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Antibacterial Activity of Phenolic Compounds in Olive Oil Extracts on Periodontopathogenic Oral Bacteria

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ABSTRACT

Phenolic compounds are secondary metabolites of plants metabolism and can be found in olive oil. They exhibit antimicrobial activity towards both gram-positive and gram-negative bacteria. However, little is known about the antibacterial activity of the compounds towards periodontopathogens. The study aimed to investigate the potential of these compounds as antibacterial agents towards pathogens, specifically *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Phenolic compounds were extracted from extra virgin olive oil (EVOO) through liquid-liquid separation using methanol:water (70:30), and hexane. It was then prepared in various concentrations to determine its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the periodontopathogens. The anti-adhesion activity was quantified using crystal violet staining while the effects on the morphology were examined through scanning electron microscopy (SEM). The MICs of the phenolic compounds on *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* were 31.25 mg/mL, 62.5 mg/mL and 125 mg/mL, respectively. The MBCs of the phenolic compounds on *A. actinomycetemcomitans* and *F. nucleatum* were 62.5 mg/mL and 125 mg/mL, respectively suggesting this compound can eradicate these bacteria. There was no bactericidal effect on *P. gingivalis*. The adhesion of all the bacteria was interrupted by the compounds at the lowest concentration (1.95 mg/mL). SEM findings showed disruption of bacterial cell surfaces such as blebs and disintegration of cells after exposure to this extract. Phenolic compounds of olive oil exhibited antibacterial activity against the tested pathogens, with bactericidal effects on *A. actinomycetemcomitans* and *F. nucleatum* and bacteriostatic effects on *P. gingivalis*.

Keywords: *Antimicrobial effect; natural antimicrobial compound; periodontal bacteria; phenolic compounds*

INTRODUCTION

Periodontal disease is an inflammatory disease affecting tooth-supporting tissue and may lead to tooth mobility and eventually loss of teeth if left untreated. This disease is indeed a global oral health burden that affects over 90% of adults in Malaysia, and it is costly (Mohd-Dom *et al.*, 2013). The incidence of periodontal disease, however, is not limited to Malaysia. Global data show that the number of people affected by this oral disease has increased by almost 70% in 2015 (Jepsen & Jepsen, 2016).

Periodontal disease is initiated by a small group of predominantly gram-negative anaerobic bacteria colonising the oral cavity (Page *et al.*, 1997; Pejčić *et al.*, 2010). Amongst the 500 species found in the oral cavity, only a small group of bacteria are frequently associated with the initiation and progression of periodontal disease. *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are commonly isolated from the sites of the periodontal lesion. These three bacteria, which are implicated in the aetiology of periodontitis (Slots, 2002; Van der Velden *et al.*, 2006), are gram-negative anaerobic or microaerophilic bacteria.

The standard care of managing periodontitis involves the patient's oral self-care and professional cleaning, which includes root surface debridement and possible surgical approaches. Antimicrobial agents may sometimes be prescribed as an adjunct to nonsurgical periodontal therapy. The most common antimicrobials are amoxicillin, metronidazole and azithromycin, and oral antiseptic such as chlorhexidine mouthwash (Lang *et al.*, 2015; Manresa *et al.*, 2018).

The use of natural antimicrobial agents from plant sources as an alternative to chemically driven antimicrobial agents has received great attention due to its low cost, biocompatibility and availability (Kensche *et al.*, 2013; Saiah *et al.*, 2016). Amongst hundreds of plant sources studied, olive oil is a plant extract with beneficial effects in eradicating pathogenic bacteria (Castro *et al.*, 2012; Sumer *et al.*, 2013). Olive oil is beneficial in eradicating gram-negative and gram-positive bacteria, including *Escherichia coli*, *Streptococcus mutans* and *Staphylococcus aureus* (Cicerale *et al.*, 2012; Laincer *et al.*, 2014). The medical field has recognised the potential of the oil in eradicating gram-negative *Helicobacter pylori* (Castro *et al.*, 2012), which can cause peptic ulcers.

