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Phytochemical, antioxidant, and antibacterial activity of *Moringa oleifera* nanosuspension against peri-implantitis bacteria: An in vitro study

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ABSTRACT

Objective: the *Moringa oleifera* leaf (MO) has active compounds that may be beneficial for peri-implantitis therapy. This research aims to analyze the phytochemical, antioxidant, and antibacterial properties of *Moringa oleifera* L. nanosuspension (MON) extract in peri-implantitis-related bacteria.

Methods: MON extract phytochemical analysis was conducted to examine active compounds such as flavonoids, saponins, quinones, alkaloids, tannins, terpenoids, and steroids. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay for antioxidant capacity was evaluated, and gas chromatography-mass spectrometry for the detection of volatile active compounds in MON extract was performed. Turax was used to create MON extract at concentrations of 1% and 2%, and then a particle size analysis was carried out. *Prevotella intermedia* (Pi), *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa), and *Fusobacterium nucleatum* (Fn) were tested for antibacterial activity of MON extract, comparing them with doxycycline as the reference drug and using the minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and diffusion zone methods.

Results: MON extract has lower antioxidant capacity than vitamin C. Flavonoids, saponins, quinones, alkaloids, tannins, terpenoids, and steroids were found in MON extract. 1% and 2% of MON extract has 10–40 d nm particle size. MIC, MBC and diffusion examination of 1% and 2% MON extract on Aa, Pg, Pi, and Fn were seen at concentrations of 25% and 12.5% with significantly different ($p < 0.05$) in vitro.

Conclusion: MON extract has potential antioxidant activity, and 1% or 2% of MON extract has antibacterial properties toward Aa, Pg, Pi, and Fn at concentrations of 25% and 12.5%, with significant differences.

1. Introduction

Dental implant prosthetic therapy is an option for supporting one to several missing teeth, with a survival rate of 96.4% for 5–10 years.[1,2] The level of public awareness of dental implants is increasing because they provide physical and psychological satisfaction.[3] Dental implants restore masticatory function, repair alveolar bone defects, and provide good aesthetics.[4] Unfortunately, plaque accumulation around the implant causes inflammation of the soft and hard tissues known as peri-implantitis with a prevalence of 45% and is considered a major cause of dental implant failure.[5,6]

Peri-implantitis complications reduce implant osseointegration in the form of progressive bone resorption of the peri-implant >3 mm, probing depth >6 mm, suppuration, and bleeding on probing.[7,8] Periodontopathogenic bacteria that cause peri-implantitis include *Actinobacillus actinomycotemcomitans* (Aa), Porphyromonas *gingivalis* (Pg), *Prevotella intermedia* (Pi), and *Fusobacterium nucleatum* (Fn).[9,10] During the century, antibiotic regimens were the main treatment for preventing infection, despite the risk of antibiotic resistance, imbalance of the oral microbiome, and allergies.[11]

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The current development of peri-implantitis treatment has shifted to using biomaterial components that are biocompatible for controlling infection and modulating tissue regeneration.[12] *Moringa oleifera* L. (MO) belongs to the *Moringaceae* family and grows abundantly in tropical and subtropical areas, such as Indonesia.[13] The tree is a fast-growing plant that is resistant to the dry season, with a drought tolerance of up to 6 months.[14,15] MO contains active compounds of flavonoids, saponins, quinones, alkaloids, tannins, terpenoids, and steroids. MO is a miracle leaf that can be used as an antibacterial, antioxidant, anti-inflammatory, antifungal, and anti-aging agent.[15] Until now, studies of the antibacterial activity of *M. oleifera* L. nanosuspension (MON) extract toward peri-implantitis-related bacteria have been limited. Because of its active compounds, MON extract may be beneficial for the development of herbal-based antibiotics to combat Aa, Pg, Pi, and Fn. Furthermore, the purpose of this study was to analyze the antibacterial properties of 1% and 2% MON extract on dental peri-implantitis-related bacteria as candidates for peri-implantitis alternative therapy, in vitro.

2. Materials and methods

2.1. Ethical clearance

This study protocol obtained the permission from the Health Research Committee of Faculty of Dental Medicine, Universitas Airlangga, Surabaya, East Java with appointment number: 683/HRECC.FODM/IX/2022.

