Research Article

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EVALUATION OF ANTI-BACTERIAL, ANTIOXIDANT, ANTI-INFLAMMATORY AND CYTOTOXICITY POTENTIAL OF MOUTH RINSE CONTAINING *PIPER BETEL* ESSENTIAL OIL

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ABSTRACT

Piper betel Linn is considered to possess important medicinal values. Minimum Inhibitory Concentration (M.I.C) of Piper betel oil was determined against common periodontal pathogens- *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia*. Using the M.I.C. results a mouthwash was formulated in three concentrations namely-0.1%, 0.3% and 0.5%. Present in vitro study was conducted to evaluate antibacterial potential of different concentrations of mouthwash against previously mentioned periodontal pathogens by determination of minimum inhibitory concentrations (M.I.C). Antioxidant, anti-inflammatory and cytotoxicity potential was also determined for the same. Antibacterial potential was determined using Disc diffusion test and Broth Microdilution method. Antioxidant and anti-inflammatory potential was determined by measurement of superoxide dismutase (SOD) activity using riboflavin-NBT assay and detection of Matrix Metallo Proteinase (MMP) -2 and MMP-9 by gelatin zymography method respectively. Cytotoxicity potential was determined using MTT cytotoxicity assay. All three concentrations of mouthwash showed effective antibacterial, antioxidant and anti-inflammatory potential. Cytotoxicity result showed viable cells indicating non-toxic nature of all three mouthwashes. Mouthwash containing *Piper betel* essential oil possesses effective antibacterial, antioxidant and anti-inflammatory potential and could be used effectively as an oral care agent.

Keywords: Antibacterial, Antioxidant, Anti-inflammatory, cytotoxicity, Piper betel essential oil

INTRODUCTION

Current research targeting microbial biofilm inhibition has attracted a great deal of attention. The search for effective antimicrobial agents against oral pathogens has led to the identification of new agents for the prevention of oral biofilm associated diseases. A variety of plant materials and phytochemicals, especially a class of essential oils, have long been found to exhibit effective antibacterial activity. The aromatic molecules derived from natural sources are being explored extensively as alternative agents in oral care products[1]. Since antiquity, betel leaves (*Piper betel Linn*) are the most valued plant (betel vine) part, and in the past were routinely used as a chewing agent to prevent halitosis. In addition, the leaves were supposed to conserve the teeth[2]**.** Irrespective of the uses, betel vine is arguably the most maligned plant. This infamous accreditation is principally due to the fact that habitual chewing of betel quid consisting of areca nut, betel leaf, catechu, slaked lime, and often tobacco causes oral cancer [3, 4]. However, contrary to the accepted belief, scientific studies have shown that tobacco [5, 6], areca nut [4,5,7-13] and slaked lime [14, 15] in the betel quid promoted carcinogenesis but, the betel leaf is devoid of mutagenic and carcinogenic effect. In fact, it has also been conclusively shown that the betel leaf and its phytochemicals namely chavibetol, chavicol, hydroxychavicol, estragole, eugenol, methyl eugenol, hydroxycatechol prevented chemical induced cancers in experimental animals[7]. In addition, the leaf also produces an aromatic volatile oil which contains phenolic compounds mainly chavichol and allylpyrocatechol[2], which have powerful antiseptic and antimicrobial properties. Betel leaves (*Piper betel*) can be used to treat halitosis by reducing the volatility of methyl mercaptan[16]. The aroma of betel leaf is due to the presence of essential oils, consisting of phenols and terpenes[2]. Preclinical experiments have also shown that betel leaf possess anti-fungal, anti-inflammatory, anti-allergic and immune modulatory effects[17]. On the basis of above properties possessed by betel leaf, the minimum inhibitory concentration (MIC) of *Piper betel* essential oil was determined against the periodontal pathogens namely*: Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia*. A mouthwash was then formulated using the obtained MIC at three different concentrations (0.1%, 0.3% and 0.5%). The present study was aimed to evaluate the

antibacterial, antioxidant, anti-inflammatory and cytotoxicity potential of these three mouthwashes in an in vitro environment.

MATERIALS AND METHODS

Piper betel essential oil was obtained from Natural Product Company, Rym exports, Mumbai, India. MIC value of this oil was determined against three periodontal pathogens, *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia* using standard disc diffusion *(*agar well diffusion*)* and broth micro dilution method in a previous study[18]. Using the MIC values for oil, three mouthwashes were prepared with different concentrations of *Piper betel* essential oil and named accordingly:

Mouthwash A: 0.1% *Piper betel* oil
Mouthwash B: 0.3% *Piper betel* oil
Mouthwash C: 0.5% *Piper betel* oil

Other ingredients that were present in mouthwashes are summarized in Table 1. All the chemicals used were of analytical grade.
The antibacterial, anti-oxidant and anti-inflammatory potential of mouthwashes were determined at Maratha Mandal's Institute of Dental Sciences and Research Centre, Belgaum, Karnataka, India. The cytotoxicity potential of mouthwashes was determined at Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, Karnataka, India.