The active component in olive oil that is suggested to exhibit antibacterial properties is the phenolic compounds (Brenes *et al.*, 2007; Hanene *et al.*, 2015). Amongst over 30 components that make up the phenolic compounds, hydroxytyrosol is one of the fractions that was mentioned to possess antibacterial properties and is responsible for the stability of olive oil (Bisignano *et al.*, 1999; Cicerale *et al.*, 2012).

In dentistry, the potential of olive oil as an antimicrobial agent towards periodontal pathogens remains in question. The experimental study by Singla *et al.* (2014) has shown a reduction in the oral bacterial count and an improvement in the gingival score after gingival massage by using olive oil. Another study has suggested that olive oil has a role in the prevention of periodontal disease through the modification of the bacterial bioadhesion to the oral mucosa (Kensche *et al.*, 2013). The incorporation of this oil into dentifrice has been suggested for the improvement of gingival health (Pretty *et al.*, 2003; Hernandez, 2006). Considering the abundant positive results of this oil in eliminating a wide range of bacteria, olive oil possibly has an antibacterial activity towards gram-negative periodontal pathogens, specifically *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*.

MATERIALS AND METHODS

Preparation of Phenolic Compounds

The phenolic compounds were extracted from crude extra virgin olive oil (EVOO), Borges™ (Spain) which has been declared by the Environmental Product Declaration (EPD) as having more than 99% of pure ingredients in the content, with no additives and preservatives (EPD, 2016). The same EVOO has been used in a previous study (Hussain *et al.*, 2014) and was reported to have antibacterial activities towards both gram-positive and gram-negative bacteria.

The extraction was carried out according to the method described by Baiano *et al.* (2009) with some modifications. Two millilitres of methanol:water (70:30 v/v) and 2 mL of hexane were added to 5 g of EVOO in a centrifuge tube. The mixture was then vortexed (MS2 Minishaker, IKA, Germany) until fully mixed. The mixed solution was centrifuged for 10 min, 4,000 g at 4°C (Sorvall ST 16R centrifuge, Thermo Scientific, USA). The sediment was then carefully removed and transferred to another centrifuge tube while the supernatant was no longer used. Another round of centrifugation was then carried out for 7 min, 30,100 g at 4°C.

Finally, the oily phase was removed leaving only the clear methanolic solution. This solution was then filtered using 0.45 µm nylon filter into a sterile universal bottle and covered with aluminium foil and kept at 2°C–8°C until further use.

Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) in the extracts was estimated by high performance liquid chromatography (HPLC) using gallic acid as the reference standard. TPC was calculated as gallic acid equivalent (GAE) in mg per g of sample weight. HPLC analysis was conducted using Breeze with dual λ absorbance detector (Waters 2487, Massachusetts, USA), isocratic HPLC pump (Waters 1515, Massachusetts, USA) equipped with an autosampler (Waters 717 plus, Massachusetts, USA) and C18 column (X-Bridge C18 5 µm, 4.6 mm × 150 mm, Waters, USA). The analysis was conducted in gradient elution method using the chromatographic conditions as shown in Table 1.

Table 1 HPLC parameter for quantification of phenolic content

Elution method	Gradient mode		
	Time (min)	Solvent A (%)	Solvent B (%)
	10	90	10
	3	70	30
	5	40	60
	3	60	40
	3	80	20
	6	90	10
Mobile phase	Solvent A: Phosphoric acid: water 0.05% Solvent B: Methanol		
Detector	UV 271 nm		
Column	C18 (5 µm, 4.6 m I.D. × 150 mm)		
Flow rate	1.0 mL/min		
Injection volume	10 µL		
Run time	30 min		

Preparation of Bacterial Culture

Bacterial strains used in this study were from the American Type Culture Collection (ATCC), (Manassas, VA, USA). The tested bacteria strain were *A. actinomycetemcomitans* (ATCC 29523), *P. gingivalis* (ATCC 33277) and *F. nucleatum* (ATCC 25586).

