

An Invitro Study Of Determination Of Anti-Bacterial, Antioxidant, Anti- Inflammatory Potential Of Piper Betel Essential Oil

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Abstract: Background: *Piper betel* Linn is considered to possess important medicinal values. Leaves are considered more valuable part and was used in past for preventing halitosis. Essential oil obtained from leaves have been tried for antibacterial potential against various oral bacteria such as *Streptococcus mutans*, *Actinomyces species* and has demonstrated effective role in suppressing plaque formation. In addition essential oil obtained from leaves also exhibits anticancer properties because of antioxidant and anti-inflammatory potential. However available literature falls short in evaluating antibacterial potential of *Piper betel* essential oil against common periodontal pathogens- *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*. Present invitro study was conducted to evaluate antibacterial potential of *Piper betel* essential oil against previously mentioned periodontal pathogens by determination of minimum inhibitory concentrations (M.I.C). Antioxidant and Anti-inflammatory potential was also determined for the same. Methodology: 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 MMP-2 and MMP-9 by gelatin zymography method respectively. Results: *Piper betel* essential oil is an effective antibacterial activity against the tested periodontal pathogens along with antioxidant and anti-inflammatory potential. Conclusion: *Piper betel* essential oil possesses effective antibacterial, antioxidant and anti-inflammatory potential and could be used effectively in formulation of oral health care product. [Uppoor A NJIRM 2015; 6(2): 37-44]

Key Words: Antibacterial, Antioxidant, Anti-inflammatory, Piper betel essential oil.

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Introduction: Current research targeting microbial biofilm inhibition has attracted a great deal of attention. The search for effective antimicrobial agents against the oral pathogens such as *Streptococcus mutans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans* has led to identification of new agents for the prevention of oral biofilm associated diseases¹. Oral diseases such as dental caries and periodontal diseases are caused by dental plaque which exists in a state of biofilm.

Use of antimicrobial agents along with mechanical plaque control is proved to be effective in preventing and treating periodontal diseases². Due to side effects associated with use of chemicals for combating growth of dental biofilm, numerous researches have been carried to obtain antimicrobial properties from plants as a suitable oral health care agent³. A variety of plant materials and phytochemicals, especially a class of essential oils, have long been found to exhibit effective antibacterial activity against these oral pathogens⁴. The aromatic molecules derived from natural

sources are being explored extensively as alternative agents in oral care products⁵.

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 halitosis⁶. The prevention of halitosis could be explained by reduction of volatility of methyl mercaptan^{7,8}. Apart from this it is also possess antibacterial, antifungal activity and causes increased flow of saliva⁹. All above properties could be attributed due to presence of aromatic volatile oil containing phenolic compounds mainly chavicol and allylpyrocatechol. Phenols and terpenes also contribute to the aroma of betel⁶.

Irrespective of these uses, betel vine is considered to be 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 cancer¹⁰.

But in contrast to this various scientific studies have shown that tobacco¹¹, areca nut¹² and slaked

lime^{13,14} present in the betel quid promoted carcinogenesis and betel leaf is devoid of mutagenic and carcinogenic effect. In addition animal studies have shown conclusively that betel leaf and its phytochemicals namely chavibetol, chavicol, hydroxychavicol, estragole, eugenol, methyl eugenol, hydroxycatechol prevented chemical induced cancers in experimental animals by several mechanism such as *free radical scavenging, antioxidant, induction of detoxification enzymes, inhibition of lipid peroxidation, anti-inflammatory, anti-mutagenic, antitumor-promoting and induction of selective apoptosis and cell death of neoplastic cells*¹⁵.