2.2. MON extract preparation

Fresh Moringa leaves were collected from Puri Kelorina Village, Ngawenombo, Kunduran, Blora, Central Java, 58255, Indonesia, which was registered by the Ministry of Agriculture, Republic of Indonesia, for use in this research. The precise GPS location for where the *MO* leaves were collected is: https://goo.gl/maps/nVmDrneWp7BWstPT9. The leaves were stored and washed with water, then dried and cut as simplices. Three hundred and 50 g of MO were macerated using ethanol 1:2 (w/v) and filtered. A vacuum rotary evaporator was utilized for filtrate evaporation at 4 °C. The thick extract was desalted and combined with ethanol, resulting in salt settling. This procedure was repeated until the white tint, indicating salt in the solvent, was no longer visible. The preparation of *MON extract* was carried out by adding 10 ml of hot aquadest into the mortar, then adding 1 g of CMC Na, and waiting for 15 min. Next, it was stirred until it became a gel mass. Then, 1 g of nipagin solution dissolved in 10 ml of distilled water was added. The extract solution (1 g and 2 g), which had been dissolved in 20 ml of 96% ethanol, was then added to the mortar and stirred until homogeneous. 100 g of distilled water was added, and then Turax was used for nanosuspension for 10 min. The preparation was stirred at 1400 rpm for 90 min.[16]

2.3. MON extract phytochemical analysis

A flavonoid test was carried out using 5 ml of the aqueous filtrate of the extract. Some dilute ammonia and concentrated sulfuric acid were added until a yellow color formed. An alkaloid test was carried out by adding a few drops of dilute hydrogen chloride (HCL), then filtering and treating it with Dragendorff's reagent until an orange-brown precipitate formed. A saponin analysis was performed by adding 2 ml of alcohol diluted with water and shaking it for 15 min until foam formed. Steroids were tested by adding a few drops of concentrated sulfuric acid to chloroform, resulting in a red color. Quinones were tested by adding 1 ml of concentrated sulfuric acid to 1 ml of the extract until a red color formed. Tannins were observed by diluting a little extract and then adding 4–5 drops of 10-trichloromethane until a blue or green color formed. Terpenoids were tested in 5 ml of extract and 2 ml of chloroform, followed by the addition of concentrated sulfuric acid until a reddish-brown layer was observed.[17]

2.4. MON Antioxidant activity examination

Antioxidant activity was evaluated via a 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. In a nutshell, 3 ml of DPPH in methanol (0.33%) was combined with 1 ml of each ethanolic solution of MON. Using a UV spectrophotometer, absorbance at 517 nm was determined after 30 min at 37 °C. All extracts and controls were tested in triplicate. Ethanol was utilized as a blank control and vitamin C as the reference drug in this assay.

2.5. GCMS analysis of MON extract

MON extract (6 cc) was transferred to a flask for gas chromatography mass spectrometry (GCMS) measurement. There was a 4-ml addition of water. In a preliminary examination, there were no appreciable differences in volatile emissions between the addition of saliva and water. By using GCMS, volatile soybean chemicals were discovered (QP; Shimadzu, Kyoto, Japan). The Tenax resin (TDTS-2010; Shimadzu) was thermally desorbed from the compounds for 5 min at 250 °C. The mass spectrometer continuously scanned from m/z 29 to 550 at a scan speed of 0.5 s/scan to obtain mass spectra using 70 eV electron impact ionization.

2.6. Antibacterial activity of MON extract to peri-implantitis bacteria

The culture of Aa (ATCC43718, UK) was determined by the agar-well diffusion method. Cell concentrations of all test organisms were adjusted to the 0.5 McFarland turbidity standard and inoculated onto MHA plates using sterile cotton swabs. The wells were pierced with a sterile 1000-L microtip, and 100-L of each crude extract was poured into the wells. Plates were incubated at 37 °C for 24 h. Pg (ATCC33277, UK) was cultured by inoculating blood agar with a single looplet of bacterial colonies and anaerobically incubating for 2 × 24 h at 37 °C with CO_2. Bacteria were then inoculated into BHI broth, and the turbidity was adjusted to standard McFarland 0.5 (1.5×10^9 CFU/ml). Pi (ATCC25611, UK) was performed by reactivating bacteria in 5 ml thioglycolate broth (BBLTM Fluid, Becton Dickinson and Company) and anaerobically incubating for 8 days at 35 °C (Anaerogen, Oxoid). Bacterial colonies were inoculated on blood agar and Wilkins-Chalgren agar. Three to five colonies were cultivated in 4 ml of BHI broth with 5 g/ml hemin and 1 g/ml menadione, then incubated anaerobically for 72 h at 35 °C (Anaerogen, Oxoid) and adjusted to the standard McFarland 0.5 (1.5 10^9 CFU/ml). Fn (ATCC22586, UK) was seeded in TSB medium and incubated anaerobically for 18–24 h at 37 °C. Bacterial colonies were collected and transferred to 3 ml of BHI media, then incubated at 37 °C for 18 h. Bacterial suspension was adjusted to the standard McFarland 0.5 (1.5×10^9 CFU/ml) and spread on nutrient agar media. The positive control, as a comparison, was given doxycycline (30 g) as the reference drug for MIC, MBC, and diffusion disk examination due to the fact that it is a bacteriostatic semi-synthetic antibiotic, which is a derivative of tetracycline with a broad spectrum. Doxycycline is widely used as a treatment for infections caused by gram-positive and gram-negative bacteria.[16,17]