A. actinomycetemcomitans was cultured on brain heart infusion agar and broth (BHI-A and BHI-B, Oxoid, Spain). The media were prepared according to the manufacturer's instructions. For *P. gingivalis* and *F. nucleatum*, the media was prepared using brain heart infusion (1.3 g) and trypticase soy (10.0 g) agar and broth (Oxoid, Spain), supplemented with 2 g yeast (Oxoid, Spain), 0.5 mg/mL cysteine (Merck, Germany), 5 mg/mL haemin (Calbiochem, USA) and 5 mg/mL menadione (Merck, Germany). The prepared culture media were kept in 2°C–8°C and used within one month.

Preparation of Control Groups

In this experimental study, the antibacterial activity of phenolic compounds was compared to normal saline as negative control and amoxicillin 0.5 mg/mL (Sigma-Aldrich, USA) as the positive control which was prepared per the guidelines described in EUCAST (2020).

Bacterial Viability Test: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

The minimum inhibitory concentration (MIC) of the phenolic compounds was determined using twofold serial dilutions of the extract in 96-well microtitre plates (Takarada *et al.*, 2004). The stock solution of the extract was prepared by adding 1 mL dimethyl sulfoxide into 250 mg phenolic compounds in a microcentrifuge tube. From this stock solution, 400 µL was collected and placed into a new tube, and 400 µL broth media was added. This solution was considered the 10^{-1} dilution. These same steps were repeated eight times until reaching the final dilution of 10^{-8}. The final concentrations of the phenolic compounds were 250–3.91 mg/mL. For the test, the aliquots (200 µL) of the extract were dispensed in 96-well microtitre plates. Then the extracts were added with an equal volume of 1×10^6 CFU/mL of tested bacteria followed with incubation for 48 h under anaerobic conditions at 37°C. In all microtitre plates, positive and negative controls were included and treated in the same manner as the extract. The absorbance rate was measured at 595 nm using microplate reader (Varioskan Thermo Scientific, USA). The MIC was recorded as the minimum concentration of the oil that showed no visible bacterial growth in the well ($OD_{595} < 0.05$).

After the measurement of MIC, 10 µL of aliquots of cultures from the wells that showed no visible bacterial growth was inoculated onto respective agar media. Then, followed by incubation under anaerobic conditions for at 48 h at 37°C for determination of minimum bacterial concentration (MBC). The MBC was defined as the lowest concentration at which the original growth was reduced by ≥ 99.9%.

Anti-adhesion Activity

The anti-adhesion activity was determined according to Chaieb *et al.* (2011) with slight modification. Bacteria and extracts at various concentrations were co-incubated into 96-well microtitre plates for 48 h, whereas the wells containing only the broth medium served as blanks. Aliquots of 1×10^6 CFU/mL of bacterial suspension (200 µL) were co-incubated with 200 µL extracts at various concentrations under anaerobic conditions for 48 hours at 37°C. The non-adhered bacterial cells were gently pipetted out, whereas the adhered bacteria were further incubated with 100 µL of 0.1% crystal violet

solution at room temperature for 15 min. The crystal violet solution was removed, and the wells were rinsed thrice with sterile distilled water. The bacterial cells that adhered were fixed with 95% ethanol and incubated at room temperature for 10 min. The solution (50 µL) was then transferred into a new 96-well microtitre plate. The optical densities (OD) of the crystal violet–ethanol solution was measured using a plate reader (Varioskan Thermo Scientific, USA) at 595 nm (OD_{595}). The results appeared as different hues of purple colour in the wells, leading to different absorbance readings obtained by the plate reader. The anti-adhesion activity of the extracts which inhibits the biofilm formation was calculated using the formula modified from Dağdelen (2016):

$$\text{Biofilm formation inhibition (\%)} = \frac{100 \times \text{Mean } (OD_{595} \text{ control} - OD_{595} \text{ treated well})}{(\text{Mean } OD_{595} \text{ control well})}$$

Scanning Electron Microscopy (SEM)

The morphological changes in the bacteria due to the phenolic compounds was examined using SEM (SEM-LEO 1450Vp, United Kingdom) in the Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia. Twelve samples were prepared. Each bacterium had four groups: (1) untreated, (2) treated with the phenolic compounds (31.25 mg/mL, 62.5 mg/mL and 125 mg/mL), (3) treated with amoxicillin 0.5 mg/mL (positive control) and (4) treated with normal saline (negative control). The concentrations of phenolic compounds in treated samples were in concordance with their concentrations of MIC.

