

Use of Traditional Indian Plants in the Inhibition of Caries-Causing Bacteria - *Streptococcus mutans*

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The aim of the study was to comparatively evaluate the antibacterial activity of six Indian plant extracts and 0.2% chlorhexidine against clinical strains of *Streptococcus mutans*, which were isolated from the plaque samples of 45 pediatric patients. Six plant extracts were prepared in three different forms, namely aqueous extracts, organic solvent-based extracts and crude (raw) extracts. The antimicrobial sensitivity testing was done by agar well diffusion method. Antimicrobial activity of the extracts was determined by measuring the mean zones of inhibition (mm) produced against the bacterial isolates. Results showed that crude garlic extract exhibited greater antibacterial activity than chlorhexidine. Aqueous extract of amla and organic solvent-based extract of ginger showed the maximum antibacterial activity against *S. mutans*, whereas aqueous extract of tulsi and organic solvent based extract of amla showed the minimum antibacterial activity. This study suggests that plant extracts like garlic in crude form, amla as aqueous infusion and ginger as alcoholic tincture have potential for the control of *S. mutans*. These extracts can be used as an alternative remedy for dental caries prevention or in the form of mouthwash, which is safe and economical.

Introduction

Dental caries is one of the most prevalent diseases in humans, second only to common cold (1). Studies have revealed that *Streptococcus mutans* average from 20-40% of the cultivable flora in biofilms removed from carious lesions (2). In addition to dental caries and related pyogenic dental infection, *S. mutans* is also a very important endocarditis agent. The participation of the microorganism in both oral and non-oral diseases has prompted interest in the knowledge of its susceptibility to antimicrobial agents. However, the antibiotics are inappropriate for routine use as antiplaque agents and should be restricted for use in medicines (3). Amongst all the antibacterial agents, chlorhexidine is a broad spectrum antimicrobial which is effective against both Gram-positive and Gram-negative microorganisms.(4)

In dentistry, chlorhexidine at 0.2% concentration became the standard international concentration for plaque control. However, it has been found to have a number of adverse side effects, like alteration in taste, staining of teeth, restorative material and the dorsum of the tongue, as well as supragingival calculus formation.

Hence, scientists are shifting their attention to folk medicine in order to find new leads for better drugs against microbial infections. Plant materials are known as source of new antimicrobial agents,

as a result search has been made to discover new antibacterial drugs of plant origin (5).

Numerous medicinal plant extracts or phytochemicals have been shown to inhibit the formation of dental biofilms by reducing the adhesion of microbial pathogens to the tooth surface, which is a primary event in the initiation and the progression to dental decay (6,7).

Aloe vera (*Aloe barbadensis* Miller) gel includes anti-inflammatory, antioxidant properties and has shown the inhibitory effects on some periodontopathic, cariogenic and opportunistic pathogenic bacteria (8).

Amla (*Emblica officinalis*) is a rich source of vitamin C. The fruit has anti-viral, antibacterial, anti-cancer and anti-allergy properties (9).

Garlic (*Allium sativum*) is one of the most extensively researched medicinal plants and its antibacterial activity depends on the allicin produced by enzymatic activity of allinase (a cysteine sulfoxide lyase) after crushing or cutting garlic clove (10), which is responsible for its antimicrobial effects.

Ginger (*Zingiber officinale*) has been widely used in herbal medicines all over the world, being a common herbal remedy for the prevention and treatment of various diseases including dental infections.(11).

Neem (*Azadirachta indica*) is the most versatile plant with immense potential. It is known to have antiallergenic, antifungal, anti-inflammatory and

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other biological activities. Neem could reduce the ability of Streptococcal bacteria to colonize on the surface of teeth (12).

Tulsi (*Ocimum sanctum*) is bestowed with enormous antimicrobial substances and is used to treat a variety of illnesses. Eugenol, ursolic acid and carvacrol, the active components of Tulsi, are responsible for its antimicrobial activity (13).

The drawback in plant extracts use, in comparison with synthetic molecules, can be the time consumption, need for elaborate apparatus to isolate and characterize active molecules. The isolation of active components faces many other challenges like inconsistency of source material, obscurity in isolating active components and the cost of extraction. In addition to that, plants like ginger and garlic have shown increased risk of bleeding whereas neem and aloe vera have shown side-effects like allergic reactions (14).

The impetus of the study was paucity of literature about the comparison of antimicrobial activity of aloe vera, amla, garlic, ginger, neem and tulsi against caries-causing micro-organisms like *S. mutans*, in addition to being feasible, cost-effective and possessing known medicinal properties. Chlorhexidine is still being used as a gold standard against which other antimicrobial agents are compared.

