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Propolis nanoemulsion extract from celebes stingless bee (*Tetragonula biroi*) phytochemistry and antibacterial analysis to periodontopathogen bacteria

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ABSTRACT

Background: Propolis from Sulawesi's stingless bees (*Tetragonula biroi*) contains antioxidants, more flavonoids than propolis from Apis bees, and the antibacterial ability.
Objective: to examine the antibacterial properties of Propolis Nanoemulsion Extract (PNE), which is extracted from the Celebes Stingless Bee (*T. biroi*), in relation to the periodontopathogen bacteria such as *Fusobacterium nucleatum* (Fn), *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa), and *Provotella intermedia* (Pi). This investigation also examines PNE's phytochemistry, particle size analysis (PSA), and zeta potential.
Methods: The maceration process with 96 % ethanol was used to create PNE from Celebes stingless bee (*T. biroi*), which was then subjected to zeta potential measurement and PSA. Phytochemical analysis was used to identify phytochemical constituents in the PNE (T. biroi). Diffusion zone, minimum bactericidal concentration (MBC), and minimum inhibitory concentration (MIC) were used to assess antibacterial efficacy against Aa, Pg, Pi, and Fn. Furthermore, the statistical analysis was used to extract the data.
Results: Phenols, alkaloids, and flavonoids were identified; however, triterpenoids and saponins were not. Between 151.28 and 182.2 diameter nanometers (d.nm) was the range of the PNE's diameter. At 1.56 % propolis (*T. biroi*) concentration, the MIC, MBC, and diffusion zone analysis performed better than at 0.76 %, with a significant difference (p:0.01; $p < 0.05$) to Aa, Pg, Pi, and Fn.
Conclusions: The highest antibacterial activity against Aa, Pg, Pi, and Fn as periodontopathogen bacteria is demonstrated by alkaloids, flavonoids, and phenols in PNE from Celebes (*T. biroi*) at a concentration of 1.56 %.

1. Introduction

Inadequate dental hygiene and patient-specific risk factors are two of the many causes of periodontal diseases. The risk factors may be either non-modifiable (like age and inheritance, which includes hereditary illnesses) or modifiable (like the smoking of tobacco, poor dental hygiene, diabetes mellitus, or pregnancy). Periodontal diseases are primarily initiated and developed by poor oral hygiene habits.[1] Inadequate dental hygiene practices can cause germs and plaque to accumulate on teeth, causing gingivitis and possibly leading to periodontitis. Extant

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research shows that there is a clear correlation between the severity and prevalence of periodontal diseases and the accumulation of tooth plaque.[2]

Insufficient dental hygiene can allow the anaerobic microorganisms that cause periodontal illnesses to spread to deeper parts of the periodontium, where they can cause much damage. *Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Provotella intermedia (Pi),* and *Fusobacterium nucleatum (Fn)* are the principal bacteria found in periodontitis.[3] These organisms cause inflammation when they are allowed to delve deeply into the periodontium because they cause the host to release inflammatory mediators and other defensive substances.[4]

In cases of periodontitis, the loss of dental support and attachment causes discomfort for movable teeth, which accelerates the disease's progression and frequently results in tooth loss. The destruction of the locations where those teeth originally resided and the conditions around those sites make tooth replacement challenging, even though there are many alternative treatment methods accessible nowadays.[5] Gingivitis is a treatable condition that only affects the gingiva, whereas periodontitis is a persistent inflammatory condition that causes attachment loss and bone loss. Severe bone loss results in a considerable reduction in tooth support.[6]

Many patients experience the onset of periodontitis gradually, which they attribute to a lack of awareness thereof or a lack of regular dental treatment—the biggest risk factor for poor oral health-related quality of life. Many patients are not compliant with appointments and visit their dentist only when they are experiencing discomfort in and around their teeth. Periodontitis often advances quietly, which causes such patients significant damage that it is too late to treat properly by the time they visit the dentist.[7] Patients who, regrettably, lose teeth as a result of dental caries or periodontal disease must adjust to a new world. They experience decreased aesthetics, decreased functioning, and, occasionally, comorbidities. Oral infections are connected to a variety of systemic diseases, including pneumonia, osteoarthritis, rheumatic diseases, inflammatory bowel disease, kidney diseases, liver diseases, metabolic syndrome, diabetes, cancer, and Alzheimer's disease, which are thought to be upregulated inflammatory mediators, cytokines, and other pathological reactions.[8]

Gingival inflammation and alveolar bone resorption result from periodontopathogenic bacteria, including *Aa, Pg, Pi,* and *Fn*, which grow in periodontal tissue as a result of the inflammation. Despite the possibility of overcoming bacterial resistance, the high cytotoxicity to cells, and the risk of long-term infection, several treatment options have been used, including mechanical debridement with a scaler, antiseptic treatment with chlorhexidine gluconate, an antibiotic regimen (mostly using tetracycline, amoxicillin, doxycycline, or metronidazole), and regenerative or surgical therapy (surgery and tissue engineering or a combination thereof).[9,10]

Propolis from a stingless bee, *Tetragonula biroi*, which is unique to Sulawesi Island, also known as Celebes Island, is a material produced by bees and is also known as "bee glue" because of its natural roles as a cementing agent for bee hives and in protecting bee larvae and honey against microbial invasion. Given Sulawesi's diversity, the components and properties of propolis are determined by the plant source, which influences its biological activity. It can aid in the production of high-quality propolis.[10] Propolis comprises wax, resin, essential oils, pollen, and organic components.[11] It has several significant minerals and trace elements, such as Fe, Mg, Ca, Na, Zn, Mn, Cu, I, and K.[12,13] A prior study also found that propolis from raw *Heterotrigona itama* had a high mineral concentration of K (974.24 mg kg^{-1}), Mg (357.99 mg kg^{-1}), and Na (273.26 mg kg^{-1}), as well as lipids (F45.60 %) and trace levels of proteins (0.18 %), fibers (0.30 %), and carbs (0.43 %). The particle sizes of ethanol-extracted raw *H. itama* propolis with different volume fractions ranged from 143.8 to 1448.0 nm.[14]

Analysis of heavy metals, including Cr, Pb, Zn, Cd, and Cu, is required to determine whether bee products are biocompatible. The total levels of heavy metals in the propolis samples from the highland areas were generally lower than those from the lowland areas. To complete the picture and identify the main mechanisms of heavy metal incorporation in beehive products, more research is required.[15] Heavy metal concentrations, such as Cd, Co, Cr, Ni, Pb, and As, were found to be two to three orders of magnitude lower than mineral contents, with dangerous heavy metals far below the maximum allowed limit. The vibrational and absorption spectrum investigations revealed the existence of aromatic chemicals such as aromatic acids, terpenes, flavonoids, and phenolic acids with amine, ester, carbonyl, alkyl, and hydroxyl functional groups. These chemicals found in propolis are thought to be bioactive. Regardless of the stingless bee species, phenolic chemicals contribute to propolis' antioxidant ability.[16]