Also aqueous extract of Piper betel oil has been shown to inhibit plaque formation by interfering with adherence and acid production of early colonizers namely *Streptococcus mutans* and *Actinomyces viscosus* species thus exerting anti cariogenic effect^{3,5,16}. But it should be noted that dental caries is a supra gingival condition, in contrast to periodontal diseases (gingivitis, periodontitis) which are sub gingival conditions that have been linked to anaerobic Gram-negative bacteria such as *Porphyromonas gingivalis*, *Actinobacillus* sp., *Prevotella* sp. and *Fusobacterium* sp.¹⁷. Available literature falls short in explaining potential of *Piper betel* essential oil against periodontal pathogens (*Actinobacillus* sp, *Prevotella* sp., *Porphyromonas* sp.).

Thus in this invitro study antioxidant, anti-inflammatory, antibacterial potential of *Piper betel* essential oil against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* which are established pathogens in periodontal disease was determined in perspective of formulating an oral hygiene product to combat dental biofilm.

Material and Methods: *Piper betel* essential oil: Pure *Piper betel* essential oil was obtained from Natural Product Company, Rym exports, Mumbai, India. The company is registered with Basic Chemicals, Pharmaceuticals & Cosmetics Export Promotion Council (CHEMEXCIL), Ministry of Commerce, Govt.Of India. Service Users of World Trade Centre, Bombay and India Indonesia Business Association.

Bacterial strains and growth conditions: The bacterial strains that were used were *Prevotella intermedia* (ATCC 25611), *Porphyromonas gingivalis* (ATCC 381) and *Aggregatibacter 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°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 the minimum inhibitory concentration of the piper betel essential oil.

Disc Diffusion test: Brain heart infusion agar after bringing to the room temperature was poured into sterile petridish. It was then inoculated with the microbial cell suspension (broth) of above mentioned organism using a swab. The turbidity of the broth was adjusted to that of a 0.5 Mac Farland turbidity standard.

Excess inoculum from the suspension was removed using a sterile cotton swab that was dipped and rotated against the wall of the liquid. To ensure an even distribution, the surface of the agar plate was swabbed three times and the plates were rotated approximately 60 degree. Hitting sides of precipitates was avoided as it creates aerosols. The inoculated plates were allowed to stand for atleast 3 minutes, but not longer than 15 minutes before making the wells. To make wells, a hollow tube of 5mm diameter was pressed on the inoculated agar plate. 5 wells on each plate were similarly made. Each of these wells was then inoculated with 20 µl *Piper betel* essential oil in the following concentrations (neat, 1:2, 1:4, 1:8 and 1:16). The plates were then incubated within 15 minutes of adding *Piper betel* oil for 18 – 24 hours at 37 °C in incubator.

Broth Microdilution: For broth microdilution thioglycollate broth was used as the medium. Nine dilutions of *Piper betel* oil were made to estimate the minimum inhibitory concentrations. First to 380µl of thioglycollate broth, 20 µl of *Piper betel* oil was added. This was considered as the initial tube.

For dilutions, only 200 µl of Thioglycollate was added in the nine tubes separately.

For dilution, 200 µl was transferred from the initial tube to the tube containing 200 µl Thioglycollate broth. This was considered as 10^{-1} dilution. From the 10^{-1} dilution tube, 200 µl of broth was transferred to the 2nd tube to make it 10^{-2} dilution. The serial dilution was repeated upto 10^{-9} dilution. From the maintained stock cultures of the microorganisms, 5 µl was mixed with 2 ml of Thioglycollate broth, and this suspension was added to each of the serially diluted tubes. The tubes were then incubated for 48 – 72 hours in an anaerobic jar at 37 ° C and then observed for turbidity.

Antioxidant potential: Antioxidant potential of piper betel oil was determined by measurement of superoxide dismutase (SOD) activity. To determine the SOD activity, the riboflavin-NBT assay was adapted from Lai¹⁸.

This method makes use of a substrate consisting of nitro blue tetrazolium chloride which reacts with superoxide ions produced upon illumination of riboflavin in the presence of methionine's an electron donor to produce a blue coloured complex called Formosan.

The SOD present in the sample acts on superoxide anion produced by riboflavin and thereby reduces the net superoxide anions in the substrate leading to decreased production of Formosan manifested by decreased intensity of blue colour formed.