Therefore, in the present research, attempt was made to compare the antibacterial susceptibility of six Indian plants (Aloe vera, Amla, Garlic, Ginger, Neem and Tulsi) with that of chlorhexidine against the clinical isolates of pioneer bacteria in dental caries - *S. mutans* - by the agar well diffusion method.

Material and Methods

The study was conducted on 45 children with age ranging from 3 to 15 years for procurement of clinical strains of *S. mutans*, who belonged to either Grade 2 or Grade 3 of caries susceptibility, obtained using the Dentocult SM strip mutans kit. Parents of selected patient were made aware of the experimental design and written informed consent was obtained before the study.

Preparation of the Extracts

Preparation of crude, aqueous and organic solvent based extracts was done by following the method of extraction, mentioned in one of our similar study (15).

Crude (raw) extracts were prepared by finely grinding the raw selected plant parts (Aloe vera leaves, Amla fruits, Garlic bulbs, Ginger rhizomes, Neem and Tulsi leaves) which were later filtered to obtain a homogenous mixture. The filtrate was then

used for antimicrobial sensitivity.

Dried powder of the selected plant parts (10 g) were heated in distilled water for 6 h at slow heat, which was later filtered and centrifuged at 5,000 rpm for 15 min to obtain aqueous extracts. The supernatant was concentrated to make the final volume one-fourth of the original volume.

Successive extraction of these powders (10 g) was done with the help of Soxhlet apparatus in different organic solvents (chloroform, acetone, ethanol) with increasing polarity of the solvent at 80 °C for 8 h. Eventually, all the extracts dissolved in various solvents were mixed together to obtain a final extract, which was later concentrated using a rotary evaporator.

The extracts were autoclaved and stored at 4 °C in airtight bottles for further use. Finally, 2 mL of stored plant extracts were dissolved in 1 L of dimethyl sulfoxide (organic solvent-based extracts) and sterile distilled water (aqueous extracts) to make the final plant extract to be tested using antimicrobial assay.

Sample Collection

The samples were collected as per the instructions given in the Dentocult SM Strip Mutans Kit. (Orion Diagnostica, Espoo, Finland) (16). A bacitracin disk was placed in the selective culture broth 15 min prior to the sample collection and the vials were closed tightly. The test strips were then inoculated according to the type of sample, i.e., square tipped test strip is for the plaque sample and round tipped test strip for the salivary sample.

Plaque samples were collected aseptically by sterile toothpicks from the interproximal site and tooth surface of the carious teeth, and were spread thoroughly but gently on the four sites of the rough surface of the square-tipped test strip.

The strips were placed with the smooth surfaces clipped and attached to the cap, in the selective culture broth. The vials were then labeled with the patient's name and age and incubated in an upright position at 37 °C for 48 h with the cap open one quarter of a turn to allow the growth of the organisms.

The adherent mutans streptococci appeared as light to dark blue, elevated colonies on each strip. The number of colony forming units on a predetermined area of the strip (10×8 mm, strip width) were counted under 10× magnification in square tipped (for plaque sample) strip. The strips were checked and grades were classified according to the density of the *S. mutans* colonies compared with the standard model chart.

The samples were then subcultured from the test strips by picking up a single blue colored colony onto

the surface of blood agar and incubated aerobically at 37 °C for 24 h to confirm the isolation of *S. mutans*. It was then phenotypically identified using standard microbiological methods like Gram staining, catalase test and other confirmatory biochemical tests. A suspension of peptone water broth was made and adjusted spectrophotometrically to an optical density of 560 nm corresponding to match the turbidity of McFarland 0.5 scale [1.5×10^8 colony-forming unit per milliliter (CFU mL⁻¹)] and later incubated at 37 °C for 24 h.

The agar well diffusion method was employed, where the freshly prepared inoculate containing the bacterial isolates in 10 mL of sterile peptone water broth (Himedia Laboratory Pvt. Ltd., Mumbai, India) was spread uniformly over the surface of 20 mL of Mueller Hinton Agar plate (Himedia) using sterile cotton swabs. Seven wells (6-plant extracts, 1-positive or negative control) of 6 mm diameter were bored and 100 µL of different extracts were dropped into each well in the plate using a micropipette. The plates were incubated at an upright position at 37 °C for 24 h. Antimicrobial activity of the extracts was determined by measuring the mean zones of inhibition in millimeter for the bacterial isolates.