Propolis resins include phenols, flavonoids, and several types of acids.[17] Flavonoids are the most abundant ingredient in propolis and have the potential to impair the permeability of bacterial cells. The presence of active chemicals in propolis influences its antibacterial action, which includes flavonoids (tt-farnesol and apigenin), polyphenols, galangin, quercetin, myrecetine, robinetin, licochalcones AB, caffeic acid phenethyl ester (CAPE), tannins, and essential oils.[18] Propolis is high in flavonoids, benzaldehyde, vitamins, amino acids, minerals, enzymes, lipids, and other chemical constituents.[19] It has been studied in biomedicine as an antibacterial agent (against both gram-positive and gram-negative bacteria), antifungal, antiamoeba, and as an antioxidant and regenerative agent.[20,21] Additionally, the propolis demonstrated unique antibacterial properties against two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacteria, with the inhibition zone varying according to the bacterial strain. This further demonstrated the differences in antibacterial active substances found in propolis from various bee species. Flavonoid molecules may be linked to the antibacterial active ingredients. As natural products, they are rich in mineral elements, have low levels of heavy metals, and have high antioxidant and antibacterial characteristics that work against both Gram-positive and Gram-negative bacteria.[16] Propolis works by altering the permeability of the bacterial membrane and by producing adenosine triphosphate (ATP), which reduces bacterial motility. Propolis inhibits nuclear factor kappa beta (NF-κB), leading to decreased levels of prostaglandin E2 (PGE2) and nitric oxide (NO_2).[22]

The presence of phenolic groups in flavonoid molecules confers antiradical action, which is amplified since radicals produced during scavenging are resonantly stabilized.[23] Each active component in the propolis has a unique mechanism for antibacterial action. Flavonoids suppress bacterial growth by adsorption via hydrogen bonding. If hydrogen bonds develop between tannins and proteins, the proteins in bacterial cells may be denatured, disrupting bacterial metabolism. Low quantities of phenol generate phenol-protein complex bonds, which are followed by phenol penetration into cells, resulting in protein precipitation and denaturation. Conversely, high concentrations of phenol cause protein coagulation and cell lysis. Propolis from trigona bees (*T. biroi*) in Sulawesi has more flavonoids than propolis from apis bees.[24,25] Propolis has extensive antibacterial action against gram-positive and gram-negative bacteria, including rods and cocci. Propolis's antibacterial impact should be assessed in terms of both its direct action on germs and its immune system stimulation, as it activates the organism's natural defenses.[26,27]

An analysis of propolis's mechanism reveals that it influences the permeability of microorganism cell membranes, disrupts membrane potential, generates ATP, and reduces bacterial motility.[23,28] This is attributed to the species-specific structure of the outer membrane of a gram-negative bacterium and to the production of hydrolytic enzymes that destroy propolis's active components.[29] One of the phenol connections discovered in propolis is CAPE, which is the active site of the flavonoids and carries out antibacterial, antioxidant, antiviral, anti-inflammatory, and antifungal activities.[30,31] Furthermore, CAPE has the ability to inhibit peptidoglycan glycosyltransferase, allowing

propolis to kill and reduce bacterial populations.[33] Several studies conclude that galangin can damage bacterial cell membranes and inhibit the activities of cyclooxygenase and lipoxygenase, lowering cyclooxygenase-2 (COX-2) isoform synthesis.[34] Stingless bee propolis (*T. biroi*) is noted to consist of 27-hydroxymangiferolic acid, 27-hydroxyisomangiferolic, (+)-Pinobanksin, and CAPE. The effects of 27-hydroxymangiferolic acid on HSP-70 activity, 27-hydroxyisomangiferolic acid on fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), runt-related transcription factor-2 (RUNX-2), alkaline phosphatase (ALP), osteocalcin, collagen type 1a1 (Coll1a1), and (+)-Pinobanksin on HSP-10 activity were demonstrated using molecular docking simulation-based models. According to *in silico* research, 27-hydroxyisomangiferolic acid from stingless bee (*T. biroi*) propolis has a greater negative binding affinity to growth factors, antioxidants, and indicators of osteoblastogenesis than 27-hydroxymangiferolic acid and (+)-Pinobanksin do.[35]

To improve its efficiency against periodontopathogen bacteria, propolis is synthesized into nanosized emulsions (20–200 nm) in order to enhance the adsorption of tiny particles with a greater surface area than normal particles. Nevertheless, most of propolis's constituents are not particularly stable and are only weakly soluble in aqueous media, making them minimally bioavailable. The utilization of nanotechnological strategies to render the hydrophobic active components of plants stable and accessible is becoming more prevalent. These medicinal plant product nano-formulations have significant therapeutic potential in relation to their ability to combat bacterial infection. This approach results in nanomedicines that can continuously release the active therapeutic component from the nanoparticle molecule, maintaining the medication's potency for an extended amount of time.[36] However, to date, there is a lack of research on Celebes stingless bee (*T. biroi*) PNE's antibacterial activity against periodontitis-related bacteria, such as *A. actinomycetemcomitans (Aa), P. gingivalis (Pg), P. intermedia (Pi),* and *F. nucleatum (Fn)*. Thus, the purpose of this study is to examine the antibacterial properties of Propolis Nanoemulsion Extract (PNE), which is extracted from the Celebes Stingless Bee (*T. biroi*), in relation to the periodontopathogen bacteria. This investigation also examines PNE's phytochemistry, particle size analysis (PSA), and zeta potential.

2. Materials and methods

2.1. Stingless bee propolis (T. biroi) nanoemulsion preparation

The propolis (*T. biroi*) was taken from Sulawesi (Celebes) Island, Indonesia, in March 2024. The distribution of *T. biroi* bees in South Sulawesi, where the plant is extensively farmed, provides the basis for the site selection. In South Sulawesi, *T. biroi* bees are extensively dispersed because of favorable climatic conditions and an abundance of bee food sources. The propolis was collected at an average temperature of around 25.30 °C and 84 % humidity. Typically, the rainy season lasts from November to April, reaching a peak in January. September marks the climax of the dry season, which runs from May to October. Primary vegetation and a variety of possible food sources are found in the location of the data collection.