Statistical Analysis

Results were expressed as mean ± standard deviations (SD). The effect of the phenolic compounds on the growth and adhesion activity of *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* was analysed using the Mann–Whitney U test ($p < 0.05$).

RESULTS

Total Phenolic Content

The extraction yield of the phenolic compounds was 25%. The amount of total phenolic determined from the extract was 73.7 mg GAE/g dry weight.

MIC and MBC Determination

Determination of MIC and MBC (Table 2) showed that the phenolic compounds inhibited the growth of all tested bacteria. The increasing trend in MIC from *A. actinomycetemcomitans* < *P. gingivalis* < *F. nucleatum* reflects the higher potency of the phenolic compounds on *A. actinomycetemcomitans*, followed by *P. gingivalis* and lastly towards *F. nucleatum.*

Anti-adhesion Activity

In general, the phenolic compounds have good antibacterial activity towards all bacteria especially *A. actinomycetemcomitans* and *P. gingivalis* because over 50% of the biofilm formation was inhibited even at the lowest concentration (1.95 mg/mL). The anti-adhesion effect towards *F. nucleatum* was pronounced at a concentration of 125 mg/mL, where at least 70% of biofilm formation was inhibited (Fig. 1).

Table 2 MIC and MBC (in mg/mL) of phenolic compounds and controls on *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*

Compound/control	*A. actinomycetemcomitans*		*P. gingivalis*		*F. nucleatum*	
	MIC	MBC	MIC	MBC	MIC	MBC
Phenolic compounds	31.25	62.5	62.5	> 250	125	125
Amoxicillin	0.5	0.5	0.5	0.5	0.5	0.5
Normal saline	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition

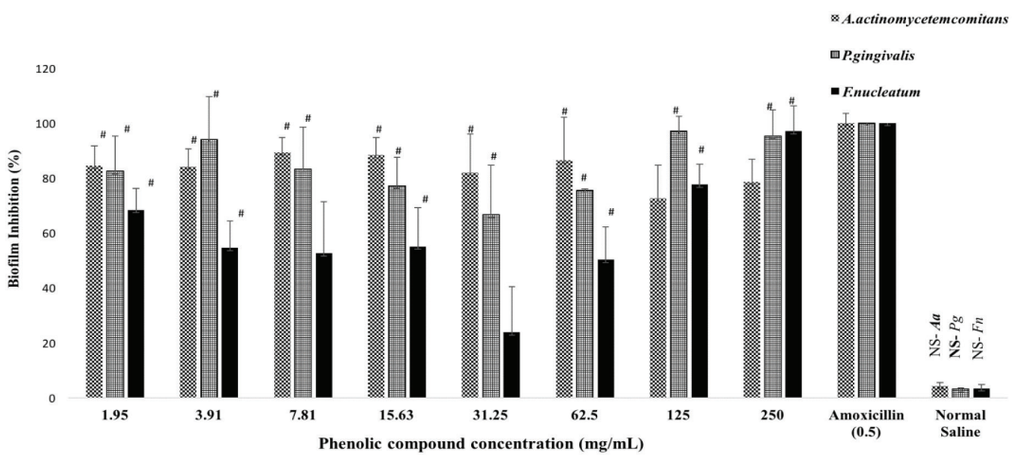

Fig. 1 Anti-adhesion activities of the phenolic compound on *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*. Amoxicillin (0.5 mg/mL) was used as the positive control and normal saline (NS) as the negative control. NS–*Aa* = Normal saline and *A. actinomycetemcomitans*; NS–*Pg* = Normal saline and *P. gingivalis*; NS–*Fn* = Normal saline and *F. nucleatum*. Error bars represent the mean ± SD of two independent experiments performed in triplicates. # = significant differences ($p < 0.05$) of anti-adhesion activity between the phenolic compounds and the negative control of each respective bacteria.

