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In-vitro Anticariogenic Activity of *P. betle* Leaf Extract Against Oral Pathogens.

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ABSTRACT

The antimicrobial activity of leaf extract of *Piper betle* against oral bacteria that cause dental caries was investigated in this report. Using agar-well diffusion method, the antimicrobial potential of the extract was tested on clinically isolated and reference strains, including *Streptococcus mutans* MTCC 497, *Lactobacillus acidophilus* MTCC 10307, *Candida albicans* MTCC 183, and *Staphylococcus aureus* MTCC 1144. The MIC the bacterial species were 0.16 mg/ml for all microorganisms tested. Subculturing microtiter wells that did not change the colour of the indicator after incubation yielded the minimum bactericidal concentration (MBC). *S. aureus* was the most susceptible of all the bacteria examined. The ability of clinically isolated *S. mutans* to develop a biofilm on the seashell surface was investigated further. In the presence of leaf extract of *Piper betle*, the biofilm reduction percent (RP%) assay was used to quantify the inhibition. the greatest reduction was observed at a concentration of 0.016 µg/ml of PE extract. From this we can conclude that extract of *Piper betle* leaves could be used as a phytotherapeutic agents to prevent biofilm formation by common oral pathogens.

Keywords: Biofilm, Oral Pathogens, Streptococci, *Piper betle*

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INTRODUCTION

Oral biofilms comprise of microbial plaque communities that are genetically distinct from each other and utilize host substratum to multiply and aggregate with each other in close proximity. This is done by strong mechanical as well as physiological and metabolic interactions. The biofilm is initiated by streptococcal species followed by other oral microorganisms to firmly establish the microbial ecosystem on the tooth surface [1]. The organisation of the biofilm is multifactorial depending upon oral hygiene, diet, salivary flow, diurnal changes and host defences [2]. The oral cavity of humans is a complex ecosystem possessing both acid-producing and acid-tolerant bacteria. Nearly 700 different bacterial species of the human oral cavity have been characterized and 200-300 species have been identified directly linked to dental plaque.

This association consists of relatively high numbers of aciduric and acidogenic bacteria like streptococci and lactobacilli. These organisms cause the demineralization of the tooth enamel resulting in dental caries. In the presence of sucrose, *Streptococcus mutans* secretes water-soluble glucan that assists in its attachment to the tooth surface. Lactobacilli are the pioneer species in the advancing front of the carious phase, particularly affecting dentine; and are involved more in the progression of the deep enamel lesion by the secretion of organic acids, oxygen peroxide, and 'bacteriocins' [3]. Other endogenous oral microorganism such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, S. mutans, Prevotellam* sp., and *Fusobacterium* sp., as well as their metabolites, play an important role in the initiation and development of certain infections. *Candida albicans* produces Secreted Aspartyl Proteinases (SAP), a factor contributing to virulence [4].

There are several ways by which the development of biofilm can be inhibited. These include the prevention of cariogenic bacteria from attaching to the substratum by the use of antimicrobial compounds, enhancing host defences and manipulation of cell signalling mechanisms. Dental formulations such as toothpastes and mouthwash contain phytochemicals extracted from different tissues of medicinal plants making it efficacious, safe, and cost effective [5]. There is a trend towards the increasing use of such plant-based formulations as against chemical-based drugs that possess several side effects [6]. One of the best ayurvedic remedies against paediatric oral disorders is the use of 'Triphala' consisting of Haritaki, Bibhitaki and amla in mouthwashes [7].

Initiation and progression of dental caries may be prevented by plaque control aiming at decreasing pathogenic flora of the supra- and subgingival regions. This is largely performed by physical methods along with adjunctive therapy like the administration of antiseptics and antibiotics [8]. The greatest issue associated with the use of antibiotics is the development of antibiotic resistance, changes in the complement of oral microbiome and lastly, teeth staining [9]. Hence it becomes imperative to use natural products either alone or in combination with oral prophylaxis methods. This is done to either eliminate and/or reduce the chance of side-effects.

