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Effect of Plant Chewing Stick Extracts on Certain Cariogenic Activities of *S.mutans*

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Abstract: ***Background & objectives*: Chewing sticks have been used for centuries to improve dental health and promote oral hygiene worldwide. Several studies have reported the antimicrobial effects of Chewing sticks on oral bacteria. The present study evaluated the effects of aqueous extracts of some Indian chewing sticks (Acacia arabica, Azadirachtaindica, Pongamiapinnata and Salvodorapersica) on cariogenic activities of S. mutans, responsible for the formation of dental caries. *Methods*: The chemical composition of different chewing sticks was studied. The effect of aqueous extracts on the growth, biofilm formation, hydrophobicity, cell-cell adhesion and acid production by the organism was investigated. *Results*: The activity of glycosyltransferase, the key enzymes of sucrose metabolism was inhibited by aqueous extracts of chewing sticks. The growth of S. mutans was also impaired by plant extracts in vitro, which yielded MIC of the order of 1580 (Acaciaarabica, Salvodorapersica), 2360 (Pongamiapinnnata) and 2500 (Azadirachtaindica) µg/ml, respectively. The plant extracts also reduced biofilm formation, hydrophobicity and acid production by the organism under in vitro conditions. Acaciaarabica and Salvodorap ersica were more efficient in inhibiting the bacterial growth compared to Pongamiapinnnata and Azadirachtaindica. *Interpretation and conclusions*: The present data suggest that plant extracts of the chewing sticks inhibit cariogenic activity of S. mutans, which may have potential application in the prevention and cure of dental caries.***

Keywords: *Streptococcus mutans;* Acid production; Adhesion; Glucosyltransferase; Chewing sticks; Plant polyphenols

1. Introduction

Dental caries is a common infectious disease in humans worldwide [1]. Bacteria present in the dental plaque play an important role in the development of dental caries [2]. Gram +ive *Mutans Streptococci*, has been recognized as primary causative agent of dental caries in humans and experimental animals [3]. Cariogenicity is strongly associated with the ability of the organism to synthesize extracellular water-insoluble glucans by the action of glucosyltransferases (GTases), together with their adhesion, acidogenic and aciduric (associated with acid tolerance) properties [4]. The glucans, so produced, enhance the pathogenic potential of dental plaque by promoting the adherence and accumulation of cariogenic streptococci on the tooth surface, and contribute to the bulk and structural integrity of the biofilm [5]. The production of acid by *S.mutans*and its power to tolerate the acidic pH favors its continual survival and colonization in the dental biofilm [4].

Plant chewing sticks have been commonly used by human population in several parts of the world as anticariogenic agents. However, the biochemical basis of such practices has remained undefined.

It has been reported that the aqueous extracts of certain Indian chewing sticks; *Acaciaarabica*, *Azadirachtaindica*, *Pongamiapinnata* and *Salvodorapersica*, are potent inhibitors of dexansucrase activity in *S. mutans in vitro* [6]. The chemical compositions of some of these plants have shown the presence of flavonoids, saponins, phenols and tannins in additions to glycosides, alkaloids and tepernoids [7]. Because of the presence of content of polyphenols, it is likely, that they impair the bacterial growth and thus cariogenic activity. In this report, we describe the effects of the aqueous extracts of *A. arabica*, *A. indica*, *P. pinnata* and *S. persica*) on certain cariogenic properties of *S. mutansin vitro.*

2. Materials & Methods

Chemicals

Agar, Brain Heart Infusion Broth (BHI), exogenous dextrose, glucose, sucrose, peptone and maelic acid, NaCl, yeast, and ethanol were obtained from Himedia Pvt. Ltd. India. All other chemical used were of analytical grade quality.

Growth of bacterial strain

A lyophilized culture of *S. mutans* (MTCC 890) was obtained from microbial type culture collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. For revival, the strain was inoculated into Snyder's medium B-87 (1% dextrose, 1% peptone, 1% yeast extract, 0.5%BHI and 2% agar, pH 7.2-7.5) and inoculated broth was incubated for 18-24 h at 37^{o}C. Culture purity was checked regularly by microscopic examination.