SEM Findings

SEM results in Figs. 2, 3 and 4 have shown morphological changes on the samples after treatment with phenolic compounds [Figs. 2(b), 3(b) and 4(b)] and amoxicillin [Figs. 2(c), 3(c) and 4(c)]. In contrast, normal cells were observed on samples which were untreated [Figs. 2(a), 3(a) and 4(a)] and treated with negative control [Figs. 2(d), 3(d) and 4(d)]. The integrity of the bacterial cell surfaces was disrupted, and the cells showed pleomorphic changes. Bleb formation was observed in Figs. 2(b), 3(c) and 4(c). The irregular cell surface morphology suggested shrinkage of the cells [Figs. 3(b), and 3(c)]. Extensive bacterial cell surface damage, including the disintegration of the cell surface, was observed in Fig. 4(b). The normal saline-treated cells were comparable with the untreated ones, showing normal bacterial morphology. These findings suggested that phenolic compounds at its MIC affected the cell surface integrity of *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*.

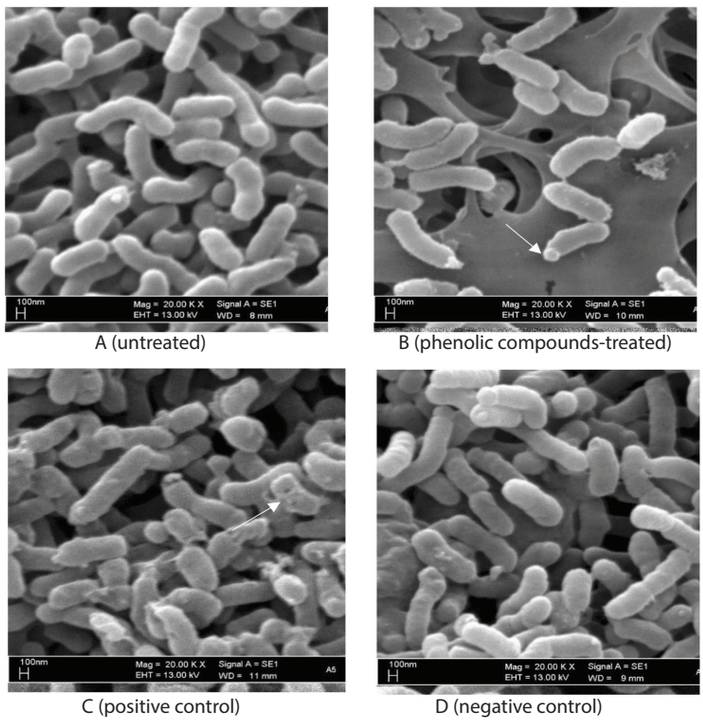

Fig. 2 SEM images of the phenolic compounds-treated *A. actinomycetemcomitans* compared with the negative (normal saline) and the positive (amoxicillin) controls at 20000× magnification. (Arrows showed bacterial cell surfaces changes such as blebs formation [B] and cell shrinkage [C] whereas [A] and [D] showed normal cell appearance).

