Evaluation of the anticariogenic effect of crude extract of Piper betle by assessing its action on salivary pH – An in vitro study

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Abstract: **Aim**: *Evaluation of anticariogenic efficacy of Piper betle by analysing its ability to resist the change in pH of human saliva containing sucrose(10%), Sodium fluoride(.05%) and distilled water serving as positive and negative control respectively.*
Method: *The hydro-alcoholic extract of Piper betle was prepared using simple maceration and its MIC and MBC against Streptococcus mutans (MTCC-497) was determined. The saliva from healthy human subjects (n= 30) were collected. After adjusting the pH, 1ml of saliva was taken in each of the test tubes marked P.B (Piper betle group), D (distilled water group) and NaF (sodium fluoride group). 10% sucrose solution(1cc) was added to the saliva in each of these test tubes. 1ml of Piper betle (5%), distilled water and NaF(.05%) were added to test tubes of their respective group. Further the pH of saliva in each group was noted with respect to time (0m, 15m, 30m, 1h, 2h, 3h, 4h, 5h, 6h). The pH of normal saliva (pH adjusted) was also noted at 0, 2, 4, and 6h. Data obtained was statistically analysed using SPSS ver10.*
Result: *P betle extract was effective in resisting salivary pH change and was comparable to that of 0.05% sodium fluoride when assessed using salivary pH model.*
Keywords: *Anticaries agent, Betel pepper, Fluoride, Maceration, Streptococcus mutans, SalivarypH.*

I.   Introduction

Piper betle known customarily as betel pepper belongs to a family of shade loving climber of Piperaceae family. It is consumed worldwide especially in Asia. In India it has been used as a masticatory for centuries. Piper betle is familiar for its wide range of ethnomedicinal properties in India and other south east Asian countries[1,2]. But the combination of Piper betle, arecanut, lime and dried tobacco leaves known popularly as "Paan" is widely consumed and is notorious for its abuse. The consumption of Pan had been widely held for the development of oral cancer. But it was striking to note that most of the patients who were pan chewers had reduced incidence of caries. Apart from the heavy staining, the carious lesions if present were usually arrested. This finding prompted us to investigate on the anticariogenic component in Pan which could be of safe clinical use against caries. Beneficial biological activities of Piper betle(PB) extracts and its chemical constituents had been reported and hence found to be a suitable candidate for the study. A study was designed to evaluate the anticariogenic potential PB extract by assessing its inhibitory action on acid production by salivary bacteria in presence of sucrose.

II.   Materials and methods

2.1 Preparation of leaf extract

Simple maceration of Piper betle(PB) leaves was done to produce the hydroalcoholic extract(50%). PB leaves were dried in hot air oven (< 50°C) for 2days following a week's shade drying. The dried leaves were then ground to fine powder and the powder(40g) was soaked in 50% ethyl alcohol(50ml) for a week in a sealed conical flask. The solution was filtered using Whatman's filter paper no1 and the solvent, allowed to evaporate completely to obtain the viscous extract. The extract was **stored** in sterile glass vials at 4°C until use.

2.2 MIC and MBC determination

MIC and MBC of the extract against Streptococcus mutans MTCC-497 [Institute of Microbial technology, Chandigarh, India] was determined.

The lyophilised culture was revived in nutrient broth and sub cultured on Mueller-Hinton agar plate. The MIC was determined by using two fold serial broth dilution method. PB extract was dissolved in distilled water to a final concentration of 20mg/ml. Serial dilution of extract was prepared in test tubes containing Mueller-Hinton broth. S mutans was grown to a stationary phase in Mueller-Hinton broth. The cell suspension was adjusted spectrophotometrically to 1-2x10^{7}CFU/ml. 0.1ml of this suspension was added to the test tubes

having serially diluted PB extract and incubated overnight. The growth was observed visually and the highest dilution at which therewas no visible growth was taken as MIC.