Preparation of plant extracts

Samples of the commonly used chewing sticks Neem (*Azadirachtaindica*), Kikar (*Acacia arabica*), *Sukhchain* (*Pongamiapinnata*)and *Salvadorapersica* were bought from the open market and were identified by their color and scent and recognized by agriculturist and vendor. The chewing sticks were shade-dried and cut into small pieces. Each successive 100 g quantity was put into a sterile screw-capped bottle containing 500 ml of 0.05 M sodium maleate buffer (pH 6.8), allowed to soak for an overnight at 4^{o}C and the mixtures were centrifuged at 10, 000 rpm for 10 min. The filtrates were then passed through a 0.2-micron filter to remove any bacterial and fungal contaminants. The solutions were concentrated by lyophilization and stored at -20°C in amber flasks.

Phenol estimation

The total phenol content of plant extracts was determined [8]. Phenol content of the tissue was expressed as tyrosine or gallic acid equivalents.

Assay of Glycosyltranferase activity

Glycosyltransferase activity was determined following the method of Mukasaet et al. [9]. Partially purified extract of *S. mutans* was used as the source of the enzyme, as described earlier[9].

Determination of Minimum Inhibitory Concentration (MIC)

MIC of aqueous extracts (*A. arabica, A. indica, P. pinnata* and *S. persica*) was determined by plate-hole assay as described by McRae et al. [10]. The MIC was considered to be the lowest concentration, which yielded a visible zone of inhibition more than 6mm. 100 µl of the culture was spread on BHI agar and allowed to dry for 10 mins. Once set, a sterilised core-borer (6 mm diameter) was used to produce wells in the agar. Increasing concentrations of plant aqueous extracts of A. *acacia*, A. *indica*, P. *pinnata*, S. *persica* 158-3000 µg/ ml were dispensed into each well. All plates were incubated overnight at 37°C under capnophilic conditions. The assays were performed in triplicate.

Effect on Sucrose-dependent and Sucrose-independent Adherence of *S. mutans* to Smooth Glass Surfaces

The effect of the sub-MIC concentrations of aqueous extracts (*A. arabica*and*S. persica* ranging from 15.8-79 µg/ ml, *A. indica* and *P.pinnata* from 15.8-118.5 µg/ ml) on the adhesion of *S. mutans* to smooth glass surface was studied as described by Hamada and Torii [9]. The bacteria were grown at 37°C for 24 h at an angle of 30° in a glass tube containing 10 ml of BHI with or without 5% (w/v) sucrose and different concentrations of plant extracts. The solvent controls included BHI with sucrose (sucrose-dependent) or without sucrose (sucrose-independent). After incubation, the glass tubes were slightly rotated and the planktonic cells were decanted. The adhered cells were then removed by adding 0.5 M of sodium hydroxide followed by agitation. The cells were washed and suspended in normal saline. The cell adherence was quantified spectrophotometrically at 600 nm. All these determinations were performed in triplicates, using untreated BHI medium as the control.

Percentage adherence = (O.D. of adhered cells/O.D. of total cells) × 100.

Effect on Cell-surface Hydrophobicity of *S. mutans*

The cell surface hydrophobicity of *S. mutans* was determined by Microbial adhesion test as described by Martin et al. [11].The cells were grown in BHI medium supplemented with 5% sucrose with different concentrations of plant extracts (*A. acacia* and *S. persica* ranging from 15.8-79 µg/ ml, *A. indica* and *P. pinnata* from 158-1200 µg/ ml) and were washed twice, suspended in sterile saline (0.85%) to adjust optical density (O.D.) to 0.3 at 600 nm. The cell suspension (3.0 ml) was placed in tubes and 0.25 ml of toluene was added. The tubes were agitated uniformly in a vortex mixer for 2 min and allowed to equilibrate at room temperature for 10 min. After separation of toluene phase from the aqueous phase, the O.D. of the aqueous phase was determined at 600 nm. Controls with *Streptococcus mutans* were run simultaneously, hydrophobic index >70% was arbitrarily classified as hydrophobic [12].