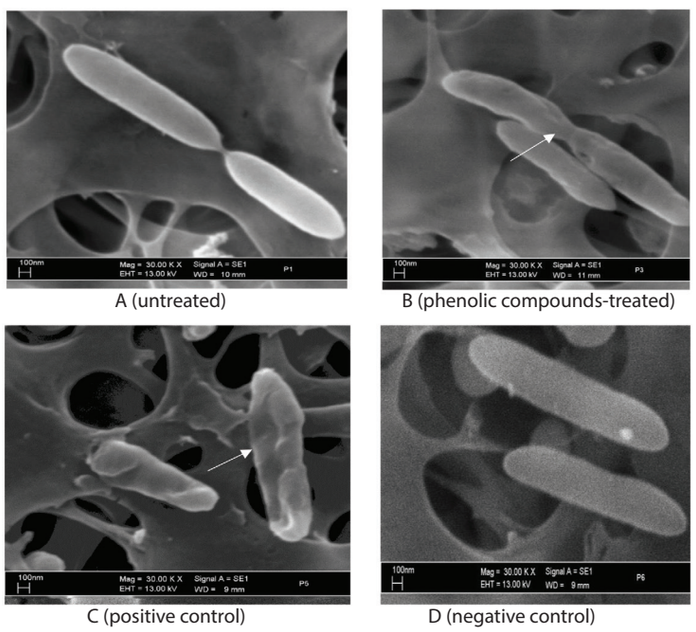

Fig. 3 SEM images of the phenolic compounds-treated *P. gingivalis* compared with the negative (normal saline) and the positive (amoxicillin) controls at 30000× magnification. (Arrows showed bacterial cell surfaces changes such as cell shrinkage [B] and blebs formation [C] whereas [A] and [D] showed normal cell appearance).

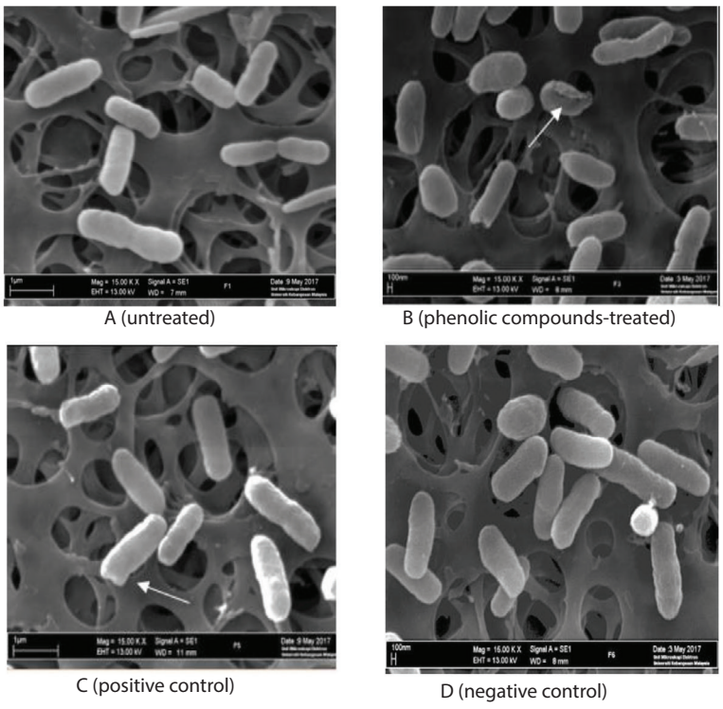

Fig. 4 SEM images of the phenolic compounds-treated *F. nucleatum* compared with the negative (normal saline) and the positive (amoxicillin) controls at 15000× magnification. (Arrows showed bacterial cell surfaces changes such as disintegration of cell [B] and blebs formation [C] whereas [A] and [D] showed normal cell appearance).

DISCUSSION

Results of this study have shown that phenolic compounds from olive oil have antibacterial effects on periodontal pathogens. To date, no study has reported on the antibacterial effect of olive oil compounds especially phenolic compounds on periodontal pathogens. Previous studies on the potency of olive oil phenolic compound towards gram-positive bacteria (*S. aureus, Bacillus subtilis* and *Listeria innocua)* have reported low MIC at 0.7–1.8 mg/mL (Pereira *et al.*, 2007; Laincer *et al.*, 2014). These findings suggest that the phenolic compound is more potent towards gram-positive bacteria than gram-negative bacteria, which have complex bacterial cell walls.

Gram-negative bacteria are more difficult to eliminate due to their several additional cell structures, such as the outer membrane that covers the cell wall externally, making the penetration of antibiotics into the bacterial cell difficult (Silhavy *et al.*, 2010). The outer membrane consists of lipopolysaccharide that acts as a barrier, which prevents hydrophobic antimicrobial agents from entering the bacterial cell (Soares *et al.*, 2012). The phenolic compounds are considered as hydrophobic compounds (Goldsmith *et al.*, 2014).