Developing countries largely depend on the use of traditional medicines for the management of various health conditions. About 80% of individuals in developing countries follow conventional home-based remedies [10]. *Piper betle* or betel leaf, belonging to the Piperaceae family is largely used across Asia against various ailments. It is mostly consumed in Asia including India and parts of Sri Lanka. It has strong antimicrobial properties that fight against pathogenic oral microflora. Betel leaf used in *paan* as a wrapping or covering for areca nut or tobacco is regarded as a great remedy to get rid of microbes that cause bad breath. Many reports suggest the chewing of betel leaf as a mouth freshener. Betel leaf when chewed can aid in combating the acids secreted by bacteria thereby reducing the risk of dental caries. Consuming too much *paan* can lead to oral cancers and therefore their intake must be regulated. It is sometimes used in cooking and imparts a raw peppery taste. Through this research paper, we aim at investigating the antimicrobial ability of betel leaf extracts in the prevention of dental caries.

MATERIALS AND METHODS

Piper betle extract preparation

Piper betle leaves were collected from the institutes botanical garden, washed well with water, air-dried, placed in a hot air oven at 50°C, and then powdered mechanically in an electric grinder. Using a rotary shaker, 20 g of the powder was immersed in 100 ml of ethanol and acetone respectively for 6 h. To remove plant particles, the crude *P. betle* extracts (PE) were filtered through Whatman filter paper no. 1 in a Buchner funnel,

and then evaporated at 40 °C. By agitation at 45 °C, the dry powder was suspended in distilled water to obtain a final concentration of 10% (w/v). The PE was sterilised using a syringe filter (0.22 m pore size) and stored at 4 °C in sterile dark bottles.

Phytochemical screening

Phytochemical investigations were carried out for all the extracts as per standard methods [11,12]. Qualitative detection of carbohydrate, proteins, amino acids, alkaloids, glycosides, saponins, phytosterols, phenols, and flavonoids were conducted in different solvent systems.

Isolation and characterization of the clinical isolate

Santosh Dental Care and RMV Dental Clinic in Bengaluru, India provided demineralized freshly extracted decayed teeth from patients with caries for this experiment. The specimens were collected in sterile screw-capped vials with 5 ml of sterile thioglycollate broth as the transport medium. For isolation, the specimens were streaked onto Brain Heart Infusion agar followed by incubation at 37 °C for 24 h. Before each isolate was identified, it was assigned a number, which was used during the entire process. Based on information from Bergey's manual of determinative bacteriology [13], the isolated species were classified as Streptococcus. There were several distinct colony features that were noted. Various biochemical tests, such as sugar fermentation test, indole, citrate utilisation and MR-VP were performed to classify the isolated subcultures [14]. Gram staining was used to identify the morphology [15].

Screening for biofilm forming ability

Tube assay: The tube staining assay, as described by Christensen et al., [16] was used to evaluate the qualitative assessment of biofilm formation. A loopful of Isolate 6 grown overnight was inoculated in 3 ml of Brain Heart Infusion broth with 1% D-glucose and incubated for 48 h at 37 °C. After incubation, the tubes were decanted carefully and rinsed with Phosphate Buffer Saline (pH 7.0), air dried, and then stained with 0.1% crystal violet. The tubes were washed with purified water to eliminate any remaining stain. The existence of a visible film on the walls and bottom of the test tube indicated the formation of biofilm [17].

Growth kinetics of isolated strains: The growth of the isolated strains both in biofilm and planktonic conditions were investigated.

Kinetics of planktonic growth**:** 50 µl of Isolate 6 grown overnight was inoculated into 5 ml of LB broth and the initial OD of this suspension was read in a spectrophotometer at 630 nm. The culture was incubated at 37 °C at 180 rpm. The OD was measured every 30 min. The planktonic growth kinetics of the isolated strains were determined by plotting time vs. absorbance.

Kinetics of biofilm formation**:** 100 µl of 1:100 dilution of Isolate 6 was inoculated into four sets of 96 well microtitre plates. The absorbance was measured using an ELISA reader at 570 nm at two-day intervals, following the same staining procedures used for screening. The biofilm formation kinetics of the isolated strain was determined by plotting their OD values against time intervals.