2.7. MIC of 1% and 2% MON extract against peri-implantitis bacteria analysis

10 ml of stock culture Aa, Pg, Pi, and Fn were mixed with 2 ml of thioglycollate (TG) broth and then added to 200 μL of antimicrobial stock solution diluted with the 101% dilution method under anaerobic conditions for 48 h. This was performed up to a serial dilution of 10^9. MIC is the lowest concentration of MON extract that does not cause turbidity.

2.8. Peri-implantitis bacteria inhibitory zones analysis

After 50%, 25%, and 12.5%, inhibitory zones were found in the Aa, Pg, Pi, and Fn culture plates. The paper discs had MON extract administration as a treatment group and doxycycline as a positive group. Using a digital caliper (Mitutoyo, Japan), the inhibitory zone was measured in millimeters and repeated for each group.[16,17]

2.9. MBC of 1% and 2% MON extract against peri-implantitis bacteria

Individually placed on agar plates, low-concentration solutions from four dilution tubes obtained using the MIC method were incubated for 48 h. MBC was the lowest MON extract concentration at which bacterial colonies perished.

2.10. Statistical analysis

The Statistical Package for Social Science (SPSS, IBM corporation, Ilinois, Chicago, US) version 20.0 for Windows was used to analyze the data. This includes analysis of variance tests for differences (ANOVA), tests for normality and uniformity ($p > 0.05$), and post hoc Tukey Honest Significant Underscore (HSD) with various significance values ($p < 0.05$).

3. Results

3.1. Phytochemical and GCMS analysis of MON extract

Flavonoids, saponins, quinones, alkaloids, tannins, terpenoids, and steroids are found in MON extract (Fig. 1A). Antioxidant tests showed EC_{50} was lower than vitamin C (Fig. 1B). GCMS examination results showed that MON extract possessed glycidol, benzoic acid, palmitic acid, and linolenic acid (Fig. 1C).

3.2. MON particle size analysis

The particle sizer analysis of MON extract at concentration 1% and 2% showed particle sizes of 10.81 d nm and 40 d nm (Fig. 2).

3.3. Antibacterial activity of MON against peri-implantitis related bacteria

The result of MIC, MBC, diffusion examination of 1% and 2% of MON extract toward Aa, Pg, Pi, and Fn were seen at concentrations of 50%, 25% and 12.5% with significant differences ($p < 0.05$). 1% MON extract showed 50% better than 25% antibacterial activity, and 2% MON extract showed 25% better than 12.5% antibacterial activity toward Aa, Pi, Pg, and Fn (Figs. 3–6).

4. Discussion

In plant research, GCMS has proven to be a helpful technique for the accurate identification of bioactive components.[18] Glycidol, benzoic acid, palmitic acid, and linolenic acid was found in the MON extract revealed by GCMS. The chemical composition discovered by the methods utilized in this investigation is quite similar to that discovered in a prior publication that possessed antibacterial and antioxidant activity of MON extract.[19,20]

This study found phytochemicals such as flavonoids, tannins, saponins, and alkaloids in MON extract. In addition, the antibacterial properties of phytochemicals such as flavonoids, saponins, and tannins have already been identified. These phytochemicals have the potential to be