Porin channels are special structures situated on the cell walls of gram-negative bacteria which act as an active efflux pump. These channels help expel antibiotics that enter the bacterial cells (Karygianni *et al.*, 2014). The porin channel also allows hydrophilic antimicrobial agents, such as amoxicillin, to diffuse through it but restricts hydrophobic antimicrobial agents (Soares *et al.*, 2012). The results of the present study show that all the three tested bacteria are sensitive to different concentrations of phenolic compounds. The lowest MIC was recorded

for *A. actinomycetemcomitans* followed by *P. gingivalis* and highest for *F. nucleatum* reflects the higher potency of phenolic compounds on *A. actinomycetemcomitans*, followed by *P.gingivalis* and lastly towards *F. nucleatum.*

The growth of *A. actinomycetemcomitans* was totally hindered at one concentration higher than the MIC of phenolic compound, whereas the bactericidal effect for *F. nucleatum* occurred at the MIC. However, even at the highest concentration of the serial dilution, only the bacteriostatic effect of the phenolic compounds was observed for *P. gingivalis*. The bactericidal effect was not observed for *P. gingivalis* within the extract range of concentration, indicating that phenolic compounds were bactericidal towards *A. actinomycetemcomitans* and *F. nucleatum* and exerted a bacteriostatic effect on *P. gingivalis.*

The inhibition of biofilm adherence to the oral hard and soft tissue surfaces is a mechanism of oral antimicrobial agents to prevent or at least delay plaque formation and maintain oral health. The incorporation of edible oil into oral antimicrobial agents, dentifrice and mouthwash has been suggested to reduce the attachment of bacteria to the acquired pellicle, reducing the re-formation of dental plaque (Pretty *et al.*, 2003). A study has suggested that the lipophilic substance of edible oils, such as olive oil, may modify the pellicle structure and modulate bioadhesion processes, resulting in good oral health maintenance (Kensche *et al.*, 2013).

In this study, the phenolic compounds have been shown to have good anti-adhesion activity. Even at the lowest extract concentration (3.91 mg/mL), at least 50% of the *A. actinomycetemcomitans* and the *P. gingivalis* biofilms were eliminated, and a concentration of 31.25 mg/mL was needed to eliminate the same percentages of biofilm for *F. nucleatum*. In another study, the effect of naturally occurring phenols towards biofilm and planktonic bacteria was investigated and it was found that biofilm was effectively eradicated by the phenolic compound (Walsh *et al.*, 2019).

The mechanism of olive oil as an antimicrobial agent remains unclear. A previous study has reported that olive oil can disrupt bacterial membrane function, which entails alterations in the membrane permeability and loss of membrane-associated enzyme function. The process is followed by the breakdown of protein and leakage from the cell (Hugo & Bloomfield, 1971; Bisignano *et al.*, 1999; Hu *et al.*, 2014). The phenolic compounds exhibit antimicrobial activity due to the presence of hydroxyl group in their chemical structure which can release a proton in the cell and affect cell membrane integrity (Laincer *et al.*, 2014).

SEM showed that all untreated samples exhibit normal cell appearance with intact and smooth cell surface, similar to those treated with normal saline (negative control). By contrast, the samples treated with the phenolic compounds as well as amoxicillin (positive control) showed evident changes in cell surface morphology, such as bleb formation and the shrinkage or rupture of cells for all tested bacteria.

These results suggest that the phenolic compounds affect the viability of *A. actinomycetemcomitans, P. gingivalis* and *F. nucleatum* by interrupting the integrity of the cell surfaces. A similar finding is reported when *Listeria monocytogenes* cell membranes were damaged at its MIC when treated with olive leaf extract (Liu *et al.*, 2017). It is suggested that the multiple components in phenolic compounds may enhance the effect of each other, making the combination a strong antibacterial agent (Pereira *et al.*, 2007). On contrary, another study reported that even as a single component, it can be a strong antimicrobial agent (Heidari-Soureshjani *et al.*, 2016).

The present study provides baseline information on the antimicrobial activity of olive oil extract, namely

the phenolic compounds towards *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*. Future research is needed to address the safety of the oil for use in humans, such as the effective and toxic doses towards cells.

Given that the resistance and virulence of strain bacteria and bacterial isolates from the oral cavity are different from one another, future studies on the antibacterial activity of olive oil and its extract on *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* isolates from the periodontal pocket is recