango (*Mangifera indica)* and betelleaves (*Piper betle)* were taken separately and shade dried and reduced to coarse powder. The powdered plant materials were then seperately extracted with ethanol by Soxhlation method. 100 g of each herbal powder was filled

in small filter paper bag and placed in thimble of Soxhlet apparatus. 500 ml of 70% ethanol was filled into the round bottom flask of the apparatus and boiled for about 12 hrs, till the solution became colourless. Then the extract was filtered, concentrated by evaporation under reduced pressure using Rotary Vaccum Evaporator, dried and stored in refrigerator in an air tight container. The percentage of yield was noted. The procedure was repeated separately for both the herbs. The extract was tested for sterility on blood agar and the plates were incubated for 24 hours at 37^oC.

Preparation of Areca nut extract

The hot water extract of *Areca catechu* was prepared by boiling 100 g nuts in 500 ml distilled water for an hour. The mixture was then filtered, the filtrate was collected in a pre-weighed porcelain dish and the water was evaporated at 60^0C by using water bath to yield residue.The percentage yield was noted. The sterility was tested.

***In vitro* antibacterial study of herbal extracts**

Disc diffusion technique [6]

The crude herbal extracts were used for the antibacterial study by disc diffusion method. Disc diffusion technique was done on Cation adjusted Mueller-Hinton agar (MHA) plate enriched with 0.6% yeast extract. 18-24 hour culture of *A. actinomycetemcomitans* grown on enriched BHI broth compared with Mc Farland 0.5 standard were swabbed over the agar plate.

One of each 4 various undiluted herbal extracts (Alcoholic extract of aloevera, betel leaf , mango leaf and boiled extract of tender areca nut) were added to set of 100 sterile disc of 6mm diameter. The discs were then placed over the enriched Muller Hinton agar and the zone of inhibition was measured in millimeters for each product after incubation under candle jar (5-10% CO_2) *A.actinomycetemcomitans* ATCC 29522 was also tested along with the clinical strains. The disc diffusion technique was done in triplicate and the mean value of the zone of inhibition in millimeters was calculated. Discs incorporated with DMSO (Dimethyl sulphoxide) the diluent was included as negative control. In addition a Doxycycline disc (30µg) the drug of choice for *A. actinomycetemcomitans* was also used in parallel as a reference antimicrobial compound. 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 [6].

Procedure of MIC detection [7, 8]

The micro broth dilution method was employed for the determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for the isolates of *A.actinomycetemcomitans* according to CLSI guidelines. The extracts which were sensitive in the disc diffusion methods were employed for MIC and MBC detection. The procedure was performed in micro titer plastic plates containing 96 wells. (Tarsons, India). The stock solution for MIC and MBC assay was prepared as follows (**Table - 2**).

Table - 2: Stock solutions of the herbal extracts:

Herbal extracts used	Stock dilution	Concentration
Ethanolic extracts of *P.betle*	1gm extract in 1ml DMSO	1gm/ml
Ethanolic extracts *M.indica*	1gm extract in 1ml DMSO	1gm/ml`
Ethanolic extract of *Aloe vera*	1gm extract in 1ml DMSO	1gm/ml

Procedure of MIC detection

- A Microtitre plate with 96 wells was used for detection of MIC. 100µl of cation adjusted enriched Mueller-Hinton broth was transferred into wells from 2 to 9. (Like B2-B9).
- 100 µl of herbal extract from stock solution was pipette into well 1 and 2 using micropipette.
- Mixed and transferred 100 µl from well 2 to 3 and continued till well 7. (Tips of the pipette were changed between wells to prevent carryover of extracts.)