250 g of propolis (*T. biroi*) was macerated in 2.5 L jars with 96 % ethanol over a period of seven days. Thereafter, the mixture was filtered, and the ethanol was evaporated at 40–50 °C using a rotary evaporator (Büchi® rotary evaporator Model R-20, Merck, Sigma-Aldrich, China). After the thick extract was heated in the oven, the ethanol was added in a quantity equal to the volume of the extract.[37] The propolis extract at 100 % concentration was diluted in dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) to vary its concentrations to 100 %, 50 %, 25 %, 12.5 %, 6.25 %, 3.125 %, 1.56 %, and 0.78 %.[37]

A UV–Vis spectrophotometer (UV-2600i/2700i, Shimadzu, Kyoto, Japan) was used to measure the Terner's phase at 650 nm in wavelength, with the distilled water used in the cuvette to calibrate as the blank control. After the residue-free, clear liquid was seen to have a transmittance value of no more than 80 %, Triplot software was used for its analysis. After polysorbate and sodium tripolyphosphate had been added to the extract, the mixture was homogenized for 60 min at 4000 rpmm to create nanoparticles. Particle size analyzers (Partica LA-960V2, Horiba Scientific, Kyoto, Japan) weree then used to quantify the zeta potential and assess the particle size diameter.[38,39]

2.2. Phytochemical analysis

A 5 ml sample of the extract's aqueous filtrate was used for the flavonoid assay. Concentrated sulfuric acid and diluted ammonia were added until a yellow hue developed. Dragendorff's reagent was applied to the stingless bee propolis (*T. biroi*) for the alkaloid test, whereas Wagner's reagent was put into the propolis in a separate tube. When Wagner's reagent produced a brown residue, it proved positive, whereas Dragendorff's reagent produced a red or brown-orange residue. After HCl 2N was shaken and added to a filtrate of propolis nanoemulsion-ammonium chloroform, 2 ml of the plant extract was added to a graduated cylinder and was shaken violently lengthwise for 15 min. The presence of saponins in the test samples was validated by a foam layer that was 1 cm or thicker. Chloroform and water were added to a warmed ethanol-extracted stingless bee propolis (*T. biroi*) nanoemulsion in order to detect triterpenoid; if the result was red or brown in color, it was triterpenoid positive.[38,39]

2.3. Periodontitis-related bacteria preparation

Fusobacterium nucleatum (*Fn*; ATCC22586, UK) was cultured in tryptic soy broth (TSB) media and was anaerobically incubated at 37 °C for 18–24 h. It was then moved into brain-heart infusion (BHI) media and was incubated for 18 h. Then, it was standardized using McFarland 0.5 and was cultured in nutrient agar. *Aggregatibacter actinomycetemcomitans* (*Aa*; *ATCC43718*, UK) was anaerobically incubated for 24 h in BHI media and was standardized using McFarland 0.5. *Porphyromonas gingivalis* (*Pg*; ATCC33277, UK) was cultured in TSB media and was anaerobically incubated at 37 °C for 18–24 h. The culture was moved into liquid BHI media and was incubated for 18 h. It was then standardized using McFarland 0.5 and was cultured in nutrient agar. *Prevotella intermedia* (*Pi*; ATCC25611, UK) was anaerobically incubated in thioglycolate broth (BBLTM Fluid, Becton Dickinson and Co.) for 8 days at 35 °C. The bacterial culture was cultured in blood agar and Wilkins-Chalgren agar for 8 days at 35 °C, which was followed by Gram staining. The bacterial sample was anaerobically cultured in hemin and menadione-added liquid BHI media, which was standardized using McFarland 0.5.[38,39]

2.4. Minimum inhibitory concentration analysis of the propolis (T. biroi) nanoemulsion extract

The stingless bee PNE was put into the liquid TSB media, BHI media, and sterile thioglycolate broth, and thenit was vortexed for ±2 min until it was homogeneous. 100 μL of the propolis solution was then removed from each medium and was combined with 100 μL of the TSB solution (containing *Fn* and *Pg*), the BHI solution (containing *Aa)*), and the thioglycolate broth solution (containing *Pi*). It was then vortexed again for ±2 min. This process was repeated 8 times. The control tube was filled with 1 mL of a standard bacteria suspension (McFarland 0.5) and distilled water for the negative control, while the positive control tube used doxycycline at a concentration of 2 mg/mL. The minimum inhibitory concentration (MIC) would be assessed quantitatively after 48 h of incubation at 37 °C anaerobically and counted using a spectrophotometer with a wavelength of 595 nm.[38,39]

2.5. Analysis of minimum bactericidal concentration of propolis (T. biroi) nanoemulsion

Following the MIC technique, four dilution tubes with antibacterial sensitivity at lower concentrations were obtained and placed in appropriate culture media to check the growth of microorganisms. Previously, the plates were incubated for 48 h in a jar or anaerobic chamber before the colonies were counted. The result was the smallest concentration where there was no growth in the media of the bacterial colony.[38,39]

2.6. Peri-implantitis in OMI-related bacterial inhibition zone analysis

Inhibition zones were found on *Fn, Pg, Aa,* and *Pi* culture plates after stingless bee propolis (*T. biroi*) nanoemulsion extract administration with concentrations of 100 %, 50 %, 25 %, 12.5 %, 6.25 %, 3.125 %, 1.56 %, and 0.78 % in each group that were placed on paper disks. The inhibition zone was measured by calculating the vertical diameter plus the horizontal diameter and dividing by two using a caliper. Inhibition zone measurements were repeated once on the horizontal and vertical sides, then added up and averaged. The results were obtained by subtracting the clear zone diameter formed around the well from the well diameter. The ability to inhibit bacterial growth was weak (<10 mm), moderate (10–15 mm), strong (16–20 mm), and very strong (>20 mm).[38,39]

2.7. Data collection for data analysis

The data were summarized and examined descriptively and inferentially. The data was displayed in a bar chart with a mean and a standard deviation. Data were analyzed using the statistical package for social sciences (SPSS) version 20.0 for Windows, which includes normality and homogeneity tests ($p > 0.05$); in the homogeneity test ($p > 0.05$), a different analysis of variance (ANOVA) test was carried out; and in the post-hoc test, Tukey's Honest Significant Different (HSD) with a different significance value of $p < 0.05$. In the homogeneity test of $p < 0.05$, the Kruskal-Wallis test and the Mann-Whitney test were carried out with different significance values of $p < 0.05$ (IBM Company, Illinois, Chicago, United States).

3. Results

The results of the phytochemical test showed that PNE positively contained alkaloids, flavonoids, and phenols (Fig. 1A). Particle Size Analyzer (PSA) is an instrument used to determine the size of a sample using the principle of dynamic light scattering. From these data, observations of the particle size of PNE showed a value of 151.28–182.2 nm, while the normal value is 10–1000 nm. In addition, the zeta potential test showed a value of −32.76 mV, while the normal value is ± 30 mV (Fig. 1B). Therefore, it can be ascertained that PNE (*T. biroi*) has a nanoparticle size and has a good zeta potential value so that it can penetrate optimally into the tissue surface.

