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In vitro Antimicrobial activity of *B. propolis* extract against *Porphyromonas gingivalis* and its potential application in the management of Periodontitis

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Abstract

Periodontitis, a bacterial-induced inflammatory disease occurs due to the colonisation of peridontopathogen Porphyromonas gingivalis accountable for causing dental plaque. *P. gingivalis* is a gram-negative, rod-shaped, obligate anaerobic bacterium isolated from subgingival plaques of admitted patients. To overcome the widespread of antibiotic resistance, the current study investigates the antimicrobial ability of ethanolic extract of B. propolis (EEP) against fifteen anaerobic strains of *P. gingivalis*. The chemical composition of *B. propolis* extract was analysed through high-resolution Gas chromatography coupled with mass spectrometry (GC-MS). Thirty different types of chemical compounds were detected in the EEP, in which diethyl phthalate, eugenol, octadecanoic acid, cis-9-hexadecenal and l-(+)-Ascorbic acid 2,6-dihexadecanoate were observed to be present in high concentrations. The EEP was applied to determine the minimum bactericidal concentrations (MBC) and minimum inhibitory concentrations (MIC) on the growth of *P. gingivalis*. The MICs and MBCs values of EEP ranges from 50 to 200 mg/ml and 50 to 100 mg/ml respectively. The growth inhibition of *P. gingivalis* was observed after 6 hrs of incubation at 200 mg/ml concentration of EEP. The scanning and transmission electron microscopy images revealed the interaction of EEP with the cell wall of *P. gingivalis*. The antimicrobial activity of EEP against the pathogenic biofilm of P. gingivalis can be used as a safe, eco-friendly and cost-effective alternative for the treatment of periodontal disease.

Key words : *Porphyromonas gingivalis*, *B. propolis*, EEP, Periodontitis, Antimicrobial ability, MIC, MB-C.

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Periodontitis is a chronic inflammatory disease mostly characterised by the loss of teeth along with the deterioration of alveolar and gingiva bone[13]. Periodontal diseases involve biofilm-induced gingivitis, specifically affecting gingiva[3], and periodontitis, distinguished by the gradual destruction of periodontium and dysbiotic plaque biofilms[16]. The presence of sucrose in dental caries originates the shift in microbiota towards aciduric and acidogenic bacteria, ultimately leading to loss of minerals from the teeth[15]. Periodontitis was reported to be caused by several pathogenic microbes including a key pathogen *Porphyromonas gingivalis*[22]. *P. gingivalis* is a gram-negative, rod-shaped, obligate anaerobic bacterium generally located in the oral and dental cavity[14]. *P. gingivalis* and its infection were reported to be associated with life-threatening diseases such as diabetes mellitus, rheumatoid arthritis, diabetes mellitus, respiratory infection, obesity and osteoporosis[9]. *P. gingivalis* can escape from the host immune system thus it can cause chronic inflammation in several peripheral organs[1].

Due to its biofilm-forming ability, *P. gingivalis* has recently been reported to develop 9-10% antibiotic resistance against various pathogenic strains[18]. To address the issue of antimicrobial drug resistance, the current research has been focused on the application of herbal-based therapy. Several medicinal plants have been identified as antimicrobial agents, an effective alternative to treat oral bacterial infections[7]. Plants have been well-recognised for their ability to synthesize phytochemicals as a secondary metabolite[2,20]. Different types of secondary metabolites were found in plants such as alkaloids, tannins, flavonoids and phenolic compounds accountable for their antimicrobial ability[15,22]. The application of *B. propolis extract* on colonising pathogenic bacteria could offer an effective strategy for preventing periodontal diseases.