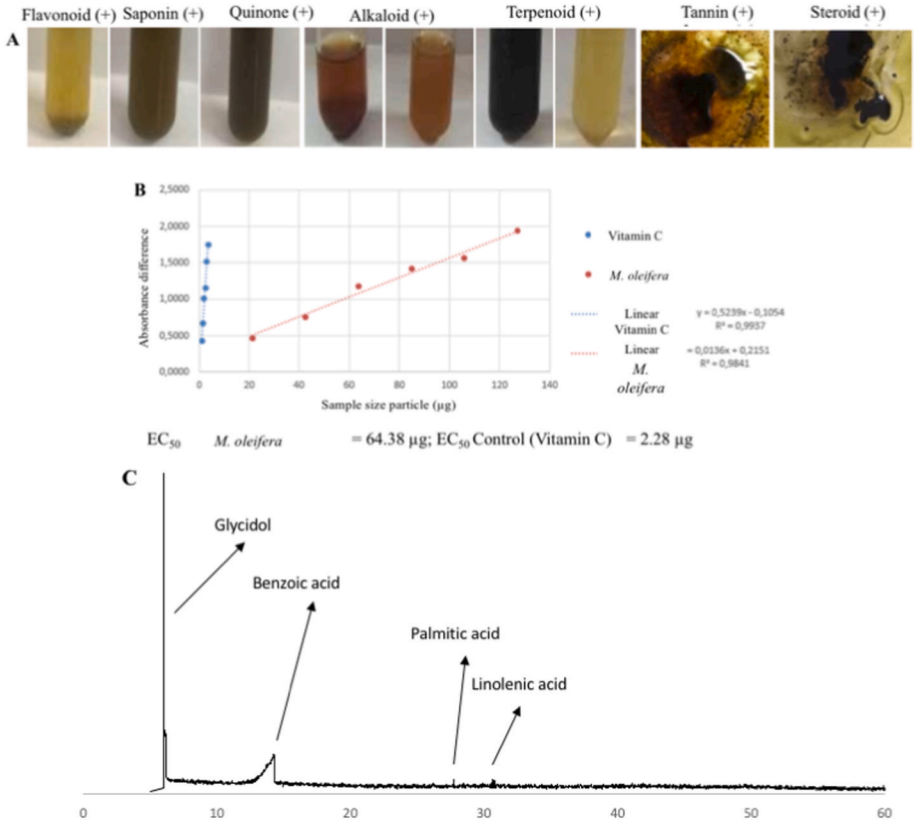

Fig. 1. The phytochemical analysis of MON extract found: (A) positive flavonoid, saponin, quinone, alkaloid, tannin, terpenoid and steroid; (B) The result of antioxidant analysis showed that MON extract possessed lower antioxidant ability compared to vitamin C as a reference drug; (C) GCMS examination results showed that MON extract possessed glycidol, benzoic acid, palmitic acid, and linolenic acid.

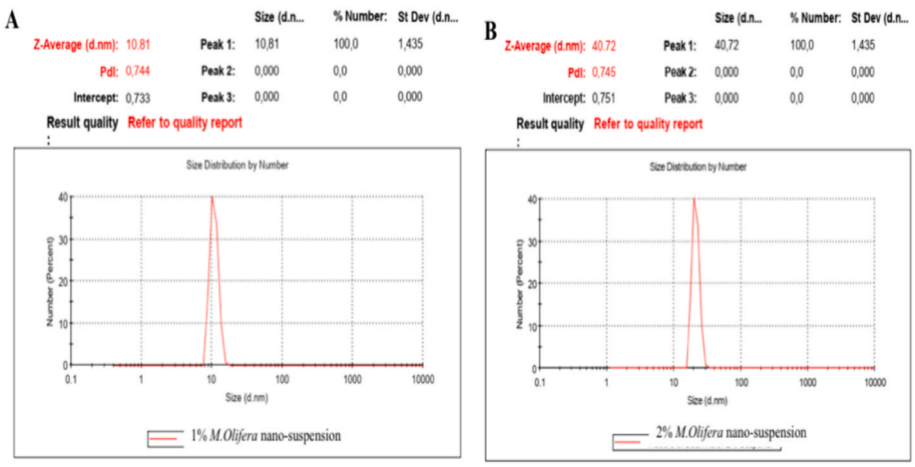

Fig. 2. Particle sizer analysis of MON extract at concentration 1% and 2% with particle size 10.81 d nm and 40 d nm.