Collection of Saliva

After masticatory stimulation by chewing a piece of parafilm, the saliva was collected from healthy individuals, who were abstained from tooth brushing and eating for 5 hours prior to collection of saliva. The collected saliva was centrifuged at 8000 g for 15 min to obtain clarified saliva and was stored at -80°C [12].The plate wells were coated with 100 µl of the clarified saliva, and were incubated at 37°C for 2 h to coat the wells by forming a thin membrane called the salivary pellicle. After incubation, the plates were rinsed thrice with 100 µl PBS before adding the bacterial culture.

Biofilm Formation Assay

Biofilm formation assay was carried out in flat bottomed 96-well microtitre plates [13]. The biofilm ability of *S. mutans* was studied in the presence and absence of salivary pellicle. Over-night culture of *S. mutans* was inoculated in fresh BHI medium containing 5% (w/v) sucrose and grown at 37°C under anaerobic condition to the mid- log phase (O.D of 1 at 600nm). The cultures were diluted to 1:100 (v/v) in pre-warmed media. The wells were first coated with saliva before the addition of bacterial suspensions. The coated plates were again incubated at 37°C for 2 h and were washed three times with PBS followed by air drying for 30 min. Bacterial suspension (200 µl) was added to the wells of both uncoated and coated plates with varying concentrations of plant extracts (*A. arabica, A. indica, P. pinnata, S. persica* from 79-800µg/ml). Blanks with media alone were run as controls. After inoculation, all the plates were incubated for 24 h at 37°C. The culture was then decanted and the plates were washed thrice with 200 µl of sterile distilled water to remove loosely bound cells. The attached bacterial cells were stained with 50 µl of 0.1% crystal violet for 15 min. After rinsing twice with 200 µl of sterile water, the bound dye was removed from the stained cells using 200 µl of 99% ethanol. Plates were then placed on a shaker for 5 min to allow full release of the dye. Biofilm formation was then quantified by measuring optical density of the suspension at 600 nm using ELISA plate reader (BioTek).

Effect on Acid Production by *S.mutans*

Effect of extracts from different chewing sticks on acid production by *S. mutans* was studied following the method of Ciardi et al [14]. 5 ml of BHI broth containing 5% (w/v) of sucrose and the different concentrations of plant extracts (*A. arabica* and *S. persica* ranging from158-790µg/ml**;** *A. indica, P. pinnata* from 158-1200 µg/ml) were inoculated with 100 µl of 18 h cultures of *S. mutans* to obtain a final inoculum of 1.5×10^4 CFU per ml and incubated at 37°C for 24 h. The pH of the bacterial broth was assessed at the onset and after 24 h of incubation. All determinations were performed in triplicates using suitable controls.

Statistical Analysis

All the results were expressed as mean ± S.D except otherwise stated. Statistical analysis of the data was performed using SPSS. A p-value <0.05 was considered significant.

3. Results

Effect on glycosyltransferase activity

The effect of the aqueous extracts of chewing sticks (*A. arabica, A. indica, P. pinnata* and *S. persica*) on glycosyltransferase activity was determined in partially purified, 60% ammonium sulfate precipitated enzyme fraction of *S. mutans*. All the plant extracts inhibited glycosyltranferase activity in *S. mutans* by 19-34% compared to controls. *A. arabica* at a concentration of 63.2µg/ml produced 34% enzyme inhibition, while 86.9 µg/ml *P. pinnata* produced 30% inhibition of the enzyme activity. *S. persica* and *A. indica* inhibited glycosyltransferase activity by 27 % and 19% at 79 µg/ml and 102.7µg/ml concentrations, respectively.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of the aqueous extracts of *Acacia arabica, Salvodorapersica, Pongamiapinnata* and *Azadirachtaindica* against *S. mutans* was found to be 1580 µg/ml and 2370 µg/ml and 2500 µg/ml respectively. A well defined zone of inhibition of cell growth was observed on BHI agar plates under the experimental conditions.

