Original Article

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Dentistry Section

Comparative Evaluation of Antibacterial Efficacy of Six Indian Plant Extracts against *Streptococcus Mutans*

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ABSTRACT

Introduction: To assess the antimicrobial efficacy of six plant extracts of Indian origin often used as traditional medicine against standard strains of *Streptococcus mutans.*

Materials and Methods: The antimicrobial activity of six plant extracts was determined by the agar well diffusion method. The minimum inhibitory concentration (MIC) for the crude (raw), Organic solvent based, aqueous extracts was determined by the agar well diffusion method.

Results: Out of all the six extracts evaluated, organic solvent based and aqueous extracts of all the extracts were found to have variable antimicrobial activities against the oral pathogen. The crude extract of Garlic was the most effective against *Streptococcus mutans* with the highest zone of inhibition (24.62 mm) followed by the aqueous extract of Amla (19.47mm) and organic solvent based extract of Ginger (18.76 mm).

Conclusion: Despite of the fact that the extracts were not pure compounds and antimicrobial results were obtained. This recommends the potency of these extracts. The figment of the derivation of antimicrobial compounds from plants seems lucrative as it will lead to the development of a phytomedicine to act against microbes.

Keywords: Antibacterial activity, Minimum inhibitory concentration, Plant extracts, *Streptococcus mutans*

INTRODUCTION

Oral diseases continue to be a major health problem worldwide[1]. *Streptococcus mutans* is the major organism implicated with dental caries and oral infections [2].

Microbial resistance to most of the antibiotics commonly used to treat oral infections (penicillins and cephalosporins, erythromycin, tetracycline and derivatives and metronidazole) has been documented [3]. The resistance of microorganisms against the traditional antibiotics needs urgent attention for the development of the new drug molecules. It is well documented from ancient times that active principles from plant origin have been used as medicines for various diseases and microbial infections [4]. A wide variety of medicinal plants used traditionally have not yet been systematically investigated against various microbial pathogens [5].

Being a common and cheap herbal remedy for the prevention and treatment of various diseases due to its incredible antimicrobial activity, selected Indian plants like Aloe vera, Amla, Garlic, Ginger, Neem and Tulsi have been widely used in Ayurvedic and Tibb-unani herbal medicines all over the world.

Aloe vera (*Aloe barbadensis miller*) is a cactus-like medicinal plant of *Liliaceae/Aloeaceae* family that grows readily in hot dry climates. As studied *in vitro* and *in vivo*, at optimum concentration, the pharmacological actions of Aloe vera gel comprise anti-inflammatory, antibacterial, antioxidant, immune boosting and hypoglycemic properties [6].

Amla (*Emblica officinalis*), a member of the family *Euphorbiaceae*, is a rich source of Vitamin C. It is rich in phenols, tannins, polyphenols, flavonoids, kaempferol, ellagic acid and gallic acid [7]. Garlic (*Allium sativum*) from the family *Alliaceae*, 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 [8].

Ginger (family *Zingeberaceae*), has been widely used in Chinese, ayurvedic and Tibb-unani herbal medicines for the prevention and treatment of various diseases including dental infections due to its incredible antimicrobial activity [9]. Neem (*Azadirachta indica*), which belongs to family *Meliaceae,* is the most versatile, multifarious trees of tropics, with immense potential [10]. Tulsi, scientifically known as *Ocimum sanctum* (family *Lamiaceae*), is a time-tested premier medicinal herb of Indian origin, worshipped by Hindus and used in Ayurvedic medicine. It is bestowed with enormous antimicrobial substances and is used to treat a variety of illnesses ranging from diabetes mellitus, arthritis, bronchitis, skin diseases, etc., [11].

The present study has been designed to assess the antimicrobial efficacy of six plant extracts (Aloe vera, Amla, Garlic, Ginger, Neem and Tulsi) against most common bacterial oral pathogen, *Streptococcus mutans*.

MATERIALS AND METHODS

The study was conducted in the Department of Microbiology, Adinath Hospital, Indirapuram, Ghaziabad in the month of October 2013.