For the determination of MBC, the culture medium inside each of the test tubes having no apparent growth were sub cultured on fresh blood agar plates incubated at 37°C for 24h. MBC was taken as least concentration that showed no growth on blood agar on subculturing.

2.3. Determination of Anticariogenic effect of Piper betle extract

Saliva samples from 30 healthy individuals were collected (individuals had no history of smoking, tobacco chewing or recent antibiotic therapy). The pH of each saliva sample was measured using Digital pH meter [(pHep® HI 96107,Hanna instruments) with a pH measuring Range: 0.0 to 14.0 pH, Accuracy: ±0.1 pH and a manual 1 point calibration.]. The pH meter was calibrated using pH buffer after each set of reading.

The pH of each saliva sample was then adjusted by adding drops of one tenth dilution of 0.1N HCl until there is drop in pH of 0.1 from the initially recorded pH. This new pH was noted as the initial pH of normal saliva. Three sets of test tubes were taken and categorised into three groups as 'PB', 'D' and 'NaF' and 1ml of Piper betle(5%), Distilled water and 0.05%NaF respectively were added into each of these test tubes. Then 1ml of pH adjusted saliva and 1ml of 10% sucrose solution was added to each one of these test tubes. All the test tubes were shaken thoroughly to mix the contents. The solution from test tubes were taken and poured in separate wells on plastic tray for ease of measuring pH. The pH of resultant saliva solution in each was recorded at time intervals 0, 15m, 30m, 1h, 2h, 3h, 4h, 5h, 6h. The pH of the Normal saliva (after adjusting PH) was measured at 0, 2h, 4h and 6h for assessing the base line change in pH of saliva without sucrose.

2.4. Statistical Analysis

Data obtained from the study was analysed using computer software, Statistical Package for Social Sciences (SPSS) version 10. Data was expressed in it as mean and standard deviation. Analysis of variance (one way ANOVA) was performed as parametric test to compare different groups as well as between different time periods. Duncan's Multiple Range (DMR) test was also performed as post comparisons to elucidate multiple comparisons between interventions. For all statistical evaluations, a two-tailed probability of value, < 0.05 were considered as significant.

III. Results

MIC and MBC of Piper betle extract was found to be 5.12mg/ml and 20.48mg/ml respectively. The mean pH in all the groups at specified time intervals was summarized in TABLE 1.

IV. Figures and Tables

Table 1. Analysis of variance of mean salivary pH comparing period of observation in three groups

F value (comparing groups)	Group IV (normal saliva)		Group III (NaF)		Group II (D)		Group I (PB)	Observation Period
	+ SD	Mean	+ SD	Mean	+ SD	Mean	Mean	
1.021	0.32	7.62^{a}	0.32	7.39^{a}	0.33	7.42^{a}	0.36	7.36^{a}	0 Hour
0.082	ND	ND	0.35	7.34^{a}	0.34	7.35^{a}	0.37	7.31^{a}	15 Minutes
0.577	ND	ND	0.33	7.30^{a}	0.32	7.21^{a}	0.37	7.27^{a}	30 Minutes
2.624	ND	ND	0.31	7.21^{b}	0.34	7.04^{a}	0.31	7.19^{ab}	1 Hour
22.011**	0.38	7.62^{c}	0.31	7.15^{b}	0.41	6.90^{a}	0.32	7.09^{b}	2 Hours
6.144*	ND	ND	0.32	7.05^{b}	0.51	6.69^{a}	0.37	6.96^{b}	3 Hours
24.973**	0.47	7.52^{c}	0.38	6.90^{b}	0.64	6.44^{a}	0.44	6.80^{b}	4 Hours
7.748*	ND	ND	0.49	6.74^{b}	0.72	6.17^{a}	0.55	6.62^{b}	5 Hours
30.607**	0.52	7.39^{c}	0.53	6.60^{b}	0.82	5.87^{a}	0.57	6.41^{b}	6 Hours
		1.931		16.131**		31.614**		19.198**	F value (comparing period of observation)

Note: In the data columns the pairing is "+ SD" then "Mean"; the leftmost column is F value (comparing groups).