Effect on Sucrose-dependent and Independent Glass Surface Adherence

The inhibitory effect of the aqueous extracts on adherence of S. mutans to glass tubes in presence and absence of sucrose is described. The plant aqueous extracts inhibited both sucrose-independent adherence (40-53%) and sucrose-dependent adherence (30-40%). However, the inhibition of sucrose-dependent adherence was more pronounced in presence of *A.*arabica and *S. persica* extracts, which yielded reduction in cell adherence by 53% and 49%, respectively at polyphenol conc. of 790 µg/ml, while *P. pinnata* and *A. indica* reduced it by 47% and 40% at a polyphenol conc. of 870 µg/ml and 1025µg/ml, respectively.

Effect on Surface Hydrophobicity of *Streptococcus mutans*

Cell surface hydrophobicity of *S. mutans* was considerably reduced in a concentration- dependent manner. The aqueous extracts of *A. Arabica* and *S. persica* at a concentration of 790µg/ml reduced hydrophobicity by over 50%, while *A. indica* and *P. pinnata* reduced it by 38- 45% at a concentration of 1025 µg/ml and 870µg/ml, respectively as compared to control. Thus cell surface hydrophobicity of *S. mutans* is also sensitive to ingredients present in the chewing stick extracts.

Effects on Biofilm Formation by *S. mutans*

All the plant extracts used inhibited biofilm formation by *S. mutans* in a dose-dependent manner. *A. arabica* and *S. persica* reduced the biofilm formation by nearly 35 % at concentrations of 474µg/ml and 790µg/ml, respectively in presence of salivary pellicle. *P. pinnata* and *A.indica* reduced biofilm formation by 29% and 23% at a concentration of 634 µg /ml and 790µg/ml, respectively. However, in the absence of salivary pellicle, the formation of biofilm was relatively small under the experimental conditions.

Effect on acid production by *Streptococcus mutans*

A significant change in *pH* of the growth medium from acidic to alkaline conditions was apparent with gradual increase in concentration of *A. arabica, S.persica* and *P. pinnata*. Aqueous extracts of *A. Arabica* and *S. persica* at a concentration of 790 µg /ml and 1185µg/ml and *P.pinnata* increased the *pH* from 4.43 in the control to *pH* 5.92, 5.77, 5.65, respectively. However, addition of *A. indica* to growth medium showed a marginal augmentation in *pH* to 5.02 under these conditions.

4. Discussion

Plant derived phenolics are of great importance as they exhibit potential health benefits. They possess various biological activities including antibacterial (Ito *et a*l. 1984) [15], anti-inflammatory (Bowden 1999) [16] anti-oxideant (Bhattacharya *et al* 2010) [17], anticancer (Borchardt et al. 2009 [18] anticariogenic effects (Milgrom *et al.*2000 [19], Luczaj *et al* 2005 [20].

In the present study, the main focus was on the effects of aqueous extracts of *A. arabica, A.*indica, *P. pinnata* and *S. Persica* which, on certain cariogenic factors associated with *S mutans*. The formation of dental biofilm in the oral cavity is the primary step leading to caries disease. The synthesis of glucans from sucrose by GTFs is one of the most important virulence properties of *S. mutans* that contributes to the development of mature dental plaques[5]. Present data showed 19-34% inhibition of glycosyltransferase activity by aqueous extracts of chewing sticks (*A. arabica, A. indica, P. pinnata* and *S. persica*). These results are similar to those of Sakanakaet al. [21] who used tannins derived from Japanese green tea extracts.