Anti-Bacterial Potential

The antibacterial potential of the three mouthwashes (A, B, C) was evaluated *in vitro* using agar disc diffusion and broth micro dilution method, against the previously mentioned periodontal pathogens.

Bacterial strains and growth conditions: The bacterial strains that were used were *P. intermedia* (ATCC 25611), *P. gingivalis* (ATCC 381) and *A. actinomycetemcomitans* (JP2). All bacteria were grown in brain–heart infusion broth supplemented with 5 µg/mL of haemin and 1 µg/mL of menadione (BHI with supplements), at 37^{0}C under anaerobic conditions (80% N2, 10% H2 and 10% CO2). The disc diffusion test and broth micro dilution tests were done to obtain the susceptibility and MIC of three mouthwashes.

Agar Disc Diffusion test: This test was used to determine the zone of inhibition (measured in mm), which showed the antibiotic susceptibility of microorganism towards the tested mouthwash. Brain heart infusion agar was used, which was inoculated with the microbial cell suspension (broth) of above mentioned organisms. 5 wells of 5mm diameter each were made on each inoculated agar plates, and were subsequently inoculated with 20 µl of mouthwash (A, B, C) in the following dilutions (1:0.75, 1:2, 1:4, 1: 8 and 1:16) and incubated according to the standard protocol[19]. The zones of growth inhibition were measured in millimeters using a caliper or ruler.

Broth Micro Dilution: MIC is considered as the lowest concentration that will inhibit the growth of the test organisms, and thioglycolate broth was used for the same in the present study. Antimicrobial susceptibility testing was performed using nine serial dilutions which were made using stock cultures of the microorganisms and broth followed by incubation for 48 – 72 hours in an anaerobic jar at 37 ° C and then observing for turbidity as part of the standard protocol[20].

Antioxidant Potential

This was checked by measurement of superoxide dismutase (SOD) activity, which was determined using the riboflavin-NBT assay[21], in which a blue coloured complex called formosan is formed. The SOD present in sample leads to decreased production of formosan manifested by decreased intensity of blue colour formed. A 50% decrease in the formation of Formosan is taken as one unit of SOD[21].

Anti – Inflammatory Potential

The anti – inflammatory potential was determined by detection of MMP-2 and MMP-9 using Gelatin Zymography. This procedure requires the preparation of 10% resolving gel and 5% stacking gel. The sample of mouthwash was prepared with addition of 5ml of tris buffer; centrifuging at 3000rpm for 15 min, and storing in -20^{0}C for further use. Before experiment the sample was centrifuged at 3000 rpm for 10-15min; supernatant formed was then used after mixing equal volume of 2x non reducing buffer into sample supernatant. It was mixed and pipetted into wells using gel loading tips.

For control 50µl of mouthwash was pre incubated with 50µl of tetracycline (300µg/ml) for 60min at room temperature. 20 µl of test sample in each well and 10 µl molecular weight marker in last well were loaded. The apparatus was run at about 50V for 15 min and then 100V until the bromophenol blue reached at the bottom of the plates.

After electrophoresis was completed the gel was washed with zymogram renaturing buffer i.e.2.5%Triton x-100 for one hour to remove sodium dodecyl sulphate from the gel and allow proteins to denature. The gel was then incubated in zymogram at 37^{0}C overnight in zymogram incubation buffer. Gel was then stained with coomassie blue R-250 for one hour, after which gels were destained with an appropriate destaining solution for about 2 hours. Appearance of white bands indicated the presence of gelatinases, lower bands indicate gelatinases-A (MMP-2) which is about 72KD while the upper bands indicate gelatinases-B (MMP-9) which runs at about 95kDa.