Zones of microbial inhibition around the wells containing the test material were measured with a pair of vernier calipers and recorded after the incubation. The shortest distance (mm) from one point of microbial growth to another around the well was considered as the 'zone of inhibition'. (The diameter of zones of inhibition comprises the diameter of each well, 6 mm, as well). The measurements were repeated four times and their mean was taken as the final measurement.

The size of the zone of inhibition determined the effectiveness of the plant extract in inhibiting the *S. mutans* growth. The plant extract's antibacterial

property is most evident when the zone of inhibition is larger. If the zone of inhibition is larger, the effect of the plant extract is high, even at lower concentrations of plant extract. The test was also confirmed using negative controls (control for solvent extract: dimethyl sulfoxide; control for aqueous extract: sterile distilled water), which had no antibacterial effect on *S. mutans*. All assays were performed under aseptic conditions and experiment was repeated three times using three separate culture plates with inocula derived from the same initial pure culture.

Statistical Analysis

All the data were entered into an Excel Spreadsheet – 2007 version (Microsoft Inc., Redmond, WA, USA) and then imported to SPSS (Statistical Package for Social Sciences) version 16.0. Data were expressed as mean \pm S.D. (standard deviation). ANOVA was used for inter-group comparisons and post hoc Bonferroni was used for intra-group comparisons.

Results

Chlorhexidine had the maximum antibacterial efficacy against *S. mutans* as a positive control. The results showed that the maximum inhibitory activity was shown by garlic, and the minimum inhibitory activity was shown by amla, whereas aloe vera, ginger, neem and tulsi showed relatively no inhibitions in crude form (Table 1).

In context of organic solvent-based extracts, ginger was most and amla was least effective of all the extracts (Table 1).

Among the aqueous extracts, amla showed the maximum inhibitory activity and tulsi showed the minimum inhibitory activity against the strains of *S. mutans* (Table 1). No zone of inhibition was observed with distilled water and dimethyl sulfoxide.

Table 1. Mean zone of microbial growth inhibition (mm) (mean \pm standard deviation) provided by different plant extracts in different formulations

Plant extracts	Number of samples	Crude form	Organic solvent form	Aqueous form	p value
Chlorhexidine (control)	45	20.07 \pm 1.13 (HS)	20.02 \pm 0.22 (HS)	20.09 \pm 0.79 (HS)	
Aloe vera	45	6.38 \pm 0.68 (R)	14.89 \pm 1.19 (S)	12.09 \pm 1.69 (S)	
Amla	45	13.22 \pm 1.53 (I)	10.27 \pm 1.23 (I)	17.96 \pm 1.67 (S)	
Garlic	45	20.87 \pm 0.89 (HS)	11.93 \pm 1.49 (I)	15.29 \pm 1.68 (S)	0.00
Ginger	45	6.16 \pm 0.36 (R)	17.58 \pm 1.36 (S)	13.82 \pm 1.12 (I)	
Neem	45	6.42 \pm 0.86 (R)	13.78 \pm 1.14 (I)	16.67 \pm 1.32 (S)	
Tulsi	45	6.31 \pm 0.55 (R)	16.53 \pm 1.25 (S)	11.07 \pm 1.37 (I)	

HS=Highly susceptible (\geq 20 mm), S=Susceptible (\geq 14 mm), I=Intermediate (8-14 mm), R=Resistant (\leq 8.0 mm).

ANOVA depicted significant difference amongst all the groups. Post hoc Bonferroni test also revealed statistically significant differences in intra group comparisons.

Discussion

A rekindled interest in the pharmaceutical importance of plants has led to the discovery and adoption of plant extracts as its global need comes from the prevalence of oral diseases, increased resistance by bacteria to antibiotics, adverse affects of certain antibacterial agents currently used in dentistry and financial considerations in developing countries (17).

The design criterion for this research was to identify the leads, determining antibacterial susceptibility and preliminary screening of the plant extracts. The results of the agar diffusion test are "qualitative" in the category of susceptibility (i.e. susceptible, intermediate or resistant) rather than quantitatively determining the minimum inhibitory concentration (MIC). However, high activity in the agar diffusion assay does not necessarily correlate to low MIC values in the microtitre plate method (18).

The major challenges associated with screening of natural products for antibacterial activity could be summarized as follows: the viscosity or hydrophobicity associated with essential oils or active components creates difficulty in obtaining a stable dispersion of the oils in aqueous media; the problems associated with the diffusion of lipophilic oil components through agar; the determination of the number of viable bacteria remaining after incubation with the oily component (broth dilution method).