3.1. Antibacterial activity of PNE towards Aa, Pg, Fn, and Pi

MIC, MBC, and diffusion test results of PNE with concentrations of 100 %, 50 %, 25 %, 12.5 %, 6.25 %, 3.125 %, 1.56 %, and 0.78 % against bacteria Aa, Pg, Pi, and Fn were seen in concentrations of 1.56 % and 0.78 % with a significant difference ($p < 0.05$). PNE with a concentration of 1.56 % showed better antibacterial activity than 0.78 % against Aa, Pg, Pi, and Fn (Figs. 2–5).

4. Discussion

Propolis, a bee product, has antibacterial properties against both gram-positive and gram-negative bacteria, as well as aerobic and anaerobic bacteria. Propolis' antimicrobial content can be acquired by

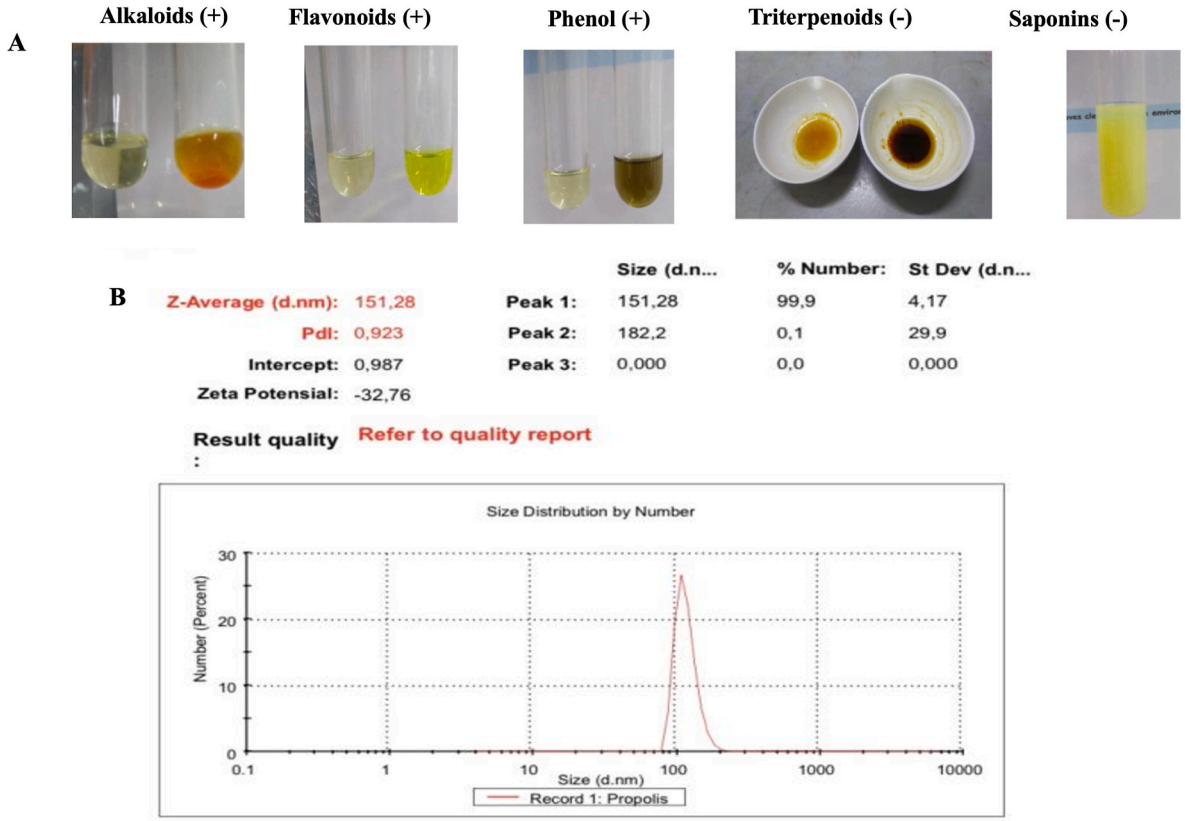

Fig. 1. (A) Stingless Bee Propolis (*T. biori*) Nanoemulsion Phytochemistry analysis and (B) Particle Size Analyzer 1 % PNE has a size of 151.28 dnm and 182.2 dnm.