The decrease in formation of Formosan is directly proportional to the amount of SOD in sample, 50% decrease in the formation of Formosan is taken as one unit of SOD.

Method:

- The test sample- betel leaf essential oil (0.1 mL) at different protein concentrations was first mixed with 2.75 mL of 67 mM phosphate buffer (pH 7.8) containing 0.01 M EDTA and 0.1 mL of 1.5 mM NBT.
- After incubation at 37 °C for 5 min, 0.05 mL of 1.2 mM riboflavin was added.

- The reaction mixture was then moved to a foil-lined box and illuminated with a 25W light tube for 15 min.
- The inhibition of NBT reduction was determined at measuring the absorbance at 560 nm by a micro titre plate reader.
- A negative control (water instead of the sample), positive control, and blank (addition of water instead of the riboflavin solution) were evaluated at the same time per micro titre plate.
- In addition, a set of standards were evaluated and these were then used to determine the SOD activity in each test sample where SOD activity was defined as that 1 unit is the amount of enzyme that provides a 50% inhibition of the riboflavin-mediated reduction of NBT.

Anti – inflammatory potential: The anti – inflammatory potential of *Piper betel* essential oil was determined by detection of MMP-2 and MMP-9 using Gelatin Zymography. This procedure requires the preparation of 10% resolving gel (10ml) and 5% stacking gel (5ml). The sample of betel leaf essential oil was prepared with addition of 5ml of tris buffer; centrifuging at 3000rpm for 15 min, and storing in -20°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 betel leaf oil 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. After this electrodes were connected. Lid was kept on tank and plug was cabled into power supply. 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 SDS (Sodium dodecyl sulphate) from the gel and allow proteins to denature. The gel was then incubated in zymogram at 37°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. The lower bands present are gelatinases-A (MMP-2) which is about 72KD while the upper bands are gelatinases-B (MMP-9) which runs at about 95KD.

Results: Antibacterial activity:

The zone of inhibition representing the antimicrobial activity of *Piper betel* essential oil at various concentrations against *Prevotella intermedia*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* is summarised in Table 1. The zones of inhibition were measured in mm.

Table 1: Zone Of Inhibitions Of Antibacterial Activity

	Neat	1:2	1:4	1:8	1:16
<i>Prevotella intermedia</i>	25m m	23m m	R	R	R
<i>Porphyromonas gingivalis</i>	24m m	22m m	R	R	R
<i>Aggregatibacter actinomycetemcomitans</i>	24m m	23m m	R	R	R

R= resistant

Aggregatibacter actinomycetemcomitans showed a zone of inhibition of 24mm and 23 mm at neat and 1:2 concentrations respectively while being resistant for other concentrations. (Figure 1)

Figure : 1



The zones of inhibition determined for *Prevotella intermedia* was 25mm; 23 mm at neat and 1: 2 concentrations respectively; for remaining concentrations it was resistant. For *Porphyromonas gingivalis* the zone was 24 mm; 22 mm at neat and 1:2 concentrations respectively and resistant for other concentrations.

Table 2 summarizes the result of broth microdilution for MIC against tested microorganisms. All the three tested microorganisms were sensitive to *Piper betel* essential oil at neat & 1:2 concentrations. They showed resistance at other concentrations.

Table 2: Broth Microdilution For MIC

	NEAT	1:2	1:4	1:8	1:16
<i>Prevotella intermedia</i>	S	S	R	R	R
<i>Porphyromonas gingivalis</i>	S	S	R	R	R
<i>Aggregatibacter actinomycetemcomitans</i>	S	S	R	R	R