Screening of Antimicrobial Activity

The antimicrobial activity of the extracts was calculated by agar well diffusion method [18]. Pure clinical isolates of Streptococcus and reference species, namely *Streptococcus mutans* MTCC 497, *Lactobacillus acidophilus* MTCC 10307, *Staphylococcus aureus* MTCC 1144, and *Candida albicans* MTCC 183, were first subcultured on Nutrient broth at 37 °C for 24 h. Both bacterial strains were grown and maintained on nutrient agar slants at 4 °C, while *C. albicans* MTCC 183 was cultured in Sabouraud Dextrose Agar at 37 °C under aerobic conditions for 24-48 h.

Two separate assay methods were used to assess the anti-microbial activity of QIE: 1) zone of growth inhibition (ZOI) using the cup-plate process, and 2) determination of minimum inhibitory concentrations (MIC) using the appropriate growth media for each microbial strain [19]. The cup-plate approach was used to assess antimicrobial activity of PE in different solvents based on its ability to inhibit the growth of the particular

microorganism on Brain Heart Infusion agar [20]. 100 µl of 24 h old inoculum of the test species at 105 CFU/ml were swabbed onto sterile media. 8 mm diameter wells were punched into the agar medium and filled with 45 µl of PE. The plates were incubated for 24 h at 37 ºC. 0.2 % chlorhexidine was used as positive control. The zone of inhibition (ZOI) was measured in mm. The tests were done in triplicates, and the mean diameter of zone of inhibition ± SD was calculated.

Determination of MIC and MBC

The two-fold serial microdilution method was used to assess the MIC of the most effective extracts at final concentrations ranging from 10 mg/ml to 0.02 mg/ml [21]. The extracts were added to sterile Mueller Hinton Broth before bacterial suspensions with final inoculum concentration of 105 CFU/ml. Each extract was tested in triplicates. The bacterial suspensions served as a positive control, while the extracts in the broth served as a negative control. The turbidity of the microtiter plate wells was interpreted as visible microbial growth. After 24 h of inoculation at 37 °C, the MIC values were calculated as the lowest concentration of extracts that displayed no turbidity. A subculture of the well that showed no visible growth in a sterile agar plate was used to establish the minimum bactericidal concentration (MBC). The MBC value was calculated using the lowest concentration that showed no visible growth on agar subculture.

Shell assay for biofilm inhibition in the presence of PE

Biofilm formation on shells was studied qualitatively because shells are made of calcium carbonate and can the mimic tooth surfaces. Before usage, the ridged and smooth shells were sterilised with 2% glutaraldehyde and washed twice with sterile distilled water. Isolate 6 grown in BHI broth was inoculated with PE. The shells were stained with 1% crystal violet for 10 min after 6 days of incubation to enable visualisation of biofilm on the tooth surface. A negative control was set up with phosphate buffered saline (PBS) instead of PE in the same way.

Microtiter Plate Assay

The microtiter plate assay was performed in 96-well polystyrene microtiter plates for the quantification of biofilm formation [22]. BHI broth with 1% D-glucose was prepared and autoclaved for sterilisation. Isolate 6 was inoculated into the BHI and incubated at 37 °C overnight. Following the addition of PE, 33 µl of broth was transferred aseptically to a 96-well microtiter plate (Tarson, India). The plate was then incubated at 37°C overnight. To check for adherence, the wells were washed with 0.85% NaCl 2-3 times and stained with a 1% crystal violet solution. The optical density was estimated at 550 nm using an ELISA plate reader. Negative controls were those microtiter wells containing only sterile LB broth, whereas positive controls were the inoculated wells. The percentage reduction was determined to assess the inhibition activity using the formula:

$$Reduction\ percent\ (RP) = \frac{[(C-B)-(T-B)]}{(C-B)}\ X\ 100$$

Where, *C* = Mean of OD of positive control wells.
B = Mean of OD of negative control wells.
T = Mean of test wells.

RESULTS AND DISCUSSION

Phytochemical screening of *P. betle*

Among all solvents used for phytochemical screening, ethanol and acetone was found to exhibit better extraction of phytochemicals (Table 1). Hence, these solvents were used for further analysis.