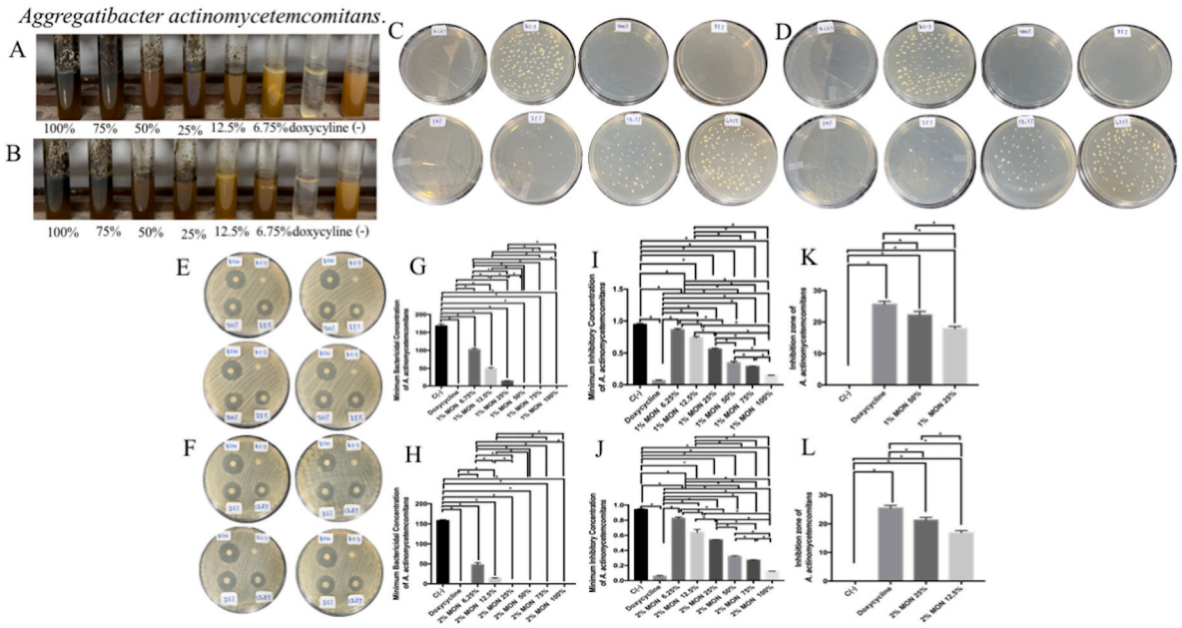

Fig. 3. The antibacterial activity of 1% and 2% MON extract toward *Aggregatibacter actinomycetemcomitans* (Aa). The MIC of Aa after administration of (A) 1% and (B) 2% MON extract showed strong antibacterial activity. The MBC of Aa after administration of (C) 1% and (D) 2% MON extract had strong antibacterial activity. (E) The inhibitory zone using disk diffusion examination on Aa after administration of (E) 1% MON extract showed 50% better than 25% antibacterial activity, and (F) 2% MON extract showed 25% better than 12.5% antibacterial activity. Doxycycline was used in this study as a reference drug. The mean and standard deviation (dv) of 1% and 2% MON result from MIC, MBC and diffusion examination (G–L). *Information: significant difference at $p < 0.05$.

exploited to develop innovative medicinal medicines with increased efficacy.[21] MON extract has antibacterial activity based on its active compound. Flavonoids and alkaloids are the major compounds of MON, at 5.42% and 5.36%.[22] Flavonoids inhibit the synthesis of nucleic acids from bacteria, cell membrane function, and energy metabolism. Flavonoids will suppress the ATPase and phospholipase enzymes leading to disruption of cell membrane permeability. Flavonoids will also form compounds with dissolved extracellular proteins that will damage the bacterial cell membrane so that membrane function is disrupted and intracellular compounds exit the bacteria.[17] Alkaloid compounds interfere with the peptidoglycan components in bacterial cells, which in turn will cause the death of bacterial cells.[23,24] This is extremely similar to our result showing that MIC, MBC, diffusion examination of 1% and 2% MON on Aa, Pg, Pi, and Fn were seen at concentrations of 50%, 25%, and 12.5% with significant differences ($p < 0.05$). This study result about antibacterial activity of MON also supported by several studies. MO ethanol extract has antibacterial effects against as *Staphylococcus aureus*, *S. epidermidis*, Pseudomonas aeruginosa, Salmonella, *Escherichia coli*.[19,21,22] The combination of 40% MO and 10% propolis as well as 10% propolis and 80% MO have better antibacterial effectiveness against Porphyromonas gingivalis biofilm than 0.7% tetracycline.[25,26] Furthermore, the particle size analyzer found MON 1% and 2% with particle sizes of 10.81 d nm and 40 d nm. This suggests that nanoparticle size MON extract may readily influence bacteria by (1) inducing cell membrane damage, (2) disrupting bacteria's metabolism, or (3) inhibiting bacteria's DNA synthesis and ATP production, which can lead to cell death.[27] The result of this study is the first to report and analyze whether MON may possess potential antibacterial activity against Aa, Pg, Pi, and Fn as peri-implantitis bacteria. Utilization of MON extract enables prevention of downstream pathways of toll-like receptors (TLRs) in recognizing pathogen-associated molecule patterns (PAMP), including lipopolysacharide (LPS), flagellin, and peptidoglycan from

