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Antimicrobial Activity Of Medicinal Plants On *Streptococcus Mutans*, A Causing Agent Of Dental Caries

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Abstract— *Streptococcus mutans* plays a significant role in dental caries and control of its activities can promote prevention of dental caries. Use of herbal agents is a notable issue in recent researches in dental caries. The study was designed to evaluate of antimicrobial activity of ethanolic extracts of leaf and bark of *Azadirachta indica*, bark of *Vitex negundo*, leaves of *Spinacia oleracea*, fruits of *Momordica charantia*, *Phyllanthus embilica*, *Piper nigrum*, and *Tamarindus indica*, rhizome of *Curcuma longa* and *Zingiber officinale* against *Streptococcus mutans*. Considerable zone of growth-inhibition was observed for the extracts of *Curcuma longa*, *Tamarindus indica* and *Phyllanthus embilica*. The MIC of the extracts ranged between concentrations of 50 mg/ml and 3.125 mg/ml and MBC ranged between concentrations of 100 mg/ml and 12.5 mg/ml. Commercially available antibiotics were used as control for comparative study. Combinations of different extracts were also used and the mixture of *Tamarindus indica* and *Curcuma longa* extracts was the most efficient. The efficiency of the extracts in a toothpaste formulation was checked with proven stability. Further study needs to be conducted to check the stability of active ingredients in commercial production condition and to prove the potential of these plant extracts to be formulated in dental care products.

Index Terms— *Curcuma longa*, Dental caries, Medicinal plants, *Phyllanthus embilica*, *Streptococcus mutans*, *Tamarindus indica*, Toothpaste,

1 Introduction

DENTAL diseases are recognized as a major public health problem throughout the world. Teeth and their supporting structure the gum (gingival) are subjected to infection by cariogenic bacteria that causes cavity and pyorrhea, which if left untreated can eventually lead to gingivitis. Recent study suggests that such chronic low-grade localized infection such as pyorrhea contribute to heart disease and coronary heart disease [1]. Among the major cariogenic bacteria, *Streptococcus* spp. specially *S. mutans* found in a greater number followed by *Actinomyces* spp. and *lactobacilli* spp. [2], [3].

During the last two decades there has been an increasing trend in search for new plant derived drugs containing the medically useful alkaloids, glycosides, polyphenolics, steroids and terpenoids derivatives. The plant genus *Phyllanthus emblica* (Euphorbiaceae) has long been used as anti-inflammatory and anti-pyretic agent by the ethnic population. The plant leaves have the anti-neutrophilic activity and anti-platelet properties in vitro. The fruit is a very rich source of vitamin C and a potent anti-oxidant [4]. The extracts also possess several pharmacological properties like anti-viral, antimutagenic, anti-allergic, antibacterial and anti-fungal activities. [5], [6], [7].

Curcuma longa is a tropical perennial herb. The rhizome has traditionally been used as a coloring agent in Asian cuisine. The active component in turmeric is curcumin, a non-water-soluble polyphenol and is quite stable in the acidic pH of the stomach. [8]. Numerous studies have shown that curcumin has antioxidant, anti-inflammatory and antimicrobial properties [9], [10]. Recent studies have also indicated that curcumin affects cellular enzymes, and angiogenesis [11], [12].

Momordica charantia (cucurbitaceae), principally eaten as vegetables, are a good source of vitamin C, vitamin A, phosphorus and iron [13]. Fruits and seeds possess medicinal properties such as anti-HIV, anti-ulcer, anti-inflammatory, anti-leukemic, antimicrobial, antitumor and antidiabetic property [14]. *Azadirachta indica* has been used as a traditional medicine in India for many centuries. Earlier studies have indicated that stem bark of neem (common name) contains strong anti inflammatory activity [15]. Various preparations of neem obtained from its different parts have been found to exert antibacterial, antimalarial, contraceptive and anti-ulcer activities. The extract from a bark containing neem stem have appeared to inhibit virulence factors of oral streptococci associated with dental plaque formation [16]. The leaves and the barks of *Vitex negundo* (Verbenaceae) are the most important in the field of medicine although almost all parts of plant are used [17]. *Spinacia oleracea* (Amaranthaceae), commonly known as spinach, is regarded as a valuable dietary source of vitamin A, non heme iron, folate, and lutein. It has also been used medicinally in treating anemia, nightblindness, tooth disorder, urinary disorder, cancer, and respiratory disorder [18]. It is considered as antioxidant, antiageing, sun protective, antipyretic and anti inflammatory agent. *Tamarindus indica*, (Fabaceae), a tropical fruit having sweet acidic taste [19], is also extensively used in Nigerian traditional medicine especially in the north-western region. It has been reported to be among the recipe in the