Table 1: Phytochemical screening of *Piper betle* leaf extracts

Phytochemical Analyzed	Test performed	Result				
		Ethanol	Methanol	Acetone	Ethyl Acetate	Water
Carbohydrates	Benedict's Test	-	-	-	-	-
	Fehling's Test	-	-	-	-	-
Proteins and Amino acids	Ninhydrin Test	+ + +	+ +	+ +	-	-
	Xanthoproteic Test	+ +	+	+	+	+ + +
Alkaloids	Mayer's Test	+	+	+	+ +	+ +
	Wagner's Test	+ +	+	+ +	+ +	+ +
	Dragendoroff's Test	+ + +	+ +	+ + +	+ + +	+ +
Phenols	Ferric Chloride Test	-	-	-	-	-
Flavonoids	Lead Acetate Test	-	-	-	-	-
Phytosterols	Salkowaski Test	-	-	-	+ +	-
Glycosides	Keller Killiani Test	-	-	+ + +	+	+

- indicates absence, + denotes average, ++ denotes abundance of phytochemicals.

Preliminary evaluation of the clinical isolate

Colony characteristics of the clinical isolate: Sample of three individuals with dental caries yielded eight isolates with different colony morphologies. Table 2 summarises their various characteristics. 75% of the dental isolates were of Gram-positive bacterial species. Streptococci were found to be the most common, which is consistent with previous research on biofilm initiation [23]. Proline-rich proteins and glycoproteins present in the pellicle of the plaque are known to bind to early streptococci colonisers [24], influencing the following stages of biofilm growth and development [25].

Table 2: Colony characteristics of microorganisms isolated from dental caries

Colony character	Colony A	Colony B	Colony C	Colony D	Colony E	Colony F	Colony G	Colony H
Size	Medium	Small	Small	Pinpoint	Pinpoint	Pinpoint	Medium	Small
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colour	White	White	Cream	White	Cream	White	White	White
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Translucent	Translucent	Translucent	Translucent	Translucent	Opaque	Opaque
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Low convex	Low convex	Low convex	Low convex	Low convex	Flat	Low convex	Low convex
Gram nature	Gram positive cocci in chains	Gram negative coccobacilli	Budding yeast	Gram positive bacilli	Gram positive cocci in chains	Gram positive cocci in chains	Gram positive cocci in chains	Gram positive cocci in chains
Haemolysis pattern of Blood agar	β-haemolytic	γ-haemolytic	β-haemolytic	γ-haemolytic	α-haemolytic	α-haemolytic	γ-haemolytic	β-haemolytic

Biochemical identification of clinical isolates: Assay techniques for fermentation abilities, activities of particular enzymes, and certain biochemical reactions are all part of a systematic method for identifying bacteria. Table 3

exhibits the qualitative findings made as a result of these experiments. Biofilm formation experiments were conducted using Isolate No. 6, which was identified as *S. mutans*.

Table 3: Biochemical tests for the identification of the clinical isolate

Isolate No.	Biochemical tests				Sugar fermentation test			Enzymes		
	Indole test	MR test	VP test	Citrate utilization test	Glucose	Lactose	Sucrose	Amylase	Catalase	Oxidase
1	+	-	+	+	+	+	+	-	-	-
2	+	-	+	-	-	-	-	-	-	-
3	+	-	-	-	-	-	-	-	-	-
4	+	+	-	-	+	+	+	+	-	-
5	+	-	+	+	+	+	+	-	-	-
6	+	-	+	+	+	+	+	-	-	-
7	+	-	+	+	+	+	+	-	-	-
8	+	-	+	+	+	+	+	-	-	-

Screening of primary biofilm formation: Biofilm formation serves as a protective mechanism in response to a variety of stressors, as it can withstand the shear forces created by saliva. Microbial species forming the biofilm are also much more resistant to pH changes, food deprivation, treatment with antibiotics and presence of reactive oxygen radicals than planktonic microorganisms. The formation is dependent on successful interaction of streptococci with receptor proteins such as salivary mucin [26]. Other interactions that play a role include activation of α–amylase, and binding to proline-rich peptides and glycosylated proline-rich glycoproteins [27]. These interactions enable the initiation and growth of biofilm on the surface of seashells. Co-adhesion with other organisms that have established adherence onto the substratum enables binding of those species that cannot bind directly to the substratum on their own. Only Isolate 6 clinical isolated and cultured form dental samples formed a biofilm on the inner walls of the test tube that could be visualized by staining with crystal violet.