- Discarded 100 µl from well 7. The 8th well, which served as a growth control, received no extract.
- Selected 4–5 isolated similar colonies of *A.actinomycetemcomitans* from an 48h culture from a enriched brain heart infusion agar (BHI) and diluted in enriched BHI broth to a turbidity comparable to that of a 0.5 McFarland turbidity standard (approximately 1.0×10^8 CFU/mL).
- This suspension was further diluted 1:100 (0.1ml of 0.5 McFarland's matched suspension + 9.9 ml of sterile BHI broth (10^6 CFU/mL) with enriched BHI broth. Within 15 min of the preparation of this inoculum, added 100 µl of bacterial broth suspension to each well except the 9th (last) well, which served as broth control (negative).
- The plates were incubated at 37^0C for 48 hours in candle jar. Placed a plastic seal over the panel to prevent dehydration during incubation.
- The results were read after 48 hours. The extract free growth control well was examined for the viability of the test isolate. This was needed to ensure the culture conditions, such as media, temperature, etc were suitable for the growth of the organism. The broth control tube included in the assay contains everything except the inoculum of test strains. This control tube should remain clear (no growth).
- MIC was considered as the lowest concentration of the antimicrobial agent that inhibited the growth of the microorganism, detected by lack of visual turbidity, matching with a negative control included with the test and recorded in microlitre per milliliter (µl/ml) or milligram per millilitre (mg/ml) depending on the nature of antimicrobial agent used.
- MIC procedure was performed in triplicate and the mean value was taken as the final value.
- A reference drug tetracycline was also tested in parallel as a positive control. (Original concentration 100 µg/ml).

Procedure of MBC detection

- From the tubes which showed no growth in MIC procedure 10µl of the broth was streaked over enriched BHI agar
- The plates were incubated at 37^oC for 48 hours in candle jar.
- The plates were observed for the presence of the growth of the bacteria. The MBC was considered as the lowest concentration of the antimicrobial agent that demonstrated a pre-determined reduction (such as 99.9%) in CFU/ml when compared to the MIC dilution.

Statistical analysis

The data were analyzed used mean, mode and standard deviation. Post hoc analysis by Bonferronis test was used to analyze the significance of antimicrobial action between each compound using SPSS-23 statistical software.

Results

i) Disc diffusion assay:

Sixty eight (68) strains of *A.actinomycetemcomitans* were subjected for the antimicrobial study using Alcoholic extracts of betel, mango and aloevera leaves and boiled extract of areca nut by disc diffusion assay. Details of the results obtained are explained in **Table - 3**.

Out of the 68 strains tested by disc diffusion method the alcoholic extracts *P.betle* showed maximum sensitivity (73.5%) followed by *M.indica* (70.6%) and *Aloevera* (19.1%). Boiled areca nut extract showed no action on tested strains of *A.actinomycetemcomitans.*

ii) MIC and MBC: (**Table - 4, 5, 6**)

In micro broth dilution the alcoholic extracts of the Aloevera, *M.indica* and *P.betle* gave a MIC range of 31.25 mg/ml to 62.5 mg/ml. MBC of the alcoholic extracts of Aloevera, *M.indica* and *P.betle* it was 62.5 mg/ml. The standard reference drug showed an MIC range of 0.78 µg/ml to 1.56 µg/ml. The MBC of tetracycline noted at 1.56 µg/ml.

Table - 3: Antimicrobial Action of herbal extracts.

Extracts	**No of strains tested**	**Grading * Sensitive isolates**			**Total No of sensitive isolates & percentage**	**Total No of resistant isolates & percentage**
		Partially active	**Active**	**Very active**		
Alcoholic extracts of *P.betle*	68	4	9	37	50 (73.5%)	18 (26.5%)
Alcoholic extracts of *M.indica*	68	3	9	36	48 (70.6%)	20 (29.4%)
Alcoholic extracts of *Aloe vera*	68	8	5	0	13(19.1%)	55(80.9%)
Boiled *Areca catechu* extract	68	0	0	0	0 (0%)	68 (100%)

Grading *:

Inactive: < 9mm, Partially active: 9-12 mm, Active: 13-18 mm, Very active: >18 mm. ($\chi 2$=181.095, p value = 0.0001, Highly significant)

Table - 4: MIC of 30 strains tested against Tetracycline.

Standard drug and initial concentration	**Number of strains showed growth against tetracycline**						
	Well 1	**Well2**	**Well 3**	**Well 4**	**Well 5**	**Well6**	**Well 7**
	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.125 µg/ml	1.56 µg/ml	0.78 µg/ml
Tetracycline (100 µg/ml)	0	0	0	0	0	0	2

Table - 5: MIC of 30 strains tested against various herbal extracts.

Alcoholic Extract and initial concentration	**Number of strains showed growth against the herbal extract**						
	Well 1	**Well 2**	**Well 3**	**Well 4**	**Well5**	**Well6**	**Well 7**
	500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml	15.625 mg/ml	7.81 mg/ml
Aloe vera (1gm/ml)	0	0	0	0	12	30	30
M.indica (1gm/ml)	0	0	0	0	15	30	30
P.betle (1gm/ml)	0	0	0	0	14	30	30

Table - 6: MBC of various herbal extracts against *A.actinomycetemcomitans*.

Herbal extract	**MIC**	**MBC**
Alcoholic extracts of *P.betle*	31.25 to 62.5 mg/ml.	62.5mg/ml
Alcoholic extracts of *M.indica*	31.25 to 62.5 mg/ml.	62.5 mg/ml
Alcoholic extracts of Aloevera	31.25 to 62.5 mg/ml.	62.5mg/ml

Discussion

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 such as clindamycin, metronidazole and amoxicillin [9]. In this context it is essential to find a novel and alternate option 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 [10]. 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 usually devoid of associated side effects of synthetic compounds.

In the present study various herbal plants and products which were used for treatment of oral ailments like caries, gingivitis, periodontitis, halitosis and tooth ache in traditional practices were tested for its antibacterial potential against *A.actinomycetemcomitans.*

Essential oil of plants were employed in dental care in the ancient times as an analgesic agent for treating toothache. It is also used as a remedy in halitosis. Mango leaves and its stem is used natural agent for tooth cleaning. Betel leaves applied with lime and other spices along with areca nut chewing is a traditional custom followed in villages of India for its stimulating and psychoactive properties. Aloevera containing preparations are used for treating gum infections.

In this study disc diffusion method was employed for the screening of the antibacterial study. The results of the disc diffusion study could be compared with Kirby Bauer's technique employing doxycycline (30 μg) disc as the control. MIC was detected using microbroth dilution in microtitre plates. MIC and MBC determination was considered more accurate than the disc diffusion methods, where the results may be influenced due to the poor diffusion of the extract into the surrounding agar medium. In addition as the bacterial cells are in direct contact with extract in broth dilution and the MIC and MBC values indicate the definite nature of the antibacterial activity of the extract as seen in the literature [11]. Tetracycline a reference drug also tested in parallel as a positive control for MIC and MBC detection.

Alcohilc extract of Piper betle, Mangifera indica and Aloe vera

Piper betle leaves are traditionally used in India, China and Thailand for prevention of oral malodor, since it has anti bacterial activity against obligate oral anaerobes responsible for halitosis. Aqueous extract of *Piper betle* has also been shown to reduce the adherence of early dental plaque bacteria. Leaves of *P.betle* have a strong pungent and aromatic flavor. They are used as a mouth freshener, for their wound healing property [12], digestive and pancreatic lipase stimulant activities in traditional medicine [13]. In the present study ethanolic leaves extract of *Piper betle* showed good antibacterial property with disc diffusion method. Among 68 isolates tested 50 isolates showed high antibacterial action (73.5%). The MIC ranged between 31.25mg/ml to 62.5 mg/ml. The MBC concentration was 62.5mg/ml. In a study by Sharma, et al. [14] when hydroxyhavicol an active compound from *Piper betle* oil was used, MIC range of 250-500 μg/ml and MBC range of 500-1000μg/ml was noticed against *A.actinomycetemcomitans*. The low concentration of MIC may be explained by the postulate that the separated active components of plant extract contain a higher concentration of bioactive compounds compared to crude form [15]. In a study by Khan, et al. methanolic and ethanolic extract of *Piper betle* showed MIC of 0.0021 mg/ml to 8.196 mg/ml against pathogenic gram positive and gram negative aerobes [16]. The variations in MIC may be described by the fact that the secondary metabolites responsible for demonstrating antibacterial activity are

greatly dependent on type of solvent system used for the extraction of metabolites from the plant sources [17]. Moreover, the geographical area and environment also affects the chemical composition of the plants and leads to the variation in activity [18].