All the selected plant products (leaves of Aloe vera, Neem, Tulsi; Fruits of Amla; Cloves of Garlic; Rhizomes of Ginger) were collected from a local market in Delhi. Plant materials were washed under running tap water and processed (Garlic was peeled, Amla and Ginger chopped into small pieces and Neem, Tulsi and Aloe vera leaves taken as such), and completely dried in the sunlight for 20 days and then homogenized to fine powder and stored in airtight bottles (Nair and Chanda) [12].

Preparation of the Extracts (Aloe Vera, Amla, Garlic, Ginger, Neem And Tulsi)

Preparation of Crude Extracts

All the fresh plant parts were collected and ground finely in a grinder to obtain a homogenous texture and filtered through Whatman filter paper no. 1. This filtrate was then used for antimicrobial sensitivity.

Preparation of Aqueous Extracts

Aqueous extract was prepared by heating ten grams of dried powder in distilled water for six hours at slow heat.

Two hours after removing from heat, it was filtered through eight layers of muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected and was concentrated to make the final

volume one-fourth of the original volume and then autoclaved at 121ºC and 15 lbs pressure before storing at 4ºC.

Preparation of Organic based extracts

Successive extraction of these powders (10 gm) was done with the help of Soxhlet apparatus in different organic solvents with increasing polarity of the solvent. The solvents included were Chloroform, Acetone, Ethanol (100 ml each). The powdered plant material was extracted with the appropriate volume of solvent at 80ºC for 8 h. After that, all the extracts dissolved in various solvents were mixed together. The final extract was concentrated by distilling off the solvent by a rotary evaporator. It was stored at 4ºC in airtight bottles for further use.

Finally, 2 ml of stored plant extracts were dissolved in 1L of Dimethyl sulfoxide (organic solvents based extracts) and Sterile Distilled water (aqueous extracts) to make the final plant extract to be tested using Antimicrobial assay.

Test Microorganisms

Oral pathogenic bacteria – *Streptococcus mutans* (MTCC*497) were obtained from Microbial Type Culture Collection, IMTECH, Chandigarh. The microorganisms were subcultured on the specific media Brain heart infusion agar and incubated aerobically at 37ºC. The media were procured from HiMedia Laboratory Pvt. Ltd., Bombay, India. Identification of all the strains was confirmed by standard biochemical and staining methods [13-15].

Screening for Antimicrobial Activity

Antimicrobial activity of the six extracts was determined by following the agar well diffusion method of Okeke [16]. In this method, pure isolates of microorganism was subcultured on the recommended specific medium at 37ºC for 24h. One hundred microlitres of inoculum of each test organism was spread onto the specific media plates (containing Mueller Hinton Agar) and 6 mm-diameter wells were bored with a sterile borer in the inoculated agar plates [17].

A 100µl volume of each extract was propelled directly into the wells (in triplicates) of the inoculated specific media agar plates for each test organism. The plates were allowed to stand for 10 min for diffusion of the extract to take place and incubated at 37ºC for 24h [18-20]. Sterile Distilled water and 20% DMSO served as the negative control, and 0.2% Chlorhexidine served as the positive controls.

The antimicrobial activity, indicated by the formation of an inhibition zone surrounding the well containing the extract and the zone of inhibition was measured in millimeters, using vernier calipers. The experiments were performed in triplicates and the mean values of the diameter of inhibition zones with ± standard deviation were calculated.

Determination of MIC [21]

MIC was determined using broth dilution method in 96-well plates using Tryptic soy broth (TSB) at 37ºC for 24 h following the method used by Chaiya et al., The extracts were dissolved in 20% DMSO (for organic solvents) and Distilled water (for aqueous extracts) to obtain 200 mg/ml, and were then diluted by serial two-fold dilutions to obtain concentrations ranging from 200 to 0.39 mg/ml. Optical density (OD) was determined in a microplate reader at 600nm before incubation (T_0). The 96-well plates were incubated at 37ºC for 24 h with constant shaking. After 24 h, the plates were again determined (T_{24}). The growth inhibition for the test wells at each dilution was determined using the formula:

$$\frac{1-(OD\ test\ well\ at\ T24-OD\ test\ well\ at\ To)}{(OD\ control\ well\ at\ T24-OD\ control\ well\ at\ To)}\times 100 = \%\ inhibition$$

The minimum concentration that showed 100% growth inhibition was indicated as MIC. The highest concentration that showed no bacterial colonies was taken as MBC (minimum bactericidal concentration).