Planktonic development and kinetics of biofilm formation: The growth pattern of Isolate F in planktonic form is as shown in Fig. 1A. Due to disintegration, the biofilm dramatically declined after 10 days as depicted in Fig. 1B.

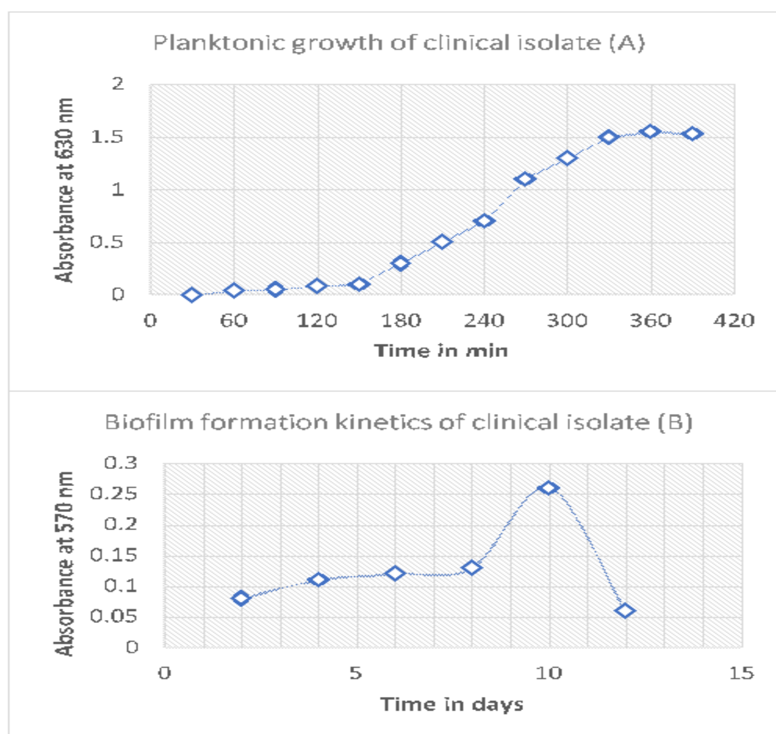

Figure 1: Planktonic growth curve of the clinical isolate (A); biofilm formation kinetics of the clinical isolate (B).

Antimicrobial activity of PE on oral pathogenic strains

Screening of antimicrobial activity: Most traditional therapists use plant material extracted mainly in water, as a solvent. When compared to aqueous extracts, many researchers have proven that plant extracts homogenized in methanol have shown greater antibacterial activity [28]. Phytochemical screening demonstrated that ethanol and acetone were better at extracting bioactives needed for the antimicrobial activity. Acetone is especially valuable as an extractant as it dissolves both hydrophilic and hydrophobic bioactive compounds and has low toxicity [29]. Table 4 depicts the antibacterial and anticandidal activities of extracts against each of the test species at 200 mg/ml concentrations. *C. albicans* was most sensitive to the extracts of PE in ethanol and acetone with a ZOI of 23 mm and 21 mm respectively. This was followed by *L. acidophilus* with the extracts of PE in ethanol and acetone showing ZOI of 12 mm and 10 mm respectively. *S. aureus* and *S. mutans* were found to be least affected by the extracts. PE possesses broad-spectrum antibacterial compounds due to the presence of hydrophilic bioactives that can be extracted by polar solvents.

Table 4: Anti-microbial activity of extracts of *P. betle* by agar diffusion test

Solvent used for extraction	Zone of Inhibition (mm)				
	S. mutans	***S. aureus***	***L. acidophilus***	***C. albicans***	***Chlorhexidine***
Ethanol	7	9	12	23	10
Acetone	8	5	10	21	7

*****ZOI of microbial growth around the well was measured (in mm). The tests were performed in triplicate, and the means of the values are shown in Table 4.

Determination of MIC and MBC: The MIC values against all bacterial species tested was 0.16 mg/ml. The results of the zone of inhibition test were associated with the MIC values. The MBC values are shown in Table 6.

Table 5: The MIC values of ethanolic PE against oral bacteria.