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treatment of cold, fevers, stomach disorders, diarrhoea, jaundice and as skin cleanser. Research has shown the pulp extract to be antibacterial [20], cholesterol and low density lipoprotein (LDL) reducing agent [21]. Tamarind leaves possess a strong in vitro antibacterial activity against more then 13 (81%) common gram positive and gram negative bacteria [22]. *Piper nigrum* (Piperaceae) is normally used to treat asthma, chronic indigestion, colon toxins, obesity, sinus, congestion, fever, intermittent fever, cold extremities, colic, gastric ailments and diarrhea [23]. Both aqueous and ethanolic extracts of black pepper have been screened for antibacterial activity against a penicillin G resistant strain of *Staphylococcus aureus*, *Bacillus cereus* and *B. subtilis* [24], [25]. The *Zingiber officinale* (Zingiberaceae) has a biennial or perennial creeping rhizome, locally called Ginger (English). The Chinese take ginger for a wide variety of medical problems such as stomachache, diarrhea, nausea, cholera, asthma, heart conditions, respiratory disorders, toothache and rheumatic complaints [25]. Ginger is a domestic remedy also known for its anti-infectant effects. Essential oil constituents were found to decrease growth rate of a variety of bacteria and fungi, including *Staphylococcus* spp. and *Candida* spp. Bactericidal activity against the highly resistant gram-negative bacteria *Pseudomonas aeruginosa* was also notable.

2 Materials and Methods

2.1 Plant Materials

Herbal plants were collected from Sher-e-Bangla Agricultural University, Dhaka. The spices, fruits and vegetables were bought from local supermarket.

2.2 Bacterial Culture

Streptococcus mutans was isolated from a patient with class-5 [26] root caries and identified through various standard procedures that included Gram staining, hemolytic pattern, MR-VP test, Sugar fermentation, Growth at 6.5% NaCl, Growth at 45ºC and bacitracin sensitivity test [27], [28], [29].

2.3 Preparation of Plant Extracts

The leaf and bark of *Azadirachta indica*, bark of *V. negundo*, leaves of *S. oleracea*, fruits of *M. charantia*, *P. embilica*, *P. nigrum*, and *T. indica*, rhizome of *C. longa* and *Z. officinale* were washed with chlorinated tap water, chopped into pieces and sundried for ten days. Those were ground into coarse powdery substance using mortar and pestle and dried again in oven at 45°C for 3 days followed by micronized to fine powder using an electric blender (Moulinex, France). All the samples were kept in clean closed glass containers and stored until use. 10.0 g of the powdered plant material was dissolved in 100 ml of 95% ethanol and incubated at 35°C for 60 h at 120 rpm in a rotator shaker. The mixture was filtered through 0.45 µm membrane filter and filtrate was kept in a water bath at 60°C to allow evaporation of the solvent. The resulting extract was stored in dark at 4°C until further use. Different concentrations (e.g. 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml etc.) of the extracts were prepared by re-dissolving the extract in 40% ethanol.

2.4 Preparation of Standerdized Inoculum

The standardization of culture was done according to clinical and Laboratory Standard Institute [30]. Pure discrete colonies of overnight *Streptococcus mutans* culture were inoculated in nutrient broth and incubated for 5 hours. Normal saline was added gradually to the culture broth so as to compare the turbidity to that of 0.5 McFarland standards corresponding to the cell concentration of approximately 2.7×10^8 cfu/ml.