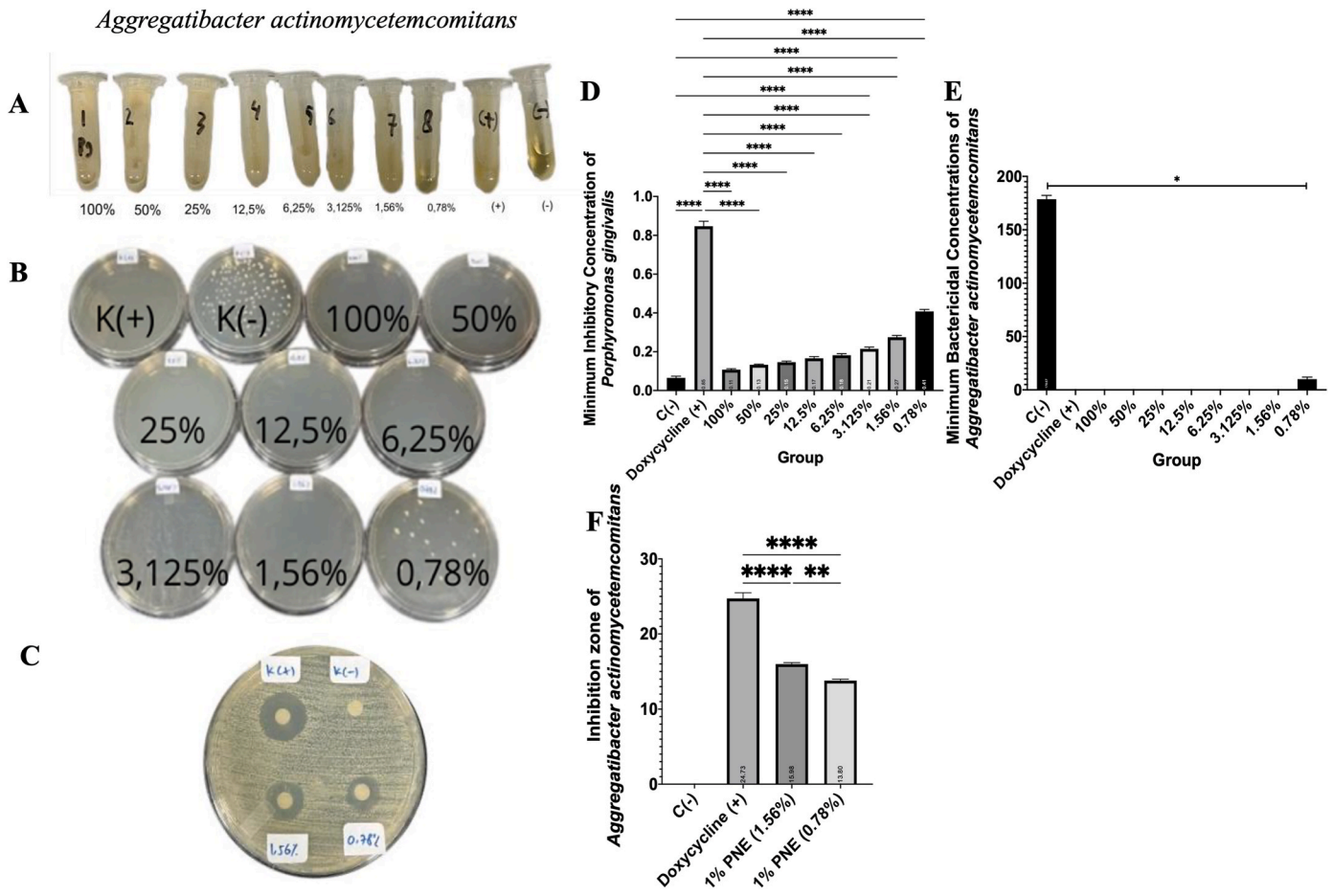

Fig. 2. Antibacterial activity of 1 % PNE against Aa at concentration 1.56 %. (A): MIC of Aa. (B): MBC of Aa. (C): Diffusion zone of Aa. Bar data chart of (D) MIC of Aa; (E) MBC of Aa; (F) Diffusion zone of Aa. Description: significant different at *p < 0.05; **p < 0.01; ****p < 0.001.

extraction. The solvent used in the extraction procedure affects the active chemicals that can be dissolved. Furthermore, propolis solvent extraction, pH, and propolis acid solution can alter its antibacterial activity, as can numerous parameters such as bee species, plant type and age, climate, and time of collection.[40,41] One of the important antibacterial products of bees is propolis. The action of propolis varies by country and is dependent on its chemical makeup. *E. coli* and *Staphylococcus aureus* bacteria were found to be highly active in response to Middle Eastern propolis. German, Irish, and Korean propolis samples simultaneously showed the lowest activity. This variation in effectiveness highlights the significance of regional factors, such as the floral sources available to bees and the environmental conditions that influence propolis composition. Understanding these differences can aid in the development of targeted antibacterial treatments derived from propolis. By identifying the specific compounds responsible for the antibacterial properties of regional propolis, researchers can enhance the efficacy of these natural remedies. Furthermore, this knowledge could lead to more sustainable practices in beekeeping and the preservation of biodiversity, ensuring that bees have access to a rich variety of floral resources.[42]

In this present study, the phytochemical test findings revealed that PNE (*T. biroi*) included flavonoids since the color changed to yellow rather than brilliant. The color shift is caused by the production of yellow flavilium salt as a result of the addition of Mg and HCl ions to the flavonoid structure, which reduces the benzopyrone core. Furthermore, PNE includes alkaloids that have developed brown deposits as a result of interaction with tetraidobismutat III particles. In addition to flavonoids and alkaloids, PNE includes phenol, which is identifiable by the production of black spots caused by the presence of enolate ions coupled with the carbon bonds of the benzene ring. PNE was produced by the maceration process. Maceration is one of the extraction processes that involves immersing the simplicia in a solvent for a set amount of time while stirring occasionally. The maceration method has several advantages, including simple equipment, a simple work technique, low operational costs, and the ability to extract thermolabile compounds. The maceration process makes it simpler for the solvent to permeate the cell wall and reach the cell cavity holding the active ingredient. The solvent utilized was 96 % ethanol, which is ideal for extracting volatile polyphenols. The choice of this solvent is based on ethanol's features as a selective solvent capable of producing secondary metabolite compounds with antibacterial activity. The ethanol concentration has a significant impact on the extraction outcomes. The usage of ethanol as a solvent can be mixed with water (stated in percent) and employed as a parameter in the extraction process. The concentration of ethanol has a significant impact on the hydrophobic strength in the dissolving process as well as the strength of hydrogen bonds or van der Waals forces of the target components. The more similar the polarity of the solvent to that of the substance included in the extracted material, the more components of the substance may be extracted, increasing the yield.[43,44]

PNE from stingless bees (*T. biroi*) has more phytochemical compounds than propolis from Apis bees.[41,45,46] The chemicals found in PNE have several antibacterial mechanisms. Flavonoid phytochemical molecules can create complex compounds with extracellular and dissolved proteins, resulting in protein denaturation and bacterial lysis. The mechanism of action of alkaloid phytochemical substances is to disrupt peptidoglycan components in bacterial cells, resulting in incomplete cell wall formation and cell death. Phenolic phytochemical substances function similarly to flavonoid compounds, denaturing proteins.[32,34]