The current study involves the analysis of *B. propolis* composition and evaluation of EEP performance by examining its in-vitro Minimal inhibitory concentration (MIC), Minimal bactericidal concentration (MBC) and antimicrobial activity against *P. gingivalis.*

Collection of B. propolis and preparation of ethanol extract :

The herbal powder of *B. propolis* sample was purchased from the local market. The ethanolic extract of *B. propolis* was prepared using the method described by[8]. The EEP solution was obtained by diluting the concentrated EEP solution in 1:1 w/v and drying until ethanol evaporation. The dried residue (5mg) was mixed with bis (trimethylsilyl) trifluoroacetamide (BSTFA) (50 ml) and dry pyridine (75 ml). This solution was heated for around 20 min at 80^{o}C and then the supernatant was evaluated through Gas chromatography coupled with mass spectrometry (GC-MS)[10].

GC-MS analysis :

The composition of organic compounds present in EEP samples was analysed by examining the respective peak area of the target compounds and the sum of peak areas in terms of percent in the chromatogram. Willey and Nist standard libraries were applied

to detect the presence of organic compounds and incorporated in the data acquisition when the comparison score was obtained higher than 90% [23].

Isolation of Porphyromonas gingivalis from subgingival plaque :

The dental plaque samples were collected from the Periodontal Department of Government Dental College and Private Dental Clinics of Nagpur district. The samples were isolated from the patients visited to hospitals using Gracey-curette number 5/6 (Hu-Friedy, Chicago, USA). The samples were inoculated on Blood agar media. The inoculated plates were incubated at 37° C for 72-76 hrs under anaerobic conditions. After incubation, the plates were observed for black-pigmented colonies representing the haemolysis zone of the blood. In all,a total of 15 strains were isolated and identified from the samples. The isolated strains were further identified by biochemical analysis such as IMViC, catalase, indole and nitrate reductase tests. The strains were preserved in glycerol stocks at -20°C for further analysis[2].

Antimicrobial susceptibility assay of B. propolis ethanolic extract against P. gingivalis :

The antimicrobial of *B. propolis* extract against *P. gingivalis* was determined by using the agar well diffusion method. Briefly, the 0.1 ml culture of *P. gingivalis* was uniformly spread with a sterilized cotton swab on the surface of blood agar. The blood agar surface was punched with 6mm diameter wells aseptically using a sterile cork borer. 50 µl of freshly prepared *B. propolis* extract was added aseptically to these wells. The plates were incubated for 5-7 days in the McIntosh and Fildes anaerobic jar. After incubation, the plates were examined for an inhibition zone surrounding the wells. A zone of inhibition validates the inhibitory effect while the absence of an inhibition zone displays no inhibitory effect of *B. propolis* extract on *P. gingivalis* growth[6].

Visualizing the mode of action of B. propolis through SEM and TEM :

The suspension of *P. gingivalis* sample No. 1 was exposed to EEP (25 mg/ml ethanol extract of propolis) for 5 min. Subsequently, the samples undergone examination through SEM and TEM [25]. The sample was centrifuged, washed two times by 0.9% w/v NaCl and ultimately suspended into the 20-fold concentration of microorganism. For SEM analysis, the microbial suspension was added to the glass slide and 2% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) was used for suspension fixation. Further, the sample was washed 3 times with 0.1 M cacodylate buffer and dehydrated with ethanolin ascending concentrations (30, 50, 70,90 and 100%) for 10 min. Liquid $CO_2$was used for sample drying. Finally, the specimen was examined by SEM[24].

For TEM analysis, the microbial suspension was fixed with 2% glutaraldehyde and 0.5% formaldehyde in 0.1 M cacodylate buffer for 30 min. Further, the sample was washed with 0.1 M cacodylate buffer (3 times) and fixed with osmium tetroxide for 1 hr followed by dehydration using ethanol and staining with uranyl acetate. During the TEM examination, the sample was embedded in

Illustrations :

Table 1.Phytochemical analysis and identification of major compounds in ethanolic extract of *B. propolis* using GC-MS