2.5 Antibacterial Susceptibility Test

Antibacterial susceptibility test was carried out by agar well and disc diffusion method [31]. The ethanolic extracts of plant materials were tested for the antibacterial effect on *S. mutans*. The diluent, 40% ethanol, was kept as negative control. Briefly, 1.0 ml of standardized inoculum was inoculated into 90 mm sterile Petri plate, 19.0 ml of autoclaved Mueller Hinton agar cooled down to 45°C was added, and the plate was rocked gently for 1 min. for even mixing of the contents. The seeded plate was then allowed to solidify. Wells were punched on the agar plate by using a sterile 6 mm in diameter cork borer and 100 µl of the each reconstituted extract of varying concentrations were poured into holes. Same amount of 40% ethanol was used as a negative control and 100 µl of pure distilled water into another hole to serve as a positive control. The extracts were allowed to get diffused into the media and then incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the diameters of zone of growth inhibition [32].

2.6 Determination of MIC and MBC

The crude plant extracts were dissolved in nutrient broth to a concentration of 100 mg/ml and other concentrations were prepared by subsequent 2-fold serial dilution up to 3.125 mg/ml. 100 µl of standard inoculum of *Streptococcus mutans* was added to each tube and incubated at 35°C for 24 h. MIC was determined as the lowest concentration of the extract in NB retarded visible growth of the bacteria. From each of the test tubes in the MIC experiment (without visible growth), 100 µl of each broth were aseptically spreaded on to a sterile MHA plate. The inoculated plates were incubated for 24 h at 37°C. After incubation, the MBC was determined as the lowest concentration of the extract of MIC tube which didn't give rise to growth on the MHA plate [33].

2.7 Determination of antibacterial activity of plant extracts in toothpaste

Semisolid toothpaste (white plus, square consumer product) was liquefied by diluting in sterile distilled water (1:4). Crude plant extracts were dissolved in the liquefied toothpaste to the concentration 100 mg/ml and then 2-fold serial diluted upto 3.125 mg/ml. Commercially available herbal toothpaste (white plus herbal, square consumer product) containing the ingredients, *Mentha arvensis, Azadirachta indica, Syzygium aromaticum* and *Ocimum sanctum,* was used as a reference. Seeded plates were prepared and wells were punched on by using a sterile 6 mm diameter cork borer. Then 100 µl of toothpaste-extract mixtures from each concentration were poured into the wells and allowed to diffused in. The plates were incubated at 37ºC for 24 h. Antibacterial activity was evaluated by measuring the diameters of zone of growth inhibition.

3 RESULTS

In this study, a variety of herbal plants, vegetables and spices were selected for the screening of antibacterial activity. The preliminary screening of ethanol extracts of the plant materials was performed by agar diffusion technique. The pH of the crude extracts was adjusted to neutrality and different working concentrations were prepared by serial dilution. All the extracts showed varying degree of antimicrobial spectrum against *Streptococcus mutans*. Samples were designated by from S1 to S10 and different concentrations by upper case alphabetes (A to G) by descending order. The result of preliminary screening has been pooled in Table 1. S4 (*S. oleracea*), S6 (*C. longa*), S9 (*P. emblica*) and S10 (*T. indica*), showed the most effectiveness against *S. mutans* and obviously in the highest concentration (100 mg/ml, designated as A). The samples S2 (*A. indica*), S3 (*V. negundo*) and S7 (*P. nigrum*) showed the least activity. All the samples showed reduction in antimicrobial activity as the concentration were reduced and showed almost no antibacterial effect beyond concentration of 12.5 mg/ml. Sample S6 (*C. longa*) showed antibacterial effectiveness exceptionally in concentration of 3.125 mg/ml (G). In this study, the inhibition zone values were interpreted as sensitive (18 mm), intermediate (14-17 mm) and resistant (<14 mm) [34].