Mango leaves possess pharmacologically and medicinally, important chemical like mangiferin, a polyphenolic antioxidant and a glucosyl xanthone, it has strong antioxidant, anti lipidperoxidation, immunomodulation, cardiotonic, hypotensive, wound healing, anti-degenerative, and anti-diabetic activities. Researchers had found antibacterial, antifungal, anti-helmintic, and anti-parasitic effects of mango leaves extract [19]. Mangiferin has also demonstrated promising therapeutic potential both in the prevention and treatment of periodontitis [20]. In the present study the crude extract of mango leaves showed good antibacterial action against *A.actinomycetmcomitans*. In disc diffusion method 48 strains showed sensitivity (70.6%). The MIC was ranged between 31.25 mg/ml to 62.5 mg/ml and the MBC value was 62.5 mg/ml. No reports are available from India regarding the in vitro studies on the antibacterial potential of mango leaves extract on *A.actinomycetemcomitans.* Shaban, et al. [21] showed aqueous and ethanol extract of leaves and stems of mango at 50 and 25 mg/mL was found effective against aerobic Gram positive and Gram negative pathogens and Candida.

Aloe vera used in dental practice for the treatment of gum diseases like gingivitis, periodontitis [22]. It reduces bleeding, inflammation and swelling of the gums. It is a powerful antiseptic in pockets where normal cleaning is difficult, and its antifungal properties help greatly in the problem of denture stomatitis [23], apthous ulcers, cracked and split corners of the mouth [24]. It is a powerful healing promoter and can be used following extractions [25].

In the present study alcoholic extracts of Aloe vera does not exhibited promising action against *A.actinomycetemcomitans.* In disc diffusion method only 13 strains were showed sensitivity. (19.1%). MIC and MBC were achieved at 125mg/ml. In a study by Fani, et al. [5] showed MIC of 25 μg/ml against reference strain of *A.actinomycetemcomitans* with fresh Aloevera gel. The difference in the solvent system used for extraction and composition of the extract material or the natural resistance showed by the isolates are the possible reason for the varying results in this study.

Boiled extract of Areca catechu extract

Changes in oral microbial flora are mainly associated with deleterious oral habits such as cigarette smoking and betel quid chewing. The effects were due to a direct inhibitory effect on the bacteria themselves or indirectly by a change in the oral environment due to host response. Researchers showed the antimicrobial effect of areca nut extract against oral bacteria, including *S.mutans*, *S.salivarius*, *C.albicans* and *F.nucleatum* and pathogenic Gram negative aerobic organisms [26, 27]. The present study was unable to confirm a direct antimicrobial effect of boiled areca nut extract on *A.actinomycetemcomitans.* According to De Miranda, et al. [27] baked and boiled nuts showed significantly more potent action than raw nut. Tannic acid content in the areca nut extract is responsible for the antimicrobial action. In a similar type of study Hung, et al.; the raw areca nut extract and the active component arecoline and tannic acid does not show an antibacterial effect on *A.actinomycetemcomitans* and other periodontal pathogens. The difference in the results with Anthikat, et al. and de Miranda, et al. may be due to the varying quantity of active ingredient in the final extract. It may also vary in areca nuts collected from different locations or types of processing of betel quid [28]. The resistance of the clinical strains of *A.actinomycetemcomitans* itself may be may be another reason for the diminished action of areca nut extract.

Conclusion

Antibacterial efficacy of extracts of various medicinal herbs reflected in our study has provided the justification for utilizing therapeutic potential of these products 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.

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