S= Sensitive

R= Resistant

Table 3: Antioxidant Activity

	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	Piper betel oil	566	8.12

Figure : 2

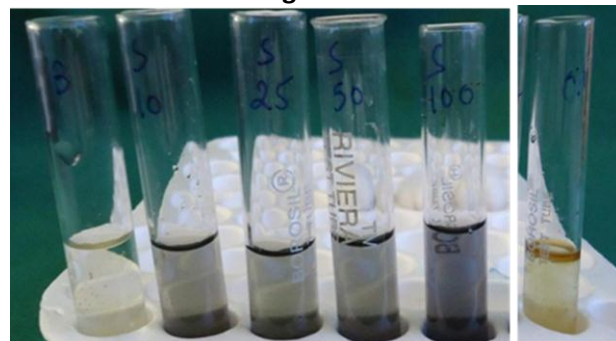


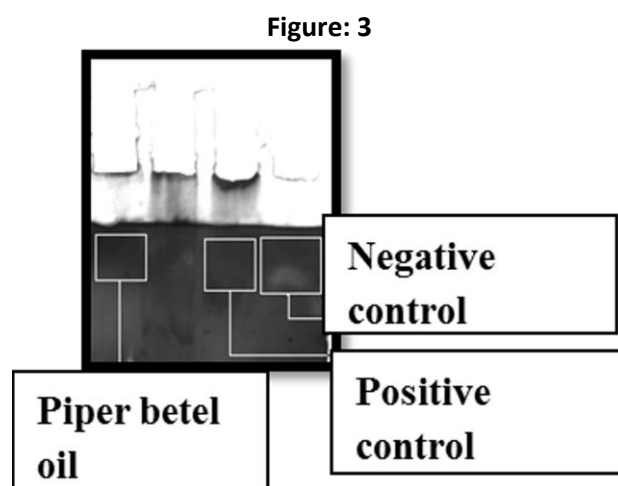
Table 3 condenses the result of antioxidant potential of *Piper betel* essential oil. The different

standard concentrations are the different concentrations of ascorbic acid, and these concentrations of ascorbic acid have been compared with the *Piper betel* oil depending on its absorbance. As an anti-oxidant, *Piper betel* oil showed 8.12% activity with absorbance at 566 nm. (Figure 2)

Table 4: Anti-Inflammatory Potential

	Sample	% of band	Anti-inflammatory activity
1	Piper betel oil	15%	85%
2	Positive Control	5%	95%
3	Negative Control	98%	-

Table 4 reviews the result of anti-inflammatory potential of betel leaf oil, wherein *Piper betel* oil showed appearance of 15% of band. After calculation for appearance of band width and thickness this 15% band corresponded for 85% anti-inflammatory activity while positive control showed 95% anti-inflammatory activity. (Figure 3)



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 immunocompromised individuals and financial considerations in developing countries¹⁷.

Different commercially available chemical agents such as cetylpyridinium chloride, chlorhexidine, amine fluorides can alter oral microbiota and have undesirable side-effects such as vomiting, diarrhea and tooth staining, presence of ethanol (in mouthwash) have been linked to oral cancer¹⁷.

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 diseases¹⁷.

Piper betel oil contains large quantity of sterols which is the bioactive molecule responsible for antibacterial activity. Sterols present in the extract interact with the bacterial cell wall and membrane altering the primary structure of cell wall and membrane which ultimately lead to pore formation and degradation of the bacterial components. As reported earlier *Piper betel* extracts containing high concentration of fatty acids like palmitic acid, stearic acid and hydroxy fatty acid esters exhibits potent antimicrobial activity against diverse pathogenic microorganisms¹⁹. High concentrations of flavonoids and polyphenols especially hydroxychavicol present in *Piper betel* oil exhibits potent antibacterial activity against oral cavity pathogens^{5,9,19}.

Various studies have evaluated antibacterial efficacy of *Piper betel* oil against common oral pathogens. The cariostatic potential of aqueous extract of *Piper betel* oil was evaluated by A.R. Fathilah et al 2003¹⁶ and Nurhayati Bt Mohd Zain 2011³. While the former showed this property in an invitro study by reduction in adherence activity of early plaque settlers namely *Strep. mitis*, *Strep. sanguinis* and *Actinomyces* sp. due to modification in complementary binding sites, the later showed downregulation of *Streptococcus mutans* gene involved in attachment and plaque formation in response to treatment with *Piper betel* aqueous extract.