Sample designation	40% ethanolic extracts of medicinal plant material	Concentrations (mg/ml)	Zone of growth inhibition (mm)
S1A	*Azadirachta indica* bark(pH 5.5)	100	15
S1B	*Azadirachta indica* bark (pH 7.0)	100	12
S1C	*Azadirachta indica* bark	50	9
S1D	*Azadirachta indica* bark	25	6
S1E	*Azadirachta indica* bark	12.5	5
S2A	*Azadirachta indica* leaf (pH 5.0)	100	13
S2B	*Azadirachta indica* leaf (pH 7.0)	100	10
S2C	*Azadirachta indica* leaf	50	8
S2D	*Azadirachta indica* leaf	25	6
S3A	*Vitex negundo* bark (pH 5.0)	100	13
S3B	*Vitex negundo* bark (pH 7.0)	100	9
S3C	*Vitex negundo* bark	50	6
S3D	*Vitex negundo* bark	25	5
S4A	*Spinacia oleracea* (pH 6.0)	100	21
S4B	*Spinacia oleracea* (pH 7.0)	100	19
S4C	*Spinacia oleracea*	50	12
S4D	*Spinacia oleracea*	25	10
S4E	*Spinacia oleracea*	12.5	6
S5A	*Momordica charantia* (pH 4.5)	100	15
S5B	*Momordica charantia* (pH 7.0)	100	14
S5C	*Momordica charantia*	50	10
S5D	*Momordica charantia*	25	8
S5E	*Momordica charantia*	12.5	6
S6A	*Curcuma longa* (pH 4.5)	100	18
S6B	*Curcuma longa* (pH 7.0)	100	17
S6C	*Curcuma longa*	50	12
S6D	*Curcuma longa*	25	9
S6E	*Curcuma longa*	12.5	10
S6F	*Curcuma longa*	6.25	9
S6G	*Curcuma longa*	3.125	8
S7A	*Piper nigrum* (pH 6.0)	100	12
S7B	*Piper nigrum* (pH 7.0)	100	12
S7C	*Piper nigrum*	50	10
S7D	*Piper nigrum*	25	9
S7E	*Piper nigrum*	12.5	6
S8A	*Zingiber officinale* (pH 5.0)	100	14
S8B	*Zingiber officinale* (pH 7.0)	100	9
S8C	*Zingiber officinale*	50	12
S8D	*Zingiber officinale*	25	12
S8E	*Zingiber officinale*	12.5	8
S8F	*Zingiber officinale*	6.25	7

The pH of the crude extracts was recorded and a sample was prepared with the highest concentration (100mg/ml) for each of the extracts with their native pH (without adjusting to neutrality). The impact of pH on antibacterial activity was best illustrated in case of sample S10A. An acidic pH induces zone of growth inhibition of 24 mm compared to 10 mm in case of neutral pH of sample S10B. This pattern was observed in all the samples which indicates, that it is mostly the acidic constituents in all the samples that induces the antibacterial effect.

The samples, mixed amongst each other to see the combined effect, have been presented in Fig. 1. The combination reduces the overall effectiveness of the individual samples and in some cases there is no activity left at all. For example, Sample S1A showing 15 mm zone of inhibition, when mixed with S4A showing 21 mm of zone of inhibition individually, produces no zone of growth inhibition. Similar results also found in mixture of S1A with S9A and S4A with S9A.

Antibiotic sensitivity pattern of the test isolate was determined against sixteen commercially available antibiotics. Amongst the antibiotics, Ciprofloxacin (5µg) and Meropenem (10µg) showed significant effectiveness against *S. mutans* whereas Amikacin (30µg) Netilmicin (30µg) Cefotaxime (30µg) and Neomycin (30µg) showed satisfactory level of effectiveness. *Streptococcus mutans* was resistant against the rest of the antibiotics, Novobiocin (30µg), Vancomycin (30µg), Clindamycin (2µg), Ceflaclor (30µg), Cephalothin (30µg), Ampicillin (10µg), Amoxycillin (10µg) and Rifampin (5µg). Though none of the herbal plants or spices showed the antibacterial activity to the level of antibiotics, it clearly shows that herbal plants and spices can be used as alternatives to antibiotics with lesser side effects.

TABLE 1
ANTIBACTERIAL EFFECT OF MEDICINAL PLANT EXTRACTS

TABLE 1 (CONTINUED)
ANTIBACTERIAL EFFECT OF MEDICINAL PLANT EXTRACTS

Sample designation	40% ethanolic extracts of medicinal plant material	Concentrations (mg/ml)	Zone of growth inhibition (mm)
S9A	*Phyllanthus emblica* (pH 6.0)	100	19
S9B	*Phyllanthus emblica* (pH 7.0)	100	14
S9C	*Phyllanthus emblica*	50	10
S9D	*Phyllanthus emblica*	25	8
S9E	*Phyllanthus emblica*	12.5	6
S9F	*Phyllanthus emblica*	6.25	6
S10A	*Tamarindus indica* (pH 1.5)	100	24
S10B	*Tamarindus indica* (pH 7.0)	100	10
S10C	*Tamarindus indica*	50	12
S10D	*Tamarindus indica*	25	11
S10E	*Tamarindus indica*	12.5	8

Note: The concentrations of plant extracts, which didn't show any zone of growth inhibition, are excluded from incorporation in the table.