Cytotoxicity Potential

For performing cytotoxicity potential, normal green monkey kidney epithelial cell lines were procured from National Centre for Cell Sciences **(**Pune, India**).** The cells were cultured with Dulbecco**'**s modified eagle**'**s medium **(**DMEM**)**, added with 10% Foetal Bovine Serum **(**FBS**)** and penicillin **(**100 µg/ml**)**, streptomycin **(**100 µg/ml**)** and amphotericin-B **(**5 µg/ml**)** and incubated in 5% CO_2 at 37 °C **(**Healforce Incubator, Shanghai, China**).**

Cytotoxicity analysis was done using MTT **[**3-**(**4,5-dimethylthiazol-2-yl**)**-2,5-diphenyltetrazolium bromide**]** cytotoxicity assay [22, 23] . For performing cytotoxicity potential, cells were plated in 96 well flat bottom micro titer plate at a density of 1 × 10^4 cells per well and cultured for 24 hours at 37ºC in 5% CO_2 atmosphere. After 24 hours, when partial monolayer was formed, medium was removed and cells were treated with different concentrations of mouthwashes prepared in maintenance medium. One set of plates were incubated for 1 hour and another for 3 hours. After the treatment, the solutions in the wells were discarded and 50 µl of freshly prepared MTT (2 mg/ml, prepared in PBS) was added to each well. The plates were shaken gently and incubated for 3 hours at 37ºC in 5% CO_2 atmosphere. After 3 hours, the supernatant was removed and 50

µl of DMSO was added to solubilize the formazan crystals formed inside the cells. Finally, the absorbance was read using a micro-plate reader (Bio-Tek, EL_X-800 MS) at a wavelength of 540 nm.

The percentage growth inhibition was calculated using the formula below:

% growth inhibition = control absorbance – test absorbance / control absorbance × 100

RESULTS

Antibacterial Potential

Disc diffusion test: With respect to zones of inhibition against *P. gingivalis*, it was evident that with increasing dilutions of all three mouthwashes, reduction in zone of inhibition was observed. Maximum zone of inhibition was observed with 1:0.75 dilutions for all three mouthwashes, with mouthwash A exhibiting maximum zone of 22mm. Against *P.intermedia* similar to that of *P.gingivalis* maximum zone of inhibition was observed with mouthwash A at 1:0.75 dilutions. For *A. actinomycetemcomitans* resistance was observed with lower dilution in comparison to other organisms for all three mouthwashes, with mouthwash C exhibiting maximum zone of inhibition at 1:0.75 dilutions. (Table 2)

Broth micro-dilution test: All the three tested micro-organisms were sensitive to mouthwashes till the 5th dilution. However, micro–organisms exhibited additional sensitivity to mouthwash C till 7th dilution. Amongst the different micro–organisms tested *P.intermedia* showed more sensitivity in comparison to the other two, with all three mouthwashes. (Table 3)

Antioxidant Potential

The different standard concentrations are the different concentrations of ascorbic acid, and these concentrations of ascorbic acid have been compared with different mouthwashes depending on their absorbance. As an anti- oxidant, mouthwash A showed more anti-oxidant potential with 9.3 % activity and absorbance at 649 nm in comparison to other two. (Table 4)

Anti- Inflammatory Potential

Mouthwash A,B and C showed appearance of 70% ,68% and 80% band respectively, which corresponded to 30%, 32% and 20% of anti-inflammatory activity. Mouthwash B exhibited maximum anti-inflammatory potential amongst the three mouthwashes. (Table 5)

Cytotoxicity Potential

More than 50 % of the cells survived with all three mouthwashes. Each mouthwash was studied for two different concentrations namely 125 and 62.5 micro liters, at two different time duration: 1hour and 3hours. All the samples were found to be nontoxic at the tested concentrations. This shows that the formulated mouthwash is safe on tissues when subjected for in-vivo studies. Amongst the different mouthwashes, mouthwash B was least cytotoxic in comparison to other two, with maximum number of viable cells both at 1 and 3 hours and at 125 and 62.5 micro liters.

Table 1: **Composition of the mouthwashes**

Ingredients	Quantity		
	Mouthwash A	**Mouthwash B**	**Mouthwash C**
Propylene Glycol	10% v	10% v	10% v
Glycerine	10% v	10% v	10% v
Tween - 80	3% v/v	3% v/v	3% v/v
Betel oil	0.1% v/v	0.3% v/v	0.5% v/v
Raspberry flavour	0.1% v/v	0.1% v/v	0.1% v/v
Sodium benzoate	0.1% w/v	0.1% w/v	0.1% w/v
Saccharin	1% w/v	1% w/v	1% w/v
Water	QS	QS	QS

Table 2: **Result of Disc diffusion test against *P. gingivalis, P.intermedia, A.actinomycetemcomitans* with zones of inhibition in mm**

Mouthwash	1:0.75			1:2			1:4			1:8			1:16		
	P.g	P.i	A.a	P.g	P.i	A.a	P.g	P.i	A.a	P.g.	P.i.	A.a	P.g.	P.i.	A.a
0.1%	22	25	15	20	15	R	17	23	R	16	15	R	10	14	R
0.3%	20	23	20	19	22	17	16	22	10	12	17	R	10	14	R
0.5%	21	25	20	18	23	18	16	21	15	13	15	R	12	15	R