The present invitro study demonstrated that *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis*, which are established periodontopathogens were sensitive to *Piper betel* oil at concentrations of neat and 1:2. This indicates that *Piper betel* oil can be tried as an alternative adjunct to chemical agents against periodontal pathogens.

In addition, *Piper betel* oil was shown to have potent antioxidant activity because of presence of polyphenol compounds like catechol, allylpyrocatechol by Pradhan et al 2013²⁰ and Nair et al 2012²¹ by using ascorbic acid and BHT as standards. They demonstrated that betel leaf extract inhibited the radiation induced lipid peroxidation process effectively because of its ability to scavenge free radicals involved in initiation and propagation steps. The extract reduced most of Fe³⁺ ions and possesses strong reductive ability. The extract also showed strong hydroxyl radical and superoxide anion radical scavenging. This can make *Piper betel* oil effective in treatment of medical conditions associated with oxidative damage such as Alzheimer's disease and cancer.

Piper betel extract was also shown to reduce cellular ageing of human diploid fibroblasts by reducing senescence-associated β -galactosidase expression, catalase activities and SOD activity by Makpol et al. 2013²². Using lipid peroxidation Sharma et al 2009⁵ also found significant antioxidant activity of hydroxyl chavicol present in *Piper betel* extract.

However for determination of antioxidant potential present study involved use of riboflavin – NBT assay (by SOD activity) which was relatively easy in comparison to that involved in Sharma et al 2009⁵ as the later required rat liver microsomes for determination of anti-oxidant potential. Also riboflavin –NBT assay has virtue of being readily standardized and independent of other enzymes and proteins, such as xanthine oxidase and cytochrome c²³. The result of present study corroborates with the finding of previous studies thus indicating antioxidant potential of *Piper betel* oil.

Apart from these, present study also exhibited anti-inflammatory potential of *Piper betel* oil apart from antibacterial and antioxidant property. This is in accordance with finding of Sharma et al 2009⁵. They found strong anti-inflammatory activity of hydroxychavicol, measured by the estimation of intracellular tumor necrosis factor alpha (TNF- α) expression in a gated population of neutrophil.

According to Pradhan et al 2013²⁰ the methanolic extract of betel leaf decrease the antibody titre and increase suppression of inflammation thus suggesting possible immunosuppressive effect of extract on cellular and humoral response. Although it should be noted that previous studies evaluated different properties of *Piper betel* using either aqueous or alcoholic extracts, but in the present study these were evaluated using *Piper betel* essential oil which is considered to be more potent in action as it presents as a concentrated hydrophobic liquid with various volatile aromatic compounds.

One of the limitation of present study includes determination of M.I.C only in planktonic state of mentioned microorganism (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*), but M.I.C remains to be determined in biofilm as bacteria in oral cavity are protected by biofilm, where they are less susceptible to antimicrobial agents than their planktonic counterparts⁵. Thus, future studies could be directed to evaluate antibacterial efficacy of *Piper betel* oil in an invivo model.

Hence within the limitations of present study it could be concluded that *Piper betel* oil demonstrated effective antibacterial activity against periodontal pathogens namely *Prevotella intermedia*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, along with antioxidant and anti-inflammatory activity. With the presence of all these properties the essential oil obtained from leaf could be used as an active pharmaceutical ingredient in formulating oral care product as an effective agent against periodontal pathogens.

Although further studies are still warranted for evaluation of antibacterial efficacy of *Piper betel* oil

in biofilm model along with antioxidant and anti-inflammatory potential in inflamed gingiva.

Conclusion: *Piper betel* essential oil possesses effective antibacterial, antioxidant and anti-inflammatory potential and could be used effectively in formulation of oral health care product.

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