Hence, in the present study, agar well diffusion method was chosen and dissolution of solvent based extracts was done in dimethyl sulfoxide (DMSO) which is miscible with both organic solvents and aqueous media. It works as a surfactant that easily gets penetrated in the agar. Since it is also an oxidizing agent, it neutralizes the antiseptic effect of ethanol. To confirm the effect of dimethyl sulfoxide, it was also evaluated against the bacterial isolates as negative control. DMSO was used as a solvent for plant extracts. It is a highly polar aprotic solvent, which is capable of dissolving both polar and nonpolar components. It has a proven safety record in humans, is an effective cell penetration enhancer and helps to bring out the properties of all the components of the herb being dissolved. DMSO showed no antibacterial activity in the agar diffusion test (19).

Successive isolation of botanical compounds

from plant material largely depends on the type of solvent used in the extraction procedure. Traditionally, water is used as a universal solvent for plant extract preparation, but Parekh and Chanda (20) found that plant extracts prepared with methanol and ethanol as solvents provided consistent antimicrobial activity. However, some studies demonstrated a comparable antimicrobial activity of aqueous extract with their alcoholic extract.

The active components of aloe vera are aloin and aloe-emodin. They inhibit protein synthesis by bacterial cells, which explains the antimicrobial activity of aloe vera (8). Whereas flavonoids present in amla have anti glucosyl-transferase activity in addition to the binding of active components to the proteins associated with the cell surface of bacteria (9). Likewise, the antibacterial activity of garlic is widely attributed to allicin, which interferes with RNA production and lipid synthesis that leads to improper formation of the phospholipid bilayer of the cell wall in both Gram positive and Gram negative bacteria (10). Ginger possesses gingerols responsible for the cell membrane rupture, which leads to the direct inhibition of the bacteria (11). Gallantoin present in neem is an important group of active components which is responsible for glucan inhibition and other virulence factors promoting plaque formation (12). The therapeutic potential of *Ocimum sanctum* has been found to be largely due to eugenol, carvacrol, ursolic acid, methyl eugenol, caryophyllene which is responsible for the protein leakage in the bacteria, contributing to its antibacterial effect (13).

Plant extracts have shown inhibitory effect on the growth of the studied bacteria. It is therefore recommended that the nature and number of the active antibacterial principles involved in each plant extract be studied in detail. Therefore, in the present investigation, all the plant extracts were prepared using water and a mixture of organic solvents as solvents, to give equal opportunities to all the active components of that particular extract to get dissolved in the solvent according to their polarities. However, there is very scarce literature on the use of raw form of plant material against some bacterial strains, but in the present investigation, the ground plant material (whole leaves of aloe vera, fruit of amla, garlic bulbs, ginger rhizomes, neem and tulsi leaves) as such (raw form) was used to evaluate the variation in the antibacterial activity after drying and dissolution in the solvents.

This study had shown that crude extract of garlic, ethanolic extract of ginger and aqueous extract of

amla possess reasonable activity against *S. mutans*. Consequently, there is a high possibility of these extracts to behave as an adjuvant in the chemical control of biofilm, which is a natural habitat for the caries causing bacteria, in association with proper oral hygiene, and controlled use of fluorides in dentifrices and other oral healthcare products such as mouthwashes.

It should be pointed out that in very few studies in the literature, the antibacterial activity of herbal extracts or mouthwashes was assayed against *S. mutans*.

All the extracts included in this study also have been generally recognized as safe (GRAS) for their intended use by U.S. Food and Drug Administration (FDA) as per 21 CFR section and 21 CFR 184.1257.

Comparisons with pertinent data from the literature indicate that, according to the methodology adopted in studies on antimicrobial activity of plants, the most diverse results can be obtained. Various clinical trials on human beings have recommended the usage of all the six extracts in different forms for the treatment of several ailments.

Similar clinical studies have revealed the role of garlic juice results were shown by Kivanc et al. (21), where fresh garlic juice had the maximum inhibitory activity against all the tested bacteria, viz *Bacillus cereus*, *B. subtilis*, *Escherichia Coli*, *Staphylococcus aureus*, etc. The results were also agree with the study by Adeshina (22) where fresh ginger juice showed no antibacterial activity against *Porphyromonas aeruginosa*, *S. aureus*, *E. coli* and with another study done by Azu et al. (23) on antimicrobial efficacy of ethanolic and aqueous extracts of ginger on *S. aureus* and *P. aeruginosa*, indicating that active components of ginger extracts dissolved easily in organic solvents rather than in distilled water.