* P< 0.01;   ** P < 0.001;   ND - Not detected

a, b, c - Means with same superscript with in each period of observation do not differ each other (Duncan's Multiple Range Test)
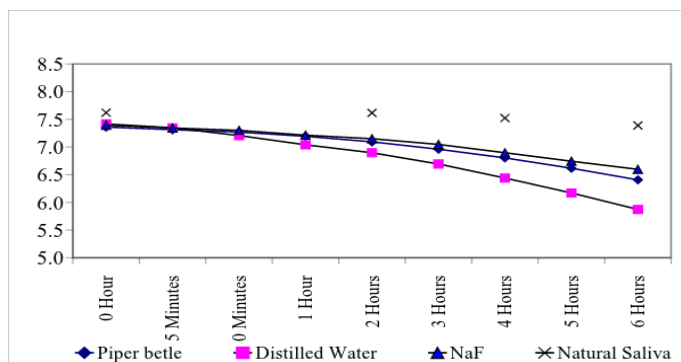
Fig1. Mean salivary pH change during different observation periods in all groups

Table 2. Percentage of subjects having < 5.5 salivary pH in each group

Percentage	Frequency	Percentage	4th Hour Frequency	5th Hour Percentage	6th Hour Frequency	Group
6.37%	2	10.00%	3	36.67%	11	Distilled water
		3.33%	1	6.67%	2	Piper betle
				3.33%	1	Sodium flouride

All the study groups showed a fall in mean salivary pH at the end of 6 hour observation period (TABLE 1). But for the normal saliva there was no significant change in pH at the end of 6hr.(p>.05)

From the mean initial pH [7.36 in **PB** group, 7.42 in **D** group, 7.39 in **Na F** group], there was significant decrease in the pH at the end of 6h (p<0.001) [6.41in **PB** group, 5.87 in **D** group, 6.60 in **NaF** group]. But the fall in pH was not the same in all the three groups. The maximum drop in the pH was observed distilled water group (pH5.87) and for PB group and NaF group the pH drop was comparable (Fig1).

On comparing the pH of different groups at particular instant of time using Duncan's post hoc analysis, there was no significant difference in pH among the various groups until an hour. After an hour there was significant difference in pH between the distilled water group and Piper betle group and also between distilled water and sodium fluoride group. No significant difference between the Piper betle group and Sodium fluoride group in terms of pH at any particular time. Significant difference was noted between normal saliva and all the three groups.

It was also observed that 36.67% of the samples in distilled water group had a salivary pH of less than 5.5 at the end of six hour compared to 6.67% in PB group and 3.33% in case of NaF (TABLE 2).

V.   Discussion

The Piper betle or betel vine is a climber having deep green heart shaped leaves known by a wide range of synonyms Nagavalli, Nagurvel, Saptaseera, Sompatra, Tambul, Tambuli, VakshaPatra, Vettilai, Voojangalataetc in different parts of India. The vine is dioecious (male and female plants are different). There are about 100 varieties of betel vine in the world, of which about 40 are found in India [1,3]

Numerous chemicals have been isolated from leaves and root of Piper betle. The array of chemicals include hydroxychavicol, chavibetol, cadinene, allylpyrocatechol, estragole, methyl eugenol, hydroxyl catechol, methyl piperbetol, piperol A, piperol B, carvachol, caryophyllene, eugenol, isoeugenol, piperine, β-sitosterol, β-sitosterylpalmitate[1,4–7]. This wide range of chemically active agents may be responsible for its wide range of pharrnacobiological actions. The PB extract was shown to have antimycotic activity(Aspergillus), antimicrobial (Vibrio cholerae, Staphylococcus aureus, Diplococcuspneumoniae and Klebsiellaaerogenes),anti protozoal(Plamodiumberghei, Leishmania) activity and it has been shown to inhibit early settlers of plaque and possess significant activity against Streptococcus mutans [8–13].