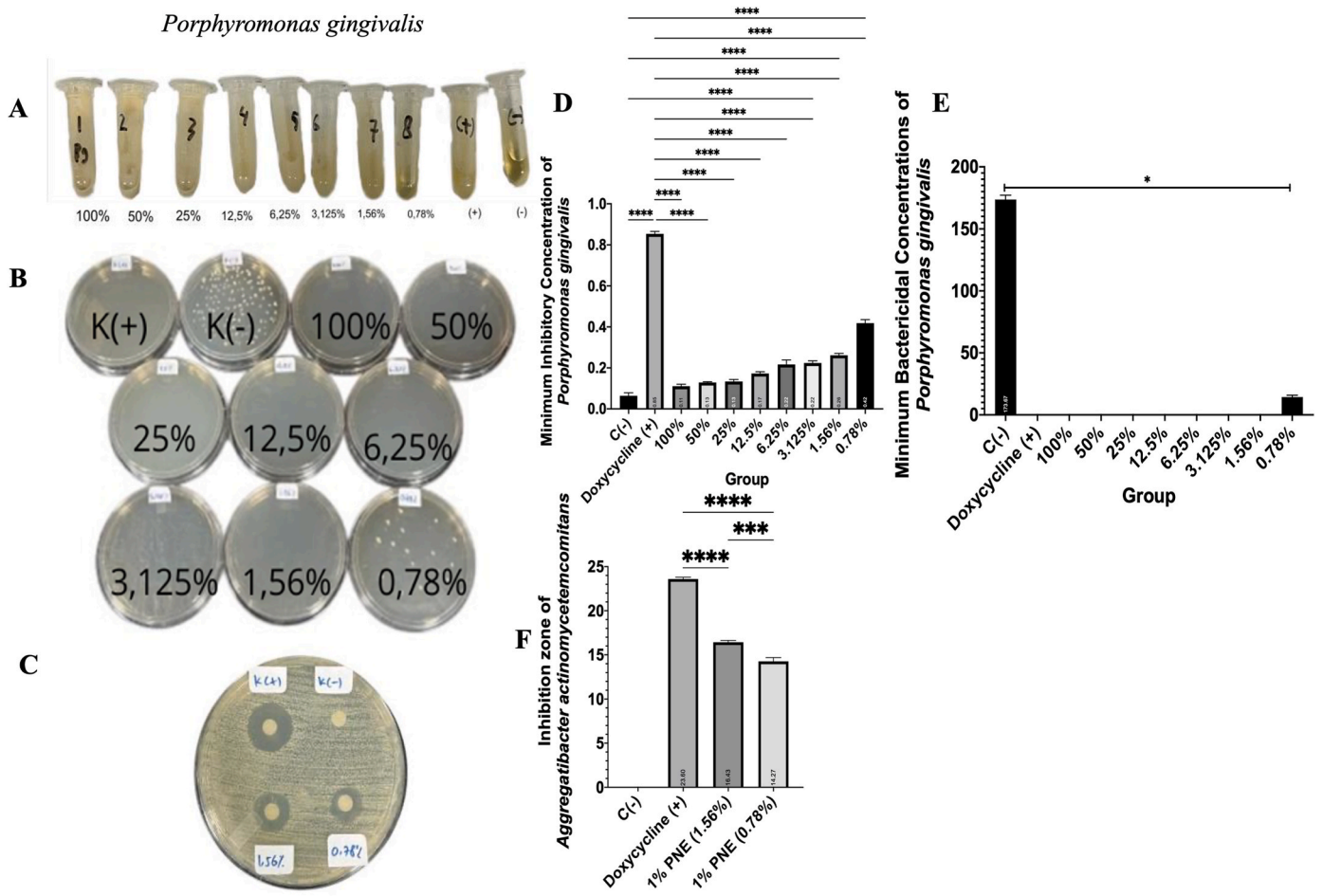

Fig. 3. Antibacterial activity of 1 % PNE against Pg at concentration 1.56 %. (A): MIC of Pg. (B): MBC of Pg. (C): Diffusion zone of Pg. Bar data chart of (D) MIC of Pg; (E) MBC of Pg; (F) Diffusion zone of Pg. Description: significant different at *p < 0.05; **p < 0.01; ****p < 0.001.

Flavonoids, phenolic compounds, polyphenols, terpenes, terpenoids, coumarins, steroids, amino acids, and aromatic acids are among the phytochemical elements of propolis that may have active properties. Bee species, geographic location, botanical supply, and environmental factors all affect the phytochemical components' makeup. Artepillin C, caffeic acid, CAPE, apigenin, chrysin, galangin, kaempferol, luteolin, genistein, naringin, pinocembrin, coumaric acid, and quercetin are a few of the biologically active substances found in propolis. These compounds have a wide range of biological and medicinal qualities, including anti-inflammatory, antioxidant, anti-cancer, cardioprotective, neuroprotective, immunomodulatory, and immuno-inflammatory agents. The potential of these phytochemicals in diverse therapeutic applications is being investigated further, with a focus on their role in enhancing health and disease prevention. As scientists explore deeper into the mechanics of these substances, they expect to discover novel natural cures and gain a better knowledge of propolis as a functional food.[47] Previous study showed antifungal activity of propolis evidenced by the reduced MIC values of aqueous extracts when compared to ethanolic extracts, all aqueous extracts of propolis from *H. Itama*, *Tetrigona binghami, Geniotrigona thoracica* demonstrated strong antifungal activity against strains of Candida albicans and *Saccharomyces cerevisiae*. All of the ethanolic and aqueous propolis extracts were fungistatic, according to the MFC values. The cytotoxicity test revealed that the propolis extracts had a modest amoebic activity against Acanthamoeba sp., significantly lower than that of chlorhexidine, whereas the brine shrimp nauplii lethality bioassay showed that the extracts were non-toxic.[48] *H. itama* had the greatest total flavonoid concentration, measuring 61.63 ± 4.53 mg QE/g propolis. At 78.79 ± 17.06 mgGAE/g propolis, *G. thoracica* had the greatest total polyphenol content. The findings of the agar diffusion method antifungal test revealed that the resistance of both fungi was in the intermediate range and that Brunei propolis had a greater inhibition zone for *C. albicans* than for *Cryptococcus neoformans.*[49]

Nanotechnology can improve the bioavailability of phytochemical compounds at PNE by increasing phytochemical component absorption. Phenolic phytochemicals, including flavonoid compounds, denature proteins. Nanotechnology can boost the bioavailability of phytochemical compounds at PNE by enhancing phytochemical component absorption. The absorption rate of nanoparticles in the human body can almost reach 100 %, whereas microparticles only reach 50 %. This is due to the increased surface area, which allows for better interaction, longer residence time, and increased penetration into tissue, resulting in more efficient cell absorption. Nano-sized propolis is more effective as an antibacterial than micro-sized propolis. In addition, nanoemulsions are thermodynamically stable.[10,46,50]

In this present study, the particle size test revealed a value of 151.28–182.2 nm, which meets the nanoparticle size criteria since it falls between the 10–1000 nm range. 33 Furthermore, the zeta potential test yielded a result of −32.76 mV, which is within the (±) 30 mV range, indicating that the solution is extremely excellent and stable. This is due to increased repulsion between particles with identical charges, which prevents particle merger. Particle size and zeta potential are important criteria in establishing a nanoparticle's stability. Surfactants create a negatively charged zeta potential, which can impair nanoemulsion stability. Nonionic surfactants are often employed in the production of nanoemulsions because they have little irritating effects when applied topically. PNE enters cells via endocytosis; nanoparticles interact with

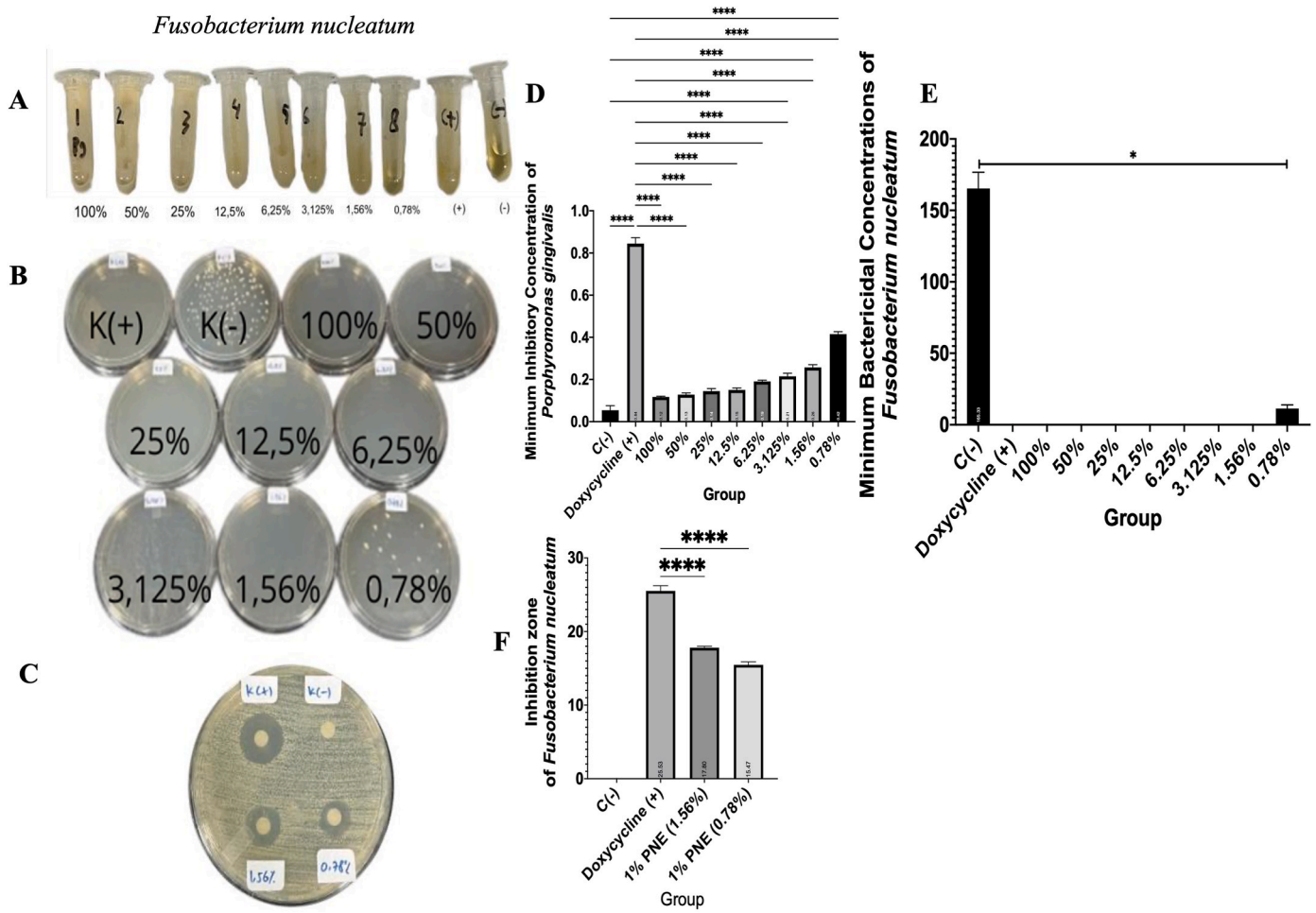

Fig. 4. Antibacterial activity of 1 % PNE against Fn at concentration 1.56 %. (A): MIC of Fn. (B): MBC of Fn. (C): Diffusion zone of Fn. Bar data chart of (D) MIC of Fn; (E) MBC of Fn; (F) Diffusion zone of Fn. Description: significant different at *p < 0.05; **p < 0.01; ****p < 0.001.

cell surface receptors, resulting in nanoparticle endocytosis. Nanoparticles will bind to early endocytic vesicles and be transported to the cytoplasm, progressively releasing PNE phytochemical components. Endocytosis is the primary mechanism by which PNE enters cells via macropinocytosis. During the macropinocytosis process, vesicles or macrospinosomes will develop and encase PNE.[50,51]

This in vitro investigation was designed to investigate the antibacterial potential of PNE (*T. biroi*) against the development of gram-negative bacteria *Aa, Pg, Pi,* and *Fn* that cause peri-implantitis. The antibacterial test of PNE was conducted by administering PNE to bacteria to determine the MIC, MBC, and diffusion zone. The MIC and MBC tests of PNE against *Aa, Pg, Pi,* and *Fn* bacteria revealed significant differences ($p < 0.05$) at concentrations of 0.78 % and 1.56 %, respectively. This demonstrates that PNE (*T. biroi)* at a concentration of 0.78 %, the lowest concentration, may suppress bacterial growth by 90 % when compared to the positive control. This happens because the 0.78 % concentration contains fewer phytochemical components than other concentrations, limiting its capacity to suppress bacterial growth. The colony count test was then used to determine the growth of *Aa, Pg, Pi,* and *Fn* bacteria in BHI medium treated with PNE at various doses. This indirect counting approach is commonly used to determine the bacterial population. The number of colonies that grow represents the number of microorganisms in the suspension; hence, the unit used in this computation is CFU (Colony Forming Unit). The colony count test technique has shown that at a concentration of 1.56 % PNE (*T. biroi*), no additional *Aa, Pg, Pi,* and *Fn* bacteria can develop, implying that PNE is capable of killing 99 % of bacteria (MBC). This demonstrates that the higher the concentration, the stronger the antibacterial properties. Furthermore, at a concentration of 1.56 % and 0.78 %, propolis' phytochemical components can destroy bacterial cell walls. The process begins with the degradation of phospholipids in the bacterial cytoplasmic membrane. H^+ ions from propolis phytochemical substances attack the phosphate group, causing the phospholipid molecules to break down into phosphoglycerol, phosphoric acid, and carboxylic acid. This causes phospholipids to lose their ability to maintain the form of the cytoplasmic membrane, resulting in cell leakage and the release of metabolic components, allowing bacteria to lyse. In this investigation, the diffusion test produced positive findings, resulting in the creation of a clean space surrounding the test disc. The diffusion test determines the concentration of PNE, which can suppress bacterial growth.[36,38,39]

The findings of this investigation revealed that the diameter of the growth inhibition region was called "strong" because when treated with 1.56 %, it inhibited bacteria by 15.98 in Aa bacteria, 16.43 in Pg bacteria, 17.80 in Fn bacteria, and 17.58 in Pi bacteria. This indicates that the biggest width of the inhibition zone corresponds to a larger inhibition zone. The findings reveal that the efficiency of antibacterial agents in inhibiting and killing bacteria is impacted by the kind of test compound, the concentration of the test compound, and the type of bacteria. At 1.56 % concentration, it contains more phytochemical components than at 0.78 % concentration, resulting in a quicker rate of diffusion into the bacterial cell wall. There was a substantial difference among the Aa, Pg, Pi, and Fn bacterial groups. This is consistent with prior studies on Lawang propolis extract against Fn bacteria, where MIC was obtained at 1.48 % and MBC at 1.54 %, indicating that PNE has almost the same