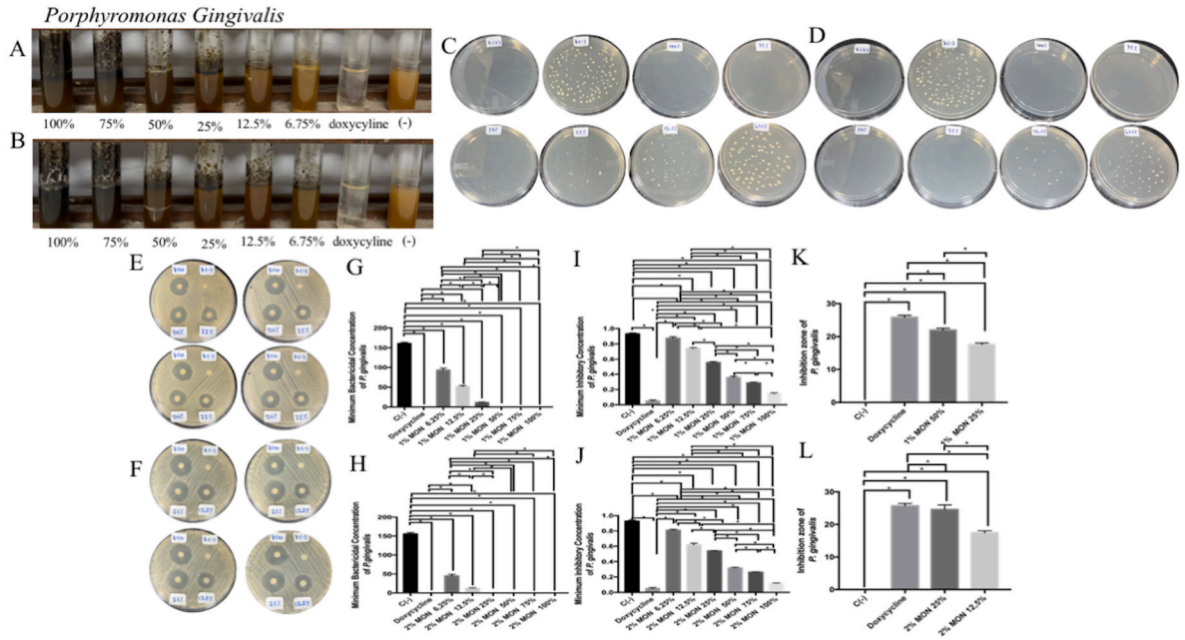

Fig. 4. The antibacterial activity of 1% and 2% MON extract toward *Porphyromonas gingivalis* (Pg). The MIC of Pg after administration of (A) 1% and (B) 2% MON extract showed strong antibacterial activity. The MBC of Pg after administration of (C) 1% and (D) 2% MON extract had strong antibacterial activity. (E) The inhibitory zone using disk diffusion examination on Pg after administration of (E) 1% MON extract showed 50% better than 25% antibacterial activity and (F) 2% MON extract showed 25% better than 12.5% antibacterial activity. Doxycycline was used in this study as a reference drug. The mean and standard deviation (dv) of 1% and 2% MON extract result of MIC, MBC and diffusion examination (G–L). *Information: significant difference at $p < 0.05$.