Sr no	Retention time	Compound	Molecular weight	Peak Area %	Peak Height %
1	6.11	Cyclohexasiloxane, dodecamethyl	444	0.43	0.82
2	6.99	Eugenol	164	10.68	9.16
3	7.89	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-Methylene	204	1.00	1.65
4	8.32	Cycloheptasiloxane, tetradecamethyl-	518	1.54	2.63
5	9.07	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	206	2.23	2.40
6	9.96	Diethyl Phthalate	222	6.48	7.38
7	10.04	Caryophyllene oxide	220	0.96	1.64
8	10.39	Cyclooctasiloxane, hexadecamethyl-	592	1.80	3.08
9	10.64	Triethyl citrate	276	2.44	2.75
10	11.77	Tetradecanoic acid	228	3.65	4.11
11	12.20	Silicone Oil	9999	2.10	3.31
12	12.87	Pentadecanoic acid	242	0.50	0.63
13	13.01	10,12-Hexadecadien-1-ol	238	1.20	0.75
14	13.49	Hexadecanoic acid, methyl ester	270	1.12	1.64
15	13.66	Acetamide (Diethylamino)-N-(2,6-Dimethylphenyl)	234	0.67	0.84
16	13.81	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,20,20-Icosamethylcyclodecasiloxane	740	2.74	3.42
17	13.95	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	11.91	11.29
18	14.08	Dibutyl phthalate	278	1.05	1.34
19	14.18	Hexadecanoic acid Ethyl Ester	284	1.78	2.63
20	14.62	9,12-Octadecadienoic acid (Z,Z)-	280	0.25	0.47
21	14.94	Heptadecanoic acid	270	0.69	0.87
22	15.11	9,12-Hexadecadienoic acid,methyl ester	266	5.96	5.67
23	15.30	Cyclooctasiloxane, hexadecamethyl	592	4.41	3.93
24	15.54	Methyl stearate	298	0.62	0.73
25	15.79	cis-9-Hexadecenal	238	12.58	8.96
26	16.01	Octadecanoic acid	284	8.87	7.31
27	16.27	Octadecanoic acid Ethyl ester	312	1.38	1.36
28	16.93	Cyclononasiloxane, octadecamethyl	666	3.23	3.34
29	19.02	1 H-Purin-6-Amine, [(2-Fluorophenyl) Methyl]-	243	3.20	2.92
30	21.88	3,5-Diisopropoxy-1,1,1,7,7,7- hexamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	546	4.54	2.96

Table-2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *B.Propolis* extracts on *P. gingivalis* isolates

Isolate	Minimum Inhibitory Concentration(MIC)	Minimum Bactericidal Concentration(MBC)
1	50 mg/ml	50mg/ml
2	Non-inhibitory	Non-inhibitory
3	200 mg/ml	100mg/ml
4	200mg/ml	100mg/ml
5	Non-inhibitory	Non-inhibitory
6	Non-inhibitory	Non-inhibitory
7	100 mg/ml	100mg/ml
8	100mg/ml	100mg/ml
9	Non-inhibitory	Non-inhibitory
10	100mg/ml	100mg/ml
11	200 mg/ml	Non-inhibitory
12	Non-inhibitory	Non-inhibitory
13	50mg/ml	100mg/ml
14	100mg/ml	100mg/ml
15	200mg/ml	100mg/ml