The MIC and MBC values are shown in Table 2 for all the samples. The MIC values ranged from 50 mg/ml to 3.125 mg/ml while MBC values ranged from 100 mg/ml to 12.5 mg/ml. The lowest MIC of 3.125 mg/ml observed in the case of *Curcuma longa*. As for MBC, concentrations below 12.5 mg/ml for any of the samples had no bactericidal effects on *S. mutans*. *T. indica* and *C. longa* has an MBC value of 12.5 mg/ml however, *A. indica* bark and *M. charantia* has no MBC effect beside the crude extract.

TABLE 2
DETERMINATION OF MIC AND MBC OF MEDICINAL PLANT EXTRACTS

40% ethanol extracts at 100 mg/ml	Zone of growth inhibition (mm)	MIC (mg/ml)	MBC (mg/ml)
Azadirachta indica bark (pH 5.5)	15	25	100
Azadirachta indica leaf (pH 5.0)	13	50	100
Vitex negundo bark (pH 5.0)	13	50	100
Spinacia oleracea (pH 6.0)	21	25	50
Momordica charantia (pH 4.5)	15	25	100
Curcuma longa (pH 4.5)	18	3.125	12.5
Piper nigrum (pH 6.0)	12	50	100
Zingiber officinale (pH 5.0)	14	12.5	50
Phyllanthus embilica (pH 6.0)	19	6.25	25
Tamarindus indica (pH 1.5)	24	6.25	12.5

Assessment of antimicrobial activity of medicinal plant extracts in commercially available tooth paste was performed. Locally produced non-herbal toothpaste (Square consumer product) was diluted to liquefy and used as matrix for the assessment of medicinal plants extracts in commercial dental care formulation. The four plant extracts e.g. S4A, S6A, S9A and S10A showed greater antibacterial efficiency and thus were used in this study. Toothpaste mixed with sample S10A showed the largest zone of growth inhibition and that was 26 mm (Fig. 1). Commercially available herbal toothpaste containing *M. arvensis, A. indica, S. aromaticum and O. sanctum,* used as a positive reference, and non-herbal paste was used as negative control. The positive reference toothpaste produced a zone of inhibition of 24 mm in diameter whereas when all the four samples S4A, S6A, S9A and S10A respectively were mixed with toothpaste, the diameter of zone of growth inhibition was showed 25 mm (Fig. 1). The observation indicated that a combination of samples S4A, S6A, S9A and S10A can be used to make herbal toothpastes.