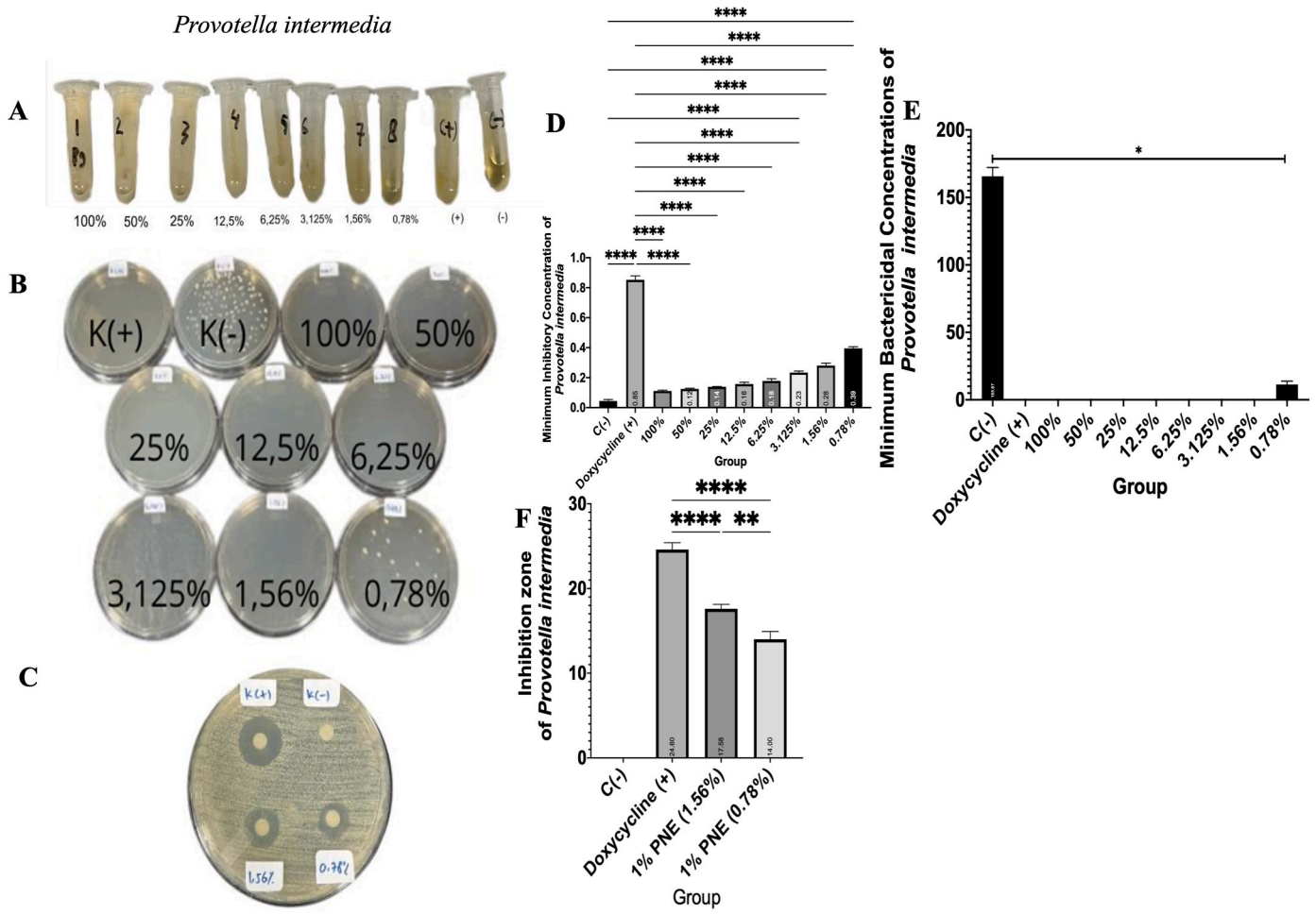

Fig. 5. Antibacterial activity of 1 % PNE against Pi at concentration 1.56 %. (A): MIC of Pi. (B): MBC of Pi. (C): Diffusion zone of Pi. Bar data chart of (D) MIC of Pi; (E) MBC of Pi; (F) Diffusion zone of Pi. Description: significant different at *p < 0.05; **p < 0.01; ****p < 0.001.

capability and is also biocompatible with the HFOB cell line, supported by previous studies.[37–39] Previous study also supported this study by revealed that the propolis particles exhibited antimicrobial properties against a variety of Gram-positive and Gram-negative bacteria. The diameters of the inhibition zones in *S. aureus, B. subtilis, and P. aeruginosa* were either superior to or comparable to those of the two common antibiotics, streptomycin and rifampicin, but not in *E. coli*. Propolis particles were found to be non-toxic to *Caenorhabditis elegans* following a 24-h exposure. These findings suggest that propolis could be a promising natural alternative for combating certain bacterial infections, particularly those caused by Gram-positive bacteria.[14]

Nevertheless, because the antibacterial activity was only examined using MIC, MBC, and diffusion zone, the study's findings are constrained. Propidium monoazide (PMA) combined with real-time PCR (PMA-qPCR) is a novel and inventive method that should be used to evaluate the antibacterial and bacteriostatic activity of PNE (*T. biroi*) against periodontopathogen bacteria.[52] Additionally, this study only used ATCC periodontopathogen bacteria; local periodontopathogen bacteria directly from the oral cavity should be used in order to more properly evaluate the antibacterial activity of PNE (*T. biroi*).

5. Conclusion

The administration of PNE (*T. biroi*) shows antibacterial activity against *Aa, Pg, Fn*, and *Pi* bacteria, demonstrating excellent antibacterial activity in the MIC test at a concentration of 0.78 %, MBC at a concentration of 1.56 %, and diffusion zone at 1.56 %. The phytochemical examination of PNE *(T. biroi)* revealed phytochemical constituents in the form of flavonoids, alkaloids, and phenols that may act as antibacterials. The PSA test of PNE (*T. biroi*) has a nanoparticle size; therefore, it may be utilized as a foundation for effective treatment biomaterials combating periodontopathogen. Further studies focused on additional pharmacological activities should be carried out to discover the extent of the medicinal properties of the Indonesian *T. biroi* propolis for herbal medicine.

Patient's and Guardian's consent

This study conducted in bacterial culture, in vitro study. Thus, Patient's/Guardian's consent is unnecessary.

Study ethical clearance

This study obtained the permission for conducting research in vitro using bacteria culture and investigation of propolis (*T. biroi*) as herbal medicine by ethical clearance committee from Faculty of Dentistry, Universitas Airlangga with appointment number: 0652/HRECC.FODM/VII/2024.

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Declaration of competing interest

The authors declare that there is no conflict of interest in this study.

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