Determination of Antiadherence Activity

Antiadherence activity was determined by glass tube surface adherence assay as in Limsong et al., [22] adapted from Murchison et al., [23]. Bacterial adhesion was scored on a score of 0 to +4 as described in the Murchison method.

STATISTICAL ANALYSIS

The results are presented as mean ± standard deviation. One-way ANOVA followed by post-hoc Bonferroni for intra-group comparisons were used for statistical evaluation. P-values less than 0.05 were considered significant.

RESULTS

The results of antimicrobial properties of crude, organic solvent based and aqueous extracts of the six Indian plants, the positive control 0.2% Chlorhexidine (positive control) and 20% DMSO, Distilled water (negative controls), are presented in [Table/Fig-1] and values of MIC of these extracts against the test pathogen are presented in [Table/Fig-2]. The antimicrobial activity of the extracts on the agar plates varied for the different solvents. Positive control (0.2% Chlorhexidine) produced significantly larger inhibition zones (22.57 mm ± 0.05) against the test bacteria. However, the negative controls produced no observable inhibitory effect.

Antibacterial Susceptibility Test: Agar Well Diffusion Method

Amongst all the six extracts – Crude extract of Garlic, Aqueous extract of Amla and Organic solvent extract of Ginger inhibited the growth of *Streptococcus mutans*; the inhibition zones varied from 6 to 25 mm at a volume of 100µl [Table/Fig-1].

MIC and MBC determination

Extracts which exhibited antibacterial activity, were further determined for MIC and MBC values. The extracts demonstrated MIC values ranging from 6.25 to 100mg/ml, and their MBC values was recorded less than 12.5mg/ml to 200mg/ml [Table/Fig-2].

Determination of Antiadherence Activity

The adherence inhibition of all extracts was determined; it was found that four extracts exhibited this activity. Data are shown in [Table/Fig-3].

DISCUSSION

Nature has bestowed a very rich botanical wealth, and a large number of diverse types of plants grow in different parts of world. Antimicrobial agents of plant origin have enormous therapeutic potential. They are effective in the treatment for infectious diseases, and simultaneously they also mitigate many of the side effects that are often associated with synthetic antimicrobials (Rios and Recio [24]. Hence, the purpose of the present study was to evaluate the role of antimicrobial agents of plant origin in inhibition of the growth of *Streptococcus mutans*.

In the present study, all the three extracts of garlic moderately inhibited the growth of *Streptococcus mutans,* showing inhibition zones from 12-24 mm and MIC as low as 6.25mg/ml. These results are in accordance with several previous studies which stated that garlic extracts could reduce the *Streptococcus mutans* count to a degree equivalent to that of 1.2% CHX mouthwash. (Fani et al., Groppo et al., Chavan et al.,) [25-27].

The results are also in synchrony with the results obtained by study done by Kivanc [28], since fresh garlic juice had the maximum inhibitory activity against all the tested bacteria, viz., *B. cereus, B. subtilis, E. coli, S. aureus* etc, although, no information about the activity against oral pathogens was provided.

The results of the present study is in accordance with the study on Amla extract by Potdar et al., [29] which assessed the invitro

Samples	Crude Extracts	Organic Solvents	Aqueous Extracts	p-value
Aloe vera	6.17±0.16	15.81±0.12	12.15±0.15	0.001
Amla	14.22±0.32	11.02±0.11	19.47±0.05	
Garlic	24.62±0.18	12.84±0.07	15.89±0.22	
Ginger	7.65±0.27	18.76±0.05	14.02±0.32	
Neem	7.79±0.05	14.30±0.22	17.09±0.17	
Tulsi	6.87±0.75	16.27±0.16	9.03±0.02	

[Table/Fig-1]: Antimicrobial efficacy of six plant extracts against *Streptococcus mutans* Chlorhexidine was evaluated as a positive control with 22.57 mm of mean diameter of inhibition zone
Distilled water for aqueous extracts and Dimethyl sulfoxide for Organic solvent based extracts was evaluated as a negative control with 6.00 mm of mean diameter of inhibition zone