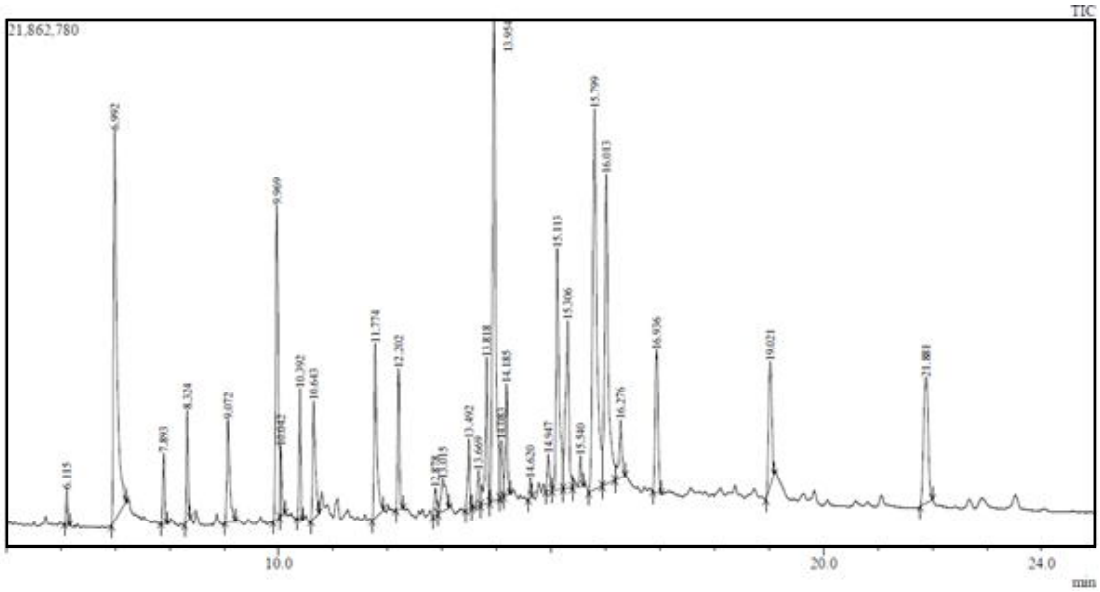


Figure 1. GC-MS chromatogram of ethanolic extract of B. *propolis*

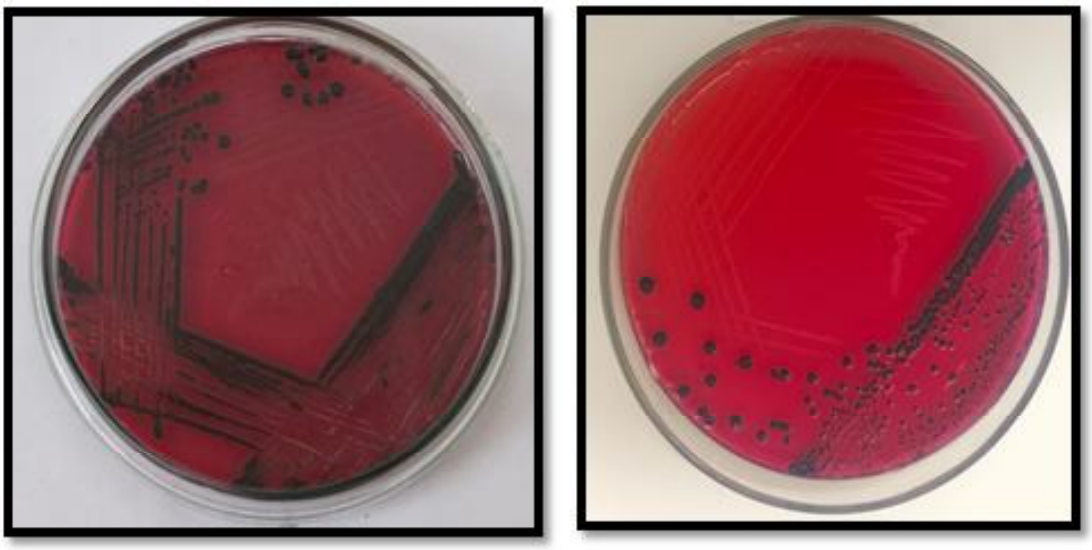


Figure 2. Black pigmented colonies of peridontopathogen *P. gingivalis* on blood agar surface after 72 hrs of incubation.