But the present results of amla extracts were in disparity with the study by Sharma et al. (24), which demonstrated the limited effectiveness of aqueous extracts of amla than its organic solvent variant against *S. aureus*, *B. cereus*, *B. subtilis* and *E. coli*. Although the bacteria used in the studies are different, all the results correspond to the theory that the active components present in this extract have the capability of destroying bacterial cell wall, which will inevitably inhibit the growth of bacteria. Hence, this plant can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals that address unmet therapeutic needs.

Although the information from all these clinical

studies are not strictly relevant to the caries control, the safe use of the extracts for the treatment of various human ailments and in food products generally implies that their use will most likely be safe for caries control. The evaluation of toxicity and allergic potential of these plant extracts was not included as part of this study. Hence, a conclusive comment on the toxicity and allergic potential of these plant extracts cannot be made. However, further studies are required to isolate and identify their active compounds and their influence on disruption of planktonic or biofilm formation to prevent and control caries and periodontal diseases.

The proposed trial of the extracts shall result in their clinical validation for the prevention of dental caries. Clinical proof of efficacy can then be used in marketing of the plant extracts as therapeutic agents. Industry and commercial partners will facilitate commercialization of these plant extracts, in the form of dry extracts or oils, should clinical efficacy be demonstrated.

In conclusion, the results obtained confirm the traditional anticipation of the antimicrobial effectiveness and therapeutic applications of the examined plants. To the best of knowledge, as few studies have been done on antimicrobial effects of Indian medicinal plants against oral pathogen (*S. mutans*), it is better that the effect of herbal extracts on other oral bacteria that have cariogenic activity be studied. Because of the antimicrobial effects of some medicinal plants, which have minimal side effects in comparison with chemical drugs, more *in vivo* and *in vitro* investigations about the oral cavity flora may be recommended. It is suggested that more research should be carried out to find plants with antimicrobial activity for producing herbal mouthwashes, which can have comparable preventive potential to the synthetic agents.

Over the ages, the whole dental fraternity has been relying on some modes of prevention of dental caries such as various gels, varnishes, mouthwashes, chemicals, etc. Besides a number of side effects associated with them, these chemotherapeutic drugs are unaffordable to a large mass of population in developing countries.

As synthetic mouthwashes and gels like chlorhexidine have the risk of ingestion in very small children, hence a natural alternative is contemplated which is safe, economical and feasible.

The observations in the present investigation can facilitate in forming the basis for further phytochemical studies to isolate active compounds,

elucidate the structures, evaluate them against a wider range of bacterial strains, dental plaque and *in vivo* models and may also be tested for their safety and efficacy to find new therapeutic principles against infectious disease. Further investigations are warranted to determine whether mouth-rinses and other oral preparations with antibacterial effects may be fabricated from these plants.

Although most of the extracts were less potent than chlorhexidine, still the plant extracts were relatively effective in inhibiting the growth of oral bacteria *in vitro*.

This was a preliminary study to evaluate the antimicrobial ability of these plant extracts against *S. mutans*. The obtained outcomes indicate that these extracts can be processed in the form of a natural antimicrobial mouth rinse alternative for patients who wish to avoid alcohol, artificial preservatives, artificial flavors and colors.

Resumo

O objetivo do estudo foi avaliar comparativamente a atividade antibacteriana de seis plantas indianas contra linhagens clínicas de *Streptococcus mutans*, que foram isoladas das amostras de biofilme dental de 45 pacientes pediátricos, com 0,2% de clorexidina. Seis extratos vegetais foram preparados em três formas diferentes, a saber, extratos aquosos, extratos à base de solventes orgânicos e extratos brutos. Os testes de sensibilidade antimicrobiana foram realizados por método de difusão em agar. A atividade antimicrobiana dos extratos foi determinada através da medição da zona de inibição, em milímetros, produzida contra os isolados bacterianos. Os resultados mostraram que o extrato de alho cru apresentou maior atividade antibacteriana do que a clorexidina. O extrato aquoso de amla e o extrato à base de solventes orgânicos de gengibre mostraram a máxima atividade antibacteriana contra *S. mutans*, enquanto o extrato aquoso de tulsi (manjeriçã) e o extrato à base de solventes orgânicos de amla mostraram mínima atividades antibacteriana. Este estudo sugere que extratos de plantas como o alho em forma bruta, amla como infusão aquosa e gengibre como tintura alcoólica tem um potencial para o controle de *S. mutans*. Estes extratos podem ser utilizados como uma via alternativa para a prevenção de cáries dentárias ou sob a forma de bochechos, que são seguros e econômicos.

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