The attachment of pathogenic bacteria to tooth surface and biofilm formation is the key element in the formation of dental caries. It has been reported that the biofilm associated bacteria are capable of tolerating antibacterial agents [22]. Therefore, a potential natural product capable of treating dental caries must be able to affect cariogenic bacteria within the biofilm. The present findings revealed that the adherence of *S. mutans* to glass surface was reduced by 30-50 % at all concentrations of plant extracts of *A. Arabica* and *S.persica* used (15.8- 79 µg/ml) and by *A. indica* and *P. pinnata* (15.8-118.5 µg/ml). There was marked a inhibition of *S. mutans* adherence to glass surface by sub-MIC concentrations of all the plant extracts used, as compared to the control. The sucrose-dependent adherence was reduced to a greater extent (40-55 %) than the sucrose independent adherence process (30- 45%). The reduction in adherence could be due to the presence of acids and tannins in the extracts of chewing sticks [23]. Since it is known that tannins have high binding affinity for certain proteins, resulting in their precipitation. Thus, bacterial proteins may interact with polyphenolic tannins by hydrogen-bonding. Alternatively, the tannins could associate with surface lipoteichoic acids, in salivary glycoproteins, resulting in bacterial aggregation. Earlier workers have reported impairment in adherence to hyderoxyapatite surface by tannins derived from Japanese green tea [19].

Hydrophobicity is one of the important properties which allow the microorganism to adhere to both living and non-living surfaces [24]. The therapeutic agents which can reduce the hydrophobic bond formation can also reduce the chances of caries formation. The results showed that aqueous extracts of chewing sticks (*A. arabica*, *A. indica*, *P. pinnata* and *S. persica*) considerably reduced the hydrophocity of *S. mutans*. Furthermore, cell-surface hydrophobicity is associated with cell-surface proteins. The observed reduction could be due to the binding of active components of plant extracts to the proteins associated with the cell surface as described by Hasan et al. [25].

Acid production by *S. mutans* plays an important role in the pathology of dental caries [4]. Tooth surface destruction is facilitated by fermentation of carbohydrates into lactic acid by *S. mutans [*26]. *pH* assay showed that sub-MIC levels of plant extracts were capable of reducing acid production. As the extract concentrations were sub-MIC, bacterial viability was unaffected suggesting that the reduction in acid production was a consequence of interference in the metabolism of sugar by bacteria. As acid production has a direct influence on the etiology of dental caries, these results are physiologically significant. Crude ethanoic extracts from *E. officinalis* is reported to reduce the acid production by *S. mutans* [22].

Thus, the effectiveness of plant extracts in inhibiting the growth of *S. mutans* is presumably due to the inhibition of biofilm formation as result of the inactivation of glycosyltransferase activity under these conditions [27]. Since extracellular polysaccharides are produced by the bacterium for its adherence to tooth surface, the inhibition of formation of these structures by polyphenols present in plant extracts, could be useful in the cure and prevention of dental caries. A similar phenomenon has been reported for *S. mutans* using scanning electron microscopy in presence of *T. ammi* seed extracts [10].

The present observations indicate that the cariogenic potential of the aqueous extracts of the chewing sticks is due to the synergistic effect of different compounds present in the plants. The antimicrobial effects of *S. persica* may be attributed to various chemicals viz. sodium chloride, potassium chloride, saponins, tannins, in addition to glycosides, alkaloids, terpenoids and of *A. arabica*, *A. indica* may be due to presence of flavonoids, saponins, phenolics and tannins, respectively [27]. Preliminary studies have shown the presence of gallic acid, catechin, quercetin and β-sitosterol by HPTLC analysis (results not shown).These findings show that the aqueous extracts of *A. arabica*, *A. indica*, *P. pinnata* and *S. persica* could be promising agents to inhibit biofilms formation and other cariogenic properties of *S. mutans*. Hence, they may have potential antiplaque characteristics.

5. Conclusion

In conclusion, the present data indicate that aqueous extracts of chewing sticks *A. arabica*, *A. indica*, *P. pinnata* and *S. persica* have bactericidal activity at high concentrations ≥ MIC and inhibitory effects against virulence factors of *S. mutans* at sub-MIC concentrations. This suggests their usefulness in the control of dental caries. The present data may be helpful in designing studies to corroborate these findings under *in vivo* conditions using the plant chewing sticks.

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