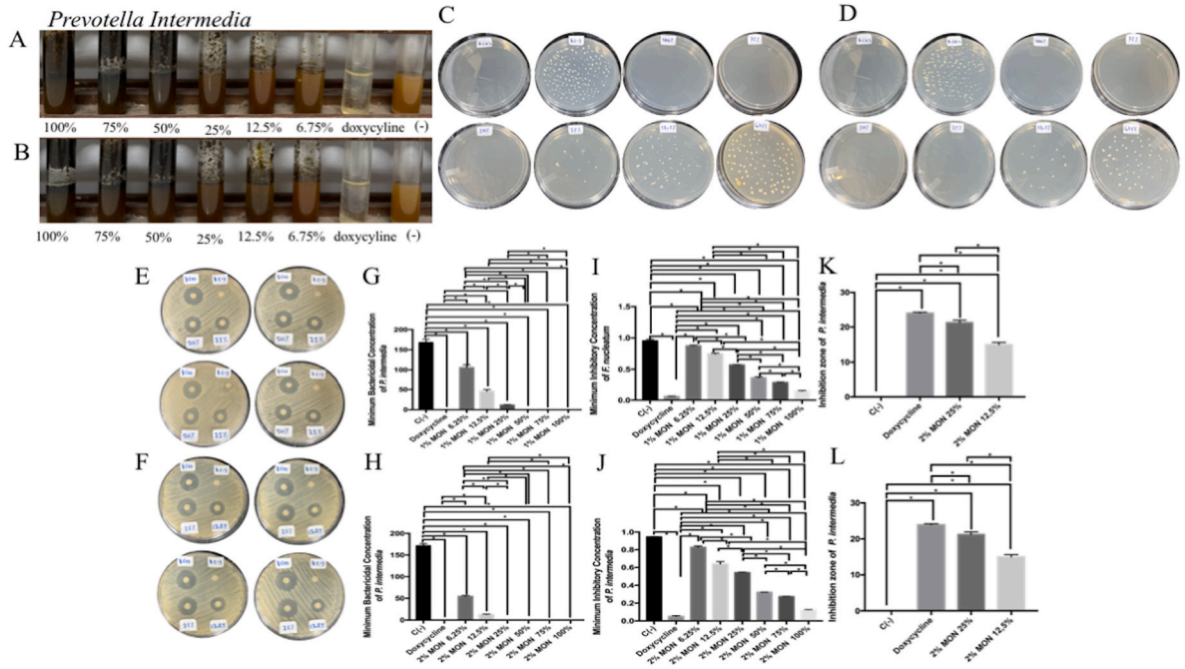

Fig. 5. The antibacterial activity of 1% and 2% MON extract toward *Prevotella intermedia* (Pi) The MIC of Pi after administration of (A) 1% and (B) 2% MON extract showed strong antibacterial activity. The MBC of Pi after administration of (C) 1% and (D) 2% MON extract had strong antibacterial activity. (E) The inhibitory zone using disk diffusion examination on Pi after administration of (E) 1% MON extract showed 50% better than 25% antibacterial activity and (F) 2% MON extract showed 25% better than 12.5% antibacterial activity Doxycycline was used in this study as a reference drug. The mean and standard deviation (dv) of 1% and 2% MON extract result of MIC, MBC and diffusion examination (G–L). *Information: significant difference at $p < 0.05$.

periodontopathogenic bacteria related to peri-implantitis. Recognition of PAMP by TLRs leads to activation of the Myeloide 88 gene (MyD88) and transforming growth factor-activated kinase 1 (TAK1). Thus, it induces IK Kinase (IKK) and IK Kinase (IKK) in order to upregulate nuclear factor kappa beta (NF-KB) expression.[28,29]

Although this study found that vitamin C has the higher antioxidant capacity than MON extract, the antioxidant ability of MON extract is quite high based on EC_{50} result. The significant different between MON and vitamin C is because vitamin C is a pure compound so its possessed a higher antioxidant capacity. Vitamin C would protect all parts of the