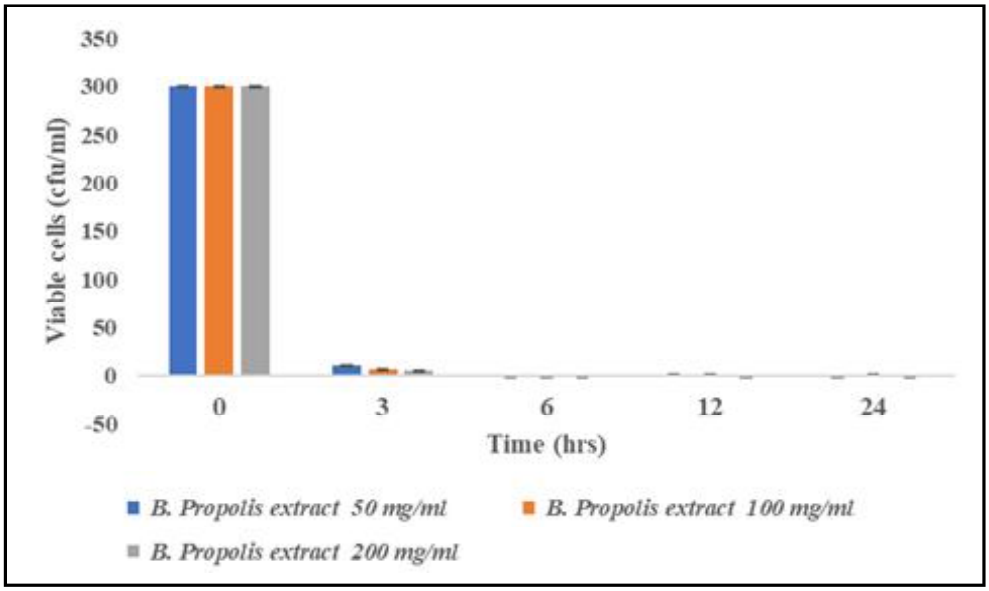
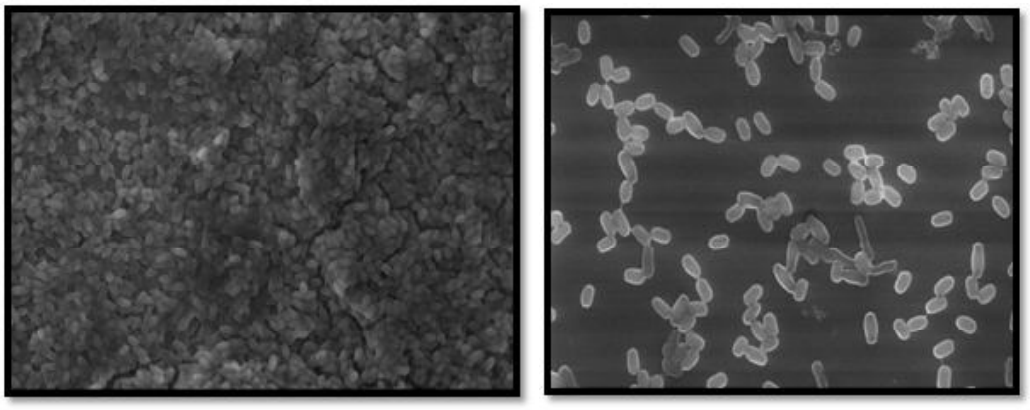
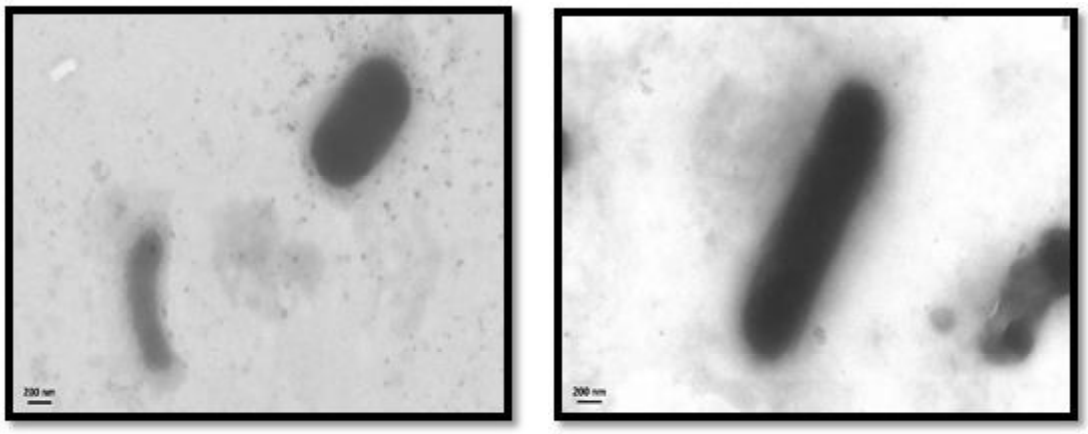