R= Resistant

Table 3: **Minimum Inhibitory Concentration against *Aggregatibacter actinomycetemcomitans (A.a.), Porphyromonas gingivalis (P.g.), Prevotella intermedia (P.i.)***

Aa	100	50	25	12.5	6.25	3.125	1.6	0.8	0.4	0.2
Mouthwash A	S	S	S	S	S	S	R	R	R	R
Mouthwash B	S	S	S	S	S	S	R	R	R	R
Mouthwash C	S	S	S	S	S	S	S	S	R	R
Pg										
Mouthwash A	S	S	S	S	S	S	R	R	R	R
Mouthwash B	S	S	S	S	S	S	R	R	R	R
Mouthwash C	S	S	S	S	S	S	S	S	R	R
Pi										
Mouthwash A	S	S	S	S	S	S	S	S	R	R
Mouthwash B	S	S	S	S	S	S	S	S	S	S
Mouthwash C	S	S	S	S	S	S	S	S	S	S

S= Sensitive, R =Resistant

Table 4: Anti-oxidant potential of different mouthwashes

SI. No.	Standard /Sample	Absorbance in nm	Concentration in %
1	Standard 0	500	0
2	Standard 1	697	10
3	Standard 2	707	25
4	Standard 3	720	80
5	Standard 4	750	100
6	Mouthwash A	649	9.3
7	Mouthwash B	628	9.01
8	Mouthwash C	620	8.89

Table 5: **Anti-inflammatory potential of different mouthwashes**

S.No.	Sample	% of band	Anti–Inflammatory activity
1.	Mouthwash A	70%	30%
2.	Mouthwash B	68%	32%
3.	Mouthwash C	80%	20%
Positive control		5%	95%
Negative control		98%	-

DISCUSSION

There is a global need for alternative prevention and treatment options and products for oral diseases that are safe, effective and economical. Available treatment strategies are limited by rise in disease incidence (particularly in developing countries), increased resistance by pathogenic bacteria to currently used antibiotics and chemotherapeutics, opportunistic infections in immune compromised individuals and financial considerations in developing countries[15].

Different commercially available chemical agents such as cetyl pyridinium chloride, chlorhexidine, amine fluorides can alter oral micro biota and have undesirable side-effects such as vomiting, diarrhea and tooth staining, presence of ethanol (in mouthwash) have been linked to oral cancer [15].

Natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals. Natural products in form of essential oils are in great demand for oral health care owing to their extensive biological properties and bioactive components which have proved to be useful against large number of diseases[15]. Antibacterial potential of Piper betel oil is attributed to the large quantity of sterols, which are bioactive molecules. Apart from this, the antibacterial property can be attributed to the presence of large amount of fatty acids like palmitic acid, stearic acid and hydroxy fatty acid esters, which cause an alteration in structure of bacterial cell wall leading to pore formation and ultimately cell death. A plethora of phytochemical extracts have shown effective antibacterial potential when used in the form of a mouthwash[24-27]. Present study also showed effective antibacterial potential in various concentrations of the piper betel mouthwash (0.1%,0.3% and 0.5%) against common periodontal pathogens namely *Aggregatibacter actinomycetemcomitans, Prevotella intermedia and Porphyromonas gingivalis*. Amongst the tested micro-organisms *Prevotella intermedia* showed more sensitivity in comparison to the other two.

The antioxidant potential[3,28,29] of piper betel oil is attributed to the presence of polyphenol compounds like catechol, allylpyrocatechol in betel leaf which inhibits the lipid peroxidation process and scavenges free radicals effectively. Antioxidant potential of different *Piper betel* mouthwashes (A, B, C) evaluated using riboflavin–NBT assay (by SOD activity) showed antioxidant potential at all three concentrations, with mouthwash A exhibiting more antioxidant potential.

The anti-inflammatory potential of piper betel oil has been explained in various studies[3, 17]. The anti-inflammatory potential of mouthwash A, B and C when evaluated showed that mouthwash B exhibited more anti-inflammatory than A and C.

Cytotoxicity analysis showed that none of the concentrations of mouthwash were toxic as more than 50% of the cells were viable both at 1 hour and 3 hours.

The present study thus showed an effective antibacterial, antioxidant and anti-inflammatory potential of mouthwash containing 0.1%, 0.3% and 0.5% Piper betel oil. The formulated mouthwashes were also safe to be used as they were not cytotoxic, thus having potential to be used as an oral care product.

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