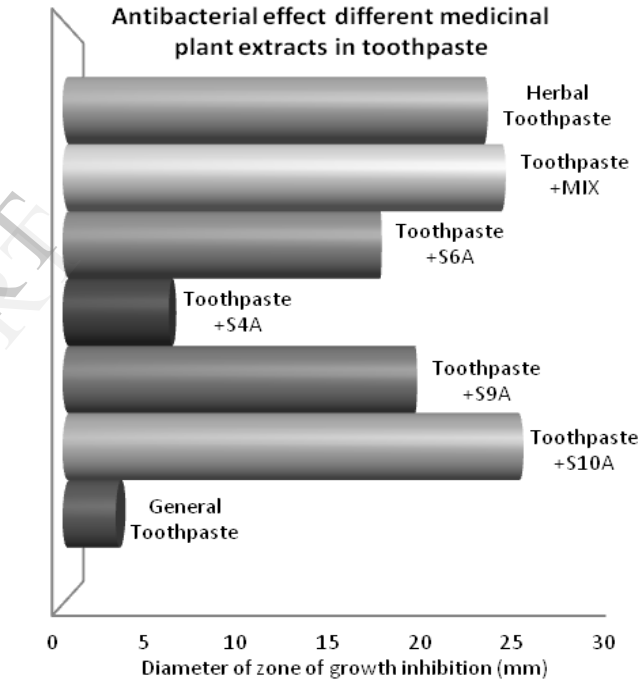

Fig. 1. Antibacterial effect of medicinal plant extracts, determined by the zone of growth inhibition, on *S. mutans* in combination with commercially available non-herbal toothpaste. A commercial herbal toothpaste was kept as positive control. The abbreviations used e.g. S4A, S6A, S9A, and S10A stand for different plant extracts corresponding to the designation in Table 1 and the abbreviation, MIX, stands for the mixture of all the four extracts.

6 DISCUSSION

Herbal medicines already form the basis of therapeutic use in the ethnic group. In recent years, there has been an increase in the use of herbal medicines in the developed world [33]. Several studies have linked presence of bioactive compounds in plant materials to antimicrobial activity [31].

A. indica or neem twigs, which provide a chewing stick and is widely used in the Indian sub-continent [35], has been reported to contain caries and plaque inhibitory substances [16].

Results from the present study demonstrated that, ethanolic extract of both *A. indica* leaf and bark is in intermediate range against caries causing *S. mutans* in its native pH. Surprisingly, in pH 7.0, *S. mutans* was found totally resistant to the pH-neutralized extract. The effect of pH observed is similar to the study conducted by El Mahmood [31]. Almas K. conducted another experiment and found no effect of neem stick on *S. mutans* [36], which may be due to the phenomenon of pH dependent activity. However, the bark was found more effective than leaves in our study. The difference in the antibacterial effect was probably because of the different percentage in the active component of phenol groups in both neem leaves and sticks [37]. Bhuiyan *et al* studied the effects of acetonic extract of neem stick on another caries causing microorganism *S. sobrinus* and found strong antimicrobial activity [38].

There is not much substantial research work done on antibacterial activity of *S. oleracea*. However study conducted in Thailand reveals that, bitter gourd helps reduce adhesion of *S. mutans* to teeth and thus prevents caries [39]. Study also shows the effectiveness of bitter gourd against a broad spectrum of microorganisms [40], [41], [42], [43]. Our study showed turmeric to be effective in inhibiting growth of *Streptococcus mutans* to an appreciative level. The crude extract of turmeric, 100 mg/ml of pH 4.5 showed a zone of inhibition of 18 mm. While at pH 7.0, the zone was 17 mm indicating that the ethanolic content of the extract had no impact on the pH. No MIC or MBC was done for the combination of the herbal agents. Our study showed Turmeric to be the best in antibacterial activity against *S. mutans*, effective as low as 3.125 mg/ml. It also showed MBC of 12.5 mg/ml, one of the least amongst all the medicinal plants tested. The findings are similar to research done elsewhere, and reporter to inhibit *S. mutans* biofilm formation; hence suppressing dental caries formation [44], [45]. Black pepper or *Piper nigrum* finds extensive use in Ayurvedic system of medicine. The MIC was determined to be 25 mg/ml and MBC of 50 mg/ml for ginger extract in our study. But it has been previously reported that the MIC and MBC value was 1.25 mg/ml and 2.5 mg/ml respectively for ethanolic extract of ginger while conducting experiment on multi drug resistant *S. mutans* [46]. Our study revealed impressive activity of *P. emblica* on *S. mutans* with MBC of 25 mg/ml and MIC of 6.25 mg/ml.

We took our best extracts from the experiment and checked the effectivity in commercial dental care product (tooth paste) formulation. Our best results were seen with *P. emblica*, *C. longa*, *S. oleracea* and *T. indica*. Toothpaste mixed with Turmeric and Spinach was not as effective individually in suppressing bacterial growth. The mixed concoction of all the four samples with toothpaste yielded a zone of 25 mm whereas the commercially available herbal toothpaste showed a zone of 24 mm.

4 Conclusion

It is obvious that present study has revealed the importance of medicinal plants to control caries causing *S. mutans*. This scientific information can serve as an important platform for the development of inexpensive, safe and effective natural medicines. The long-range goal of this study is to develop applications of such materials for use as chemotherapeutic agents in oral hygiene products.

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