The extracts of Piper betle has shown to possess multitude of pharmacological effects which include cardioprotective action, antiplatelet and anti-inflammatory action, antioxidant and free radical scavenging action, neuroprotective and hepatoprotective action, antiulcer activity, antidiabetic activity and antimutagenic action[6,7,14–18].PB extract was shown to be nontoxic in one preclinical study. The present study was an attempt at finding out the possible role of Piper betle in the prevention of caries through its action on salivary pH.

The role of plaque in the development of caries is well established[19]. Stephan established by his classic study a fall in plaque pH covering the enamel following sucrose challenge and that the plaque pH would gradually return back to the resting levels within 30-60 minutes. This is mainly due to the saliva present in the oral cavity[20–22]. The bicarbonate and phosphate ions from saliva can diffuse into the plaque and neutralize the acid or the excess acid in plaque can diffuse into the saliva following concentration gradient and thus pH of

plaque is brought back to the resting level. So plaque-salivary interface plays a key role in modifying dental plaque pH and hence, directly affect ionic composition of plaque fluid which in turn would affect the mineral component of enamel.

The salivary secretion rate in an individual falls to almost zero during sleep. Total volume of saliva (0.8-1.2ml) spread out to a thin film on teeth and mucosa [23]. The fermentable sugar remnants will remain dissolved in this amount of saliva. But if the concentration is large it will cause stimulation of salivary secretion with resultant dilution of the substance and its gradual elimination. This is the rationale for selecting 1ml of saliva and 10% sucrose solution for the study. Assuming the average sleep duration of an individual to be a minimum of 6hrs, the secretion of saliva during this time will be negligible and hence its buffering capacity is also low. A small quantity of saliva will remain stagnant in the oral cavity during this time. This is reason for selecting 6hr as the observation period for recording pH change of saliva.

Saliva in the oral cavity is not sterile. It contains numerous microbes and these microbes too would metabolise sugar and produce acid, not alone the plaque bacteria. The only difference is that an organised microbial ecosystem exists in plaque whereas free floating planktonic forms exist in saliva. So, if sucrose is available in the saliva during night time the salivary pH also will fall as inferred from our study. It was inferred from the study that 36.67% of the salivary sample(table2) without NaF or Piper betle developed a pH of less than 5.5 or the critical pH. This fall in pH of saliva may have direct effect as well as indirect effect (through plaque) on the equilibrium of ionic composition at tooth-saliva; tooth-plaque and plaque-saliva interface and hence will enhance the caries demineralising activity as well as tooth erosion. This aspect played by saliva in dental caries hasn't been looked upon in literature. Even though huge amount of literature can be found about protective effects of saliva in dental caries the role played by the free microbes in saliva seems to be ignored. This study may be first of its kind which put a light on the caries facilitating action of saliva and this nocturnal salivary pH drop in presence of fermentable sugars can also explain some cases of teeth erosion and sensitivity.

The saliva will act as a reservoir of microorganism for the formation of plaque. Salivary factors modify the quantity and quality of the plaque so formed. So the salivary pH will also have significant effect on the quality of plaque. According to Takahashi and Nyvad, prolonged exposure of plaque to low pH would result in acid induced adaptation and acid induced selection of low pH non-mutans bacteria within the heterogenous plaque ecosystem. This shift towards acidogenicity in plaque might cause disturbances in demineralisation-remineralisation dynamics and induce initiation and progression of caries. Further fall in pH will provide selective survival advantage to aciduric bacteria such as S.mutans and lactobacilli [24]. So the plaque derived from low pH saliva will be significantly acidogenic and the probability of developing caries will be more. From the above it can be concluded that by preventing a fall in salivary pH we can reduce the formation of acidogenic plaque and hence reduce the initiation and progression of carious lesion.