Concentration (mg/ml)	Microorganism				Control	
	S. mutans	***S. aureus***	***L. acidophilus***	***C. albicans***	**Positive**	**Negative**
5.00	-	-	-	-	+	-
2.50	-	-	-	-	+	-
1.25	-	-	-	-	+	-
0.63	-	-	-	-	+	-
0.31	-	-	-	_	+	-
0.16	+	+	+	+	+	-
0.08	+	+	+	+	+	-
0.04	+	+	+	+	+	-
0.02	+	+	+	+	+	-
0.01	+	+	+	+	+	-

Table 6: The MBC values of ethanolic PE against oral bacteria.

Strain	Extract concentration (mg/ml)					
	5.00	2.50	1.25	0.63	0.31	0.16
S. mutans	-	-	-	-	ND	ND
S. aureus	-	-	-	-	-	-
L. acidophilus	-	-	-	+	ND	ND
C. albicans	-	-	-	-	+	ND

Qualitative inhibition of biofilm on seashell: Isolate F demonstrated slight consistent darkening and random darker spotting on the smooth shell ridged shell surfaces respectively after 6 days of incubation. Upon treatment with crystal violet, deep purple colouration was seen in the negative control while the shells treated with PE

appeared mildly purple (Fig. 2). This indicates that PE has ability to inhibit streptococcal biofilm formation. Similar results were obtained in the presence of methanolic extracts of galls of *Quercus infectoria* [30].

Figure 2: Inhibition of Streptococcal biofilm by ethanolic extract of *P. betle,* with ridged shells (a) and smooth shells (b).

Biofilm inhibition in microtiter plates: The inhibition of streptococcal biofilm formation was expressed as percentage reduction by treating the biofilm in microtiter plates with increasing concentrations of ethanolic PE (Fig. 3). With increasing concentrations of PE extract, the reduction percentage was found to increase.

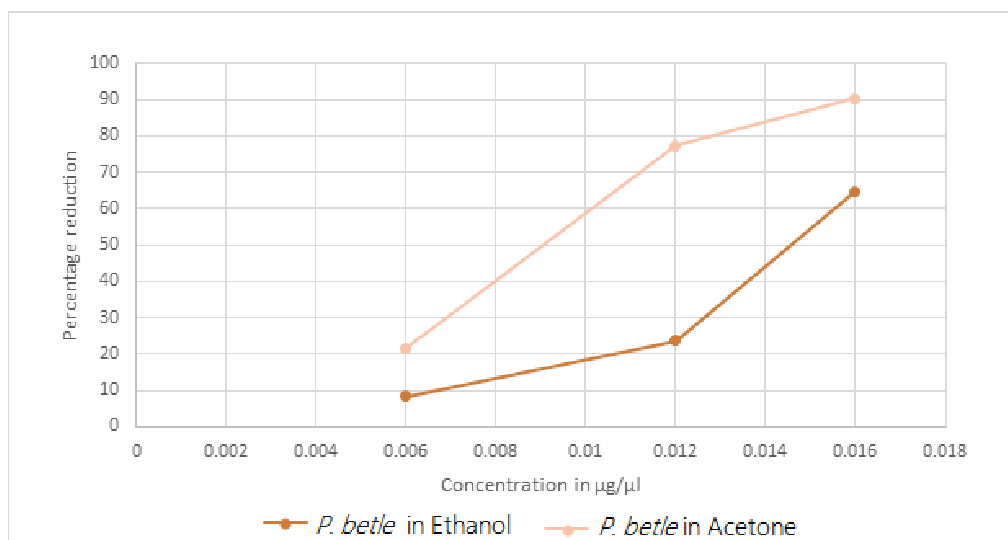

Figure 3: Comparison of reduction percentage of ethanol and acetone extracts of *P. betle* on inhibition of Streptococcal biofilm.

CONCLUSION

This research sheds light on the use of extracts of *P. betle* in the treatment of oral disease caused by a bacterial infection. *S. mutans* was isolated from dental caries for its biofilm forming in the presence of sucrose. The ethanol and acetone extract of *P. betle* in was both found to contain a potent bioactive that inhibited the growth and multiplication of common oral pathogens. The PE extract performed marginally better than chlorhexidine. As a result, it can be formulated for dental use. However, further research is required to see how PE affects oral microorganisms when grown as a mixed culture possessing coordinated and complicated behavioural strategies.

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Conflicts Of Interest

The authors declared no competing interests.

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