Samples	Crude Extracts		Organic Solvents		Aqueous Extracts		p-value
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	
Aloe vera	-	-	25	100	50	100	0.001
Amla	25	50	50	100	<2.5	25	
Garlic	6.25	12.5	<50	100	<25	50	
Ginger	-	200	12.5	25	<50	100	
Neem	-	200	25	50	12.5	50	
Tulsi	-	-	<25	50	100	200	

[Table/Fig-2]: Minimum inhibitory concentration of all the six extracts against *Streptococcus mutans*

Plant species	Volume of Crude extract (µl)			Volume of Organic solvent based extract (µl)			Volume of Aqueous extract (µl)		
	5	10	20	5	10	20	5	10	20
Aloe vera	0	0	0	+2	+2	+2	+1	+1	+1
Amla	+3	+3	+3	+1	+1	+1	+4	+4	+4
Garlic	+4	+4	+4	+1	+1	+1	+2	+2	+2
Ginger	+1	+1	+1	+4	+4	+4	+2	+2	+2
Neem	+2	+2	+2	+2	+2	+2	+3	+3	+3
Tulsi	0	0	0	+3	+3	+3	0	0	0

[Table/Fig-3]: Antiadherence activity of Crude, Aqueous and Organic solvent-based extracts against *Streptococcus mutans*

antimicrobial property of distilled water, ethanolic and acetonic extracts of *E.officinalis* (Amla) against *Enterococcus faecalis, Candida albicans and Streptococcus mutans.* All the three *E.officinalis* extracts were effective against all the three microorganisms. In another study conducted by Saeed and Tariq [30], the effectiveness of antimicrobial potential of aqueous infusions and aqueous decoctions of *E. officinalis* (Amla) was confirmed against 186 bacterial isolates belonging to 10 different genera.

Past study reports have also shown that ethanol extracts were more effective than water extracts [31,32]. Concentration of antibacterial compounds may be low in water extract or antibacterial compound(s) or all of the identified components from plants active against microorganisms may not be extracted in aqueous extract. Aromatic or saturated organic compounds are mostly obtained through initial ethanol or methanol or organic solvent extraction [33]. However, some studies demonstrated that the antimicrobial activity of the aqueous extract used were as strong as their alcoholic extract (Muangsan and Senamontee; Rani and Kullar [34,35], or even better effects of their corresponding organic solvent extracts (Al-Bayati and Sulaiman; Aqil and Ahmad, Dogruoz et al.,) [36-38].

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.

In addition, 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.

Simultaneously, post analysis of the results of the present study, it would not be an understatement that compounds responsible for antibacterial activity of some plants like Amla and Garlic can be lost during the extraction method where continuous heat is applied to the crude material or chances of insolubility of those compounds in the organic solvents can also be a responsible factor. This is in agreement with another report by O'Hara et al., [39] where following the heat treatments, the activities of balsam pear and garlic extracts were lost, while the active components in ginger, Japanese ginger appeared.

Although the bacteria used in the studies are different, all the results correspond to this 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 for discovery of bioactive natural products that may serve in the development of new pharmaceuticals.

The analysis of the previous reports supports the anti-cariogenic properties of polyphenols on cariogenic Streptococci, probably due to: a direct effect of polyphenols against *Streptococcus mutans*; an interaction of polyphenols with microbial membrane proteins inhibiting the adherence of bacterial cells to the tooth surface; and the inhibition of glucosyl transferase and amylase in bacteria.

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 the marketing of the plant extracts as therapeutic agent. Industry and commercial partners will facilitate commercialisation of these plant extracts, in the form of dry extracts or oils, should clinical efficacy be demonstrated.

CONCLUSION

The extracts showed significant activity against the investigated microbial strains, which is promising. These extracts were not pure compounds and in spite of it, antimicrobial results were obtained. This recommends the potency of these extracts. The figment of the derivation of antimicrobial compounds from plants seems lucrative as it will lead to the development of a phytomedicine to act against microbes. Isolation and purification of phytoconstituents from these plants may yield significant novel antimicrobials, as plant based antimicrobials have enormous therapeutic potential as they can serve the purpose without any adverse effects that are often associated with synthetic compounds.

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