Figure 3. Cytotoxicity of *B. propolis* extracts at different concentrations (50-200 mg/ml) against *P. gingivalis* sample No.1 analysed after 0,3,6,12 and 24 hrs of incubation. The experiment was performed in triplicates. Bar display mean ±SD.



(A) (B)

Figure 4. Scanning electron microscopy images of *P. gingivalis* without any exposure (A) and with 5 min exposure to 100 mg/ml of *B. propolis* extract (B). Bar (A&B) 2µm.



(A) (B)

Figure 5. Transmission electron microscopy image of *P. gingivalis* without any exposure (A) and with 5 min exposure to 100 mg/ml of *B. propolis* extract (B).

epoxy resin (Araldite) and the ultrathin section was mounted on filmed Cu grids, post-stained with lead citrate[24].

Statistical analysis :

The statistical analysis of data was performed by Statistical Package for Social Science (SPSS) 18.0 software. The data obtained from the inhibition of microorganisms by EEP was compared by the Covariance analysis test. The p-values less than or equal to 0.05 were considered to be statistically significant.

Analysis of the chemical constituents present in B. propolis extract :

GC-MS analyses were performed to detect major chemical compounds in the *B. propolis* extract. 30 different compounds were found in the extract. Among the identified compounds the octadecanoic acid, l-(+)-Ascorbic acid 2,6-dihexadecanoate,eugenol, diethyl phthalate andcis-9-hexadecenal were found in the highest concentration of 8.87, 11.91, 10.68, 6.48 and 12.58 respectively Table-1. The significant peaks with their corresponding retention times are demonstrated in Figure 1.

Porphyromonas gingivalis from subgingival plaque :

The dental plaque isolates shown a black pigmented colony on the surface of blood agar revealing the presence of haemolytic zones around the strain Figure 1. Out of all, 15 strains contained haemolysis ability and hence were selected for further analysis. The biochemical analysis demonstrates the catalase-negative, indole-positive, urease-negative and glucose fermentation-positive ability of the potent strains. Based on 16S rRNA gene sequencing, the strain was identified as *P. gingivalis* up to the species level.

Antimicrobial susceptibility assay of B. propolis ethanolic extract against P. gingivalis :

The MICs and MBCs of EEP range from 50 to 200 mg/ml and 50 to 100 mg/ml respectively Table-2. 96% aqueous ethanol was taken as control that did not inhibit the growth of *P. gingivalis*. MICs and MBCs were confirmed to be statistically significant ($p= 0.001$, $p=0.0008$) respectively. It has been observed the *B. propolis extract* was more effective against samples No. 1,3,4,7,8,10, 11,13,14 and 15 of *P. gingivalis* Table-2. Of all, sample No. 1 showed the highest antimicrobial activity against *P. gingivalis*, hence selected for further analysis.

Further, the number of viable cells (CFU) of *P. gingivalis* was analysed after exposure to *B. propolis* extract at different concentrations (50 -200 mg/ml) and the growth was observed after 0,3,6,12 and 24 hrs of incubation. It was observed that the treatment of *B. propolis extract* at 200 mg/ml for 6 hrs inhibits the growth of *P. gingivalis* Figure 3.