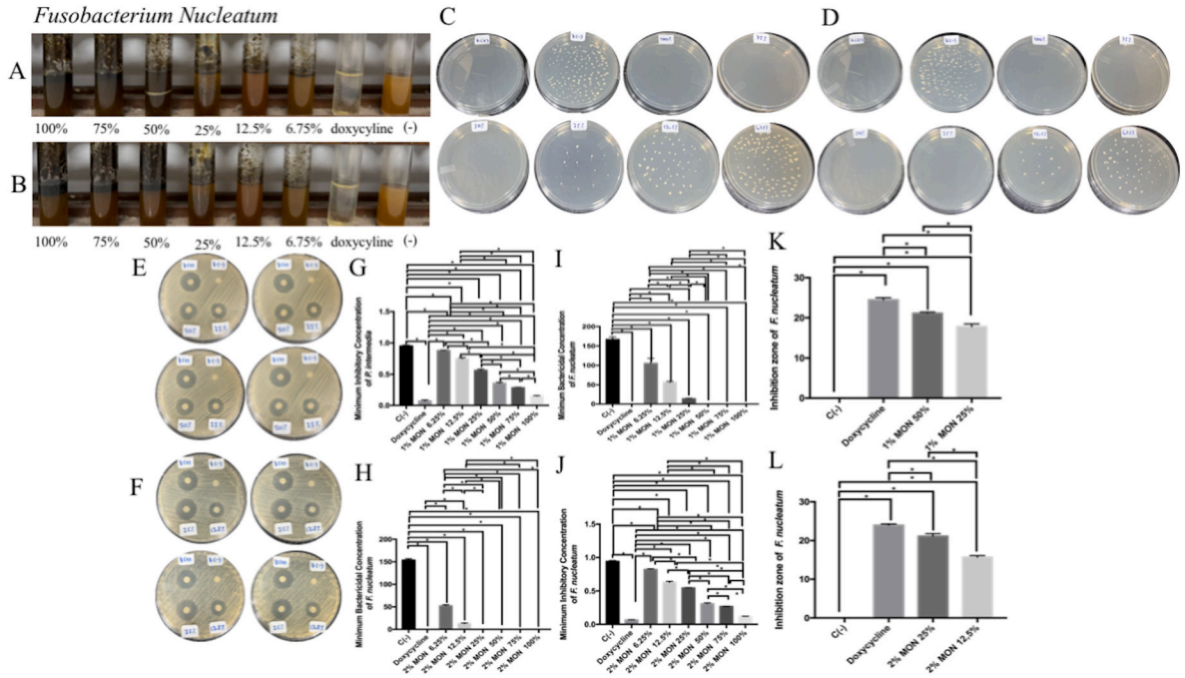

Fig. 6. The antibacterial activity of 1% and 2% MON extract toward *Fusobacterium nucleatum* (Fn) The MIC of Fn after administration of (A) 1% and (B) 2% MON extract showed strong antibacterial activity. The MBC of Fn after administration of (C) 1% and (D) 2% MON extract had strong antibacterial activity. (E) The inhibitory zone using disk diffusion examination on Fn after administration of (E) 1% MON showed 50% better than 25% antibacterial activity and (F) 2% MON extract showed 25% better than 12.5% antibacterial activity. Doxycycline was used in this study as a reference drug. The mean and standard deviation (dv) of 1% and 2% MON extract result of MIC, MBC and diffusion examination (G–L). *Information: significant difference at $p < 0.05$.

cells, organs, and tissues against oxidative damage and oxidative stress at the same time without destroying any of the numerous normal and beneficial functions of ROS. Indeed, supplementation with antioxidants has often resulted in no effect or even adverse disease outcomes.[30] MON contains vitamin C and vitamin E derivatives as antioxidants and is able to upregulate heat shock protein 70 (HSP-70) as their presence is able to inhibit activation of NF-KB as an inflammatory pathway. Down-regulation of NF-KB manifests in inhibition of matrix metalloproteinase-9 (MMP-9) by modulating tissue inhibitor matrix metalloproteinase-1 (TIMP-1). This condition leads to the stability of extracellular matrix (ECM), thus preventing further soft tissue destruction.[31–34]

The current study uses MON extract to propose a novel alternative therapy based on herbal medicine for peri-implantitis. 1% MON extract demonstrated 50% greater antibacterial activity than 25%, while 2% MON demonstrated 25% greater antibacterial activity than 12.5% against Aa, Pg, Fn, and Pi as peri-implantitis related bacteria. Although the outcomes of this study show a potential impact, the study's disadvantage is that all findings were acquired in vitro in idealized settings, and whether the same results can be produced clinically is still dubious. As a result, in vivo and clinical research with various research methods are required to confirm the use of 1% or 2% MON extract (concentrations of 25% and 12.5%), particularly in terms of rute of administration, metabolism, dosage optimization, toxicity, and adverse effects in the event of peri-implantitis. However, this study result has several limitations such as this study only used ATCC peri-implantitis related bacterias, only conducted in vitro setting with limited investigation method. Further study is necessary to be done with various investigation methods, in vivo setting and using wild type of peri-implantitis related bacterias from patients isolate.

5. Conclusion

MON has potential antioxidant activity, and 1% or 2% of MON has antibacterial properties toward Aa, Pg, Pi, and Fn at concentrations of 25% and 12.5% in vitro, with significant differences. MON can be considered for peri-implantitis alternative therapy because, as a natural element, it contains additional components that act as anti-bacterial, anti-inflammatories and antioxidants, both of which play a part in peri-implantitis treatment. However, further study is still needed with various methods of examination and an in vivo setting.

Declaration of competing interest

The authors should declare if exist or not conflict of interest with the data contained in the manuscript.

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