By assessing the fall in salivary pH following a sucrose challenge we can assess the cariogenic potential of that particular individual. If there is an appreciable fall in salivary pH we can assume the individual to have a high caries susceptibility. In caries susceptibility test we are actually measuring the acidogenic microbes present in saliva (eg- Lactobacillus colony count test). By recognising the importance of fall in salivary pH and its influence on development and progression of carious lesion we chose this model for the preliminary evaluation of anticariogenic potential of P.betle extract.

The results of the study indicate that the Piper betle(5%) possess significant potential to reduce acid production by salivary microbes. The effectiveness Piper Betle was comparable to that of Sodium fluoride which is an established agent which inhibits the acid production.

The potential for the Piper betle in the prevention of caries was deduced from the observation of low caries incidence among betel leaf users in the form of pan. For evaluating a plant for its antimicrobial or chemical properties the active principles present in it should be extracted. Extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular marc. Simple maceration using 50% ethanol in distilled water was used to prepare the extract. The hydroalcoholic solution would dissolve the water soluble and aromatic components from the leaves. The extract obtained was semisolid in consistency with dark brown colour and had an aromatic odour.

Even though many organisms are involved in the development of caries, Streptococcus mutans is proposed to be the principal organism involved in the initiation of caries. Nalina et al have demonstrated antimicrobial action for P betle extract against Streptococcus mutans. P betle has also shown to inhibit growth, acid production, cell associated glucosyltransferase, adherence of S mutans in a concentration dependant manner [12]. So S.mutans (MTCC-497) was used to find out the effective concentration of the P.betle extract to be used in the study. The concentration of extract used was adjusted so that the effective concentration of Piper betle in test solution is below the bactericidal concentration so as to assess the inhibitory effect of extract on acid production by bacteria rather than its cidal effect.

The saliva collected was stimulated saliva as indicated by the high initial pH. The buffering capacities of saliva of each of the individuals differ. So, each sample was titrated with small quantity of Hydrochloric acid

until there was pH drop of 0.1 from the intial value. The acid was neutralised by the salivary buffer until its buffering capacity was overcome. It was done to reduce the variability between different salivary samples.

From the observation it can be inferred that the salivary pH change is gradual, but significant in all the three groups unlike the plaque pH behaviour. This may be due to the difference in the number microbes present or due to the difference in the metabolic activity of the microbes or it may be due to the pH inhibitory factors in saliva. The NaF(.05%) or Piper betle(5%) does not cause complete inhibiton of acid production by the bacteria in the saliva. This may be owing to difference in susceptibility of various microbes in saliva to NaF and Piper betle or to their respective concentration used. The study demonstrated Piper betle(5%) to be as effective as NaF(0.05%), in restricting the fall in salivary pH following sucrose challenge. This confirms the anticariogenic potential of Piper betle.

T.Nalina and Z.H.A.Rahim has reported the presence of fatty acids, hydroxy fatty acids and hydroxychavicol in the aqueous extract of Piper betle [25].The fatty acids present in P.betle might interfere with glycolytic enzymes of bacteria and hence interefere with the acid production. The hydroxychavicol, a major component of Piper betle extract is shown to inhibit acid production and insoluble glucan synthesis by interfering with glucosyltransferase enzyme [26]. The P.Betle extract has shown to increase the membrane permeability and coagulation of nucleoid, which was proposed as the mechanism for its antibacterial properties [25]. May

VI.   Conclusion

Piper betle appears to be a hidden treasure pregnant with numerous alkaloids having favourable biological effects that have been overlooked in the past. Piper betle was shown to be effective in inhibiting the acid production by the salivary bacteria thus proves to be an effective anticariogenic agent. Further studies need to be done to isolate the active ingredients which may be more potent and effective in prevention and control of dental caries and related oral disease. The role of planktonic salivary microflora in relation to development of caries, erosion and sensitivity needs to be analysed critically which has not been given emphasis in the past.

Acknowledgements

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