SEM and TEM images of P. gingivalis after exposure to B. propolis extract :

SEM and TEM images were obtained after exposing *P. gingivalis* to100 mg/ml of EEP. The SEM images suggested the interaction of *B. propolis* with the cell wall of *P. gingivalis*. The planktonic cells *of P. gingivalis* get dispersed after exposure to ethanolic extract of *B. propolis* Figure 4 (A) & (B). The TEM images demonstrated the presence of vesicles outside of cells and after exposure to *B. propolis* the cell wall integrity gets damaged Figure 5 (A) & (B).

Periodontal disease is mostly known to be caused by aggregation of anaerobic Gram-negative rods such as *Bacteroides, P. gingivalis*, *A. actinomycetemcomitans, Fusobacterium Tannerella forsythus* and *Prevotella* species. These periodontopathogens could form complex biofilms on the hard tissues of the oral cavity, sometimes

detrimental to humans[21]. More than 500 bacterial species were reported to be involved in colonisation, adhesion and aggregation of dental plaque that accounts for periodontal disease[21]. In the present study, 15 *P. gingivalis* was isolated as an opportunistic periodontopathogen from the pockets of patients suffering from periodontitis[11,19]. Good oral hygiene was observed to be more susceptible to the colonisation of *P. gingivalis*[17]. Several chemotherapeutic agents and traditional methods have been used to treat periodontal disease. For example, chlorhexidine mouthwash was commonly utilised as a standard anti-plaque agent. It was reported to cause a burning sensation in the oral cavity, discolouration of the tongue and teeth and sometimes cause loss of teeth. Most of the bacterial species developed resistance against antibiotics and it has been an alarming condition for the treatment of biofilm-forming bacteria[14]. To address the issue, there is a need to exploit plant-derived agents[28]. The plant extracts were reported to demonstrate antimicrobial, anti-inflammatory and antioxidant properties against pathogenic biofilms and can be used as a safe, eco-friendly and cheap alternative for the treatment of periodontal disease[6,28].

The present study analysed the antimicrobial ability of EEP against periodontopathogen *P. gingivalis*. *B. propolis* has been identified to have antimicrobial, anti-inflammatory and immunostimulant properties[34]. Similarly, *B. propolis* has been reported to demonstrate antibacterial action against *Pseudomonas* spp., *Lactobacillus plantarum, S. cerevisiae* and *Fusarium oxysporum*[22]. Also, the study by Gebara *et al*., 1996 exhibited the antibacterial activity of *B. propolis* against periodontopathic bacteria[8]. It is impossible to detect the antimicrobial activity of *B. propolis* by only analysing its organic and inorganic composition, as the composition of propolis may vary from region to region[16]. The solubility of propolis will increase in the presence of hydrophilic compounds at higher concentrations. Specifically, a higher concentration of flavonoids favours higher solubility of propolis[10].

B. propolis extract was exhibited to be more active towards Gram-positive bacteria as compared to Gram-negative[9]. In contrast, very few studies exhibited the antimicrobial ability of *B. propolis* against the major gram-negative pathogen *P. gingivalis*. The present study demonstrates the excellent inhibitory activity of EEP against gram-negative bacteria *P. gingivalis*. The MIC and MBC values of EEP ranged from 50 to 200 mg/ml and 50 to 100 mg/ml. The Gram-positive anaerobic bacteria was reported to have MIC values lower than Gram-negative anaerobic bacteria ($p < 0.05$)[10]. According to SEM and TEM images, the microbial cell wall is the main target of EEP. The *B. propolis* demonstrated the membrane-rupturing effect against *P. gingivalis*[33].

In all, the present work demonstrated the isolation and screening of *P. gingivalis* from subgingival plaque samples exhibiting haemolytic ability. Also, the antimicrobial ability of the ethanolic extract of *B. propolis* was reported against *P. gingivalis*. The ethanolic extract of *B. propolis* has revealed its effectiveness in the prevention and treatment of *P. gingivalis* infection. Further research could involve the production of

mouthwash containing *B.propolis*, as a potential product for application against *P. gingivalis* (biofilm pathogens) infection.

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Conflict of interest :

The authors do not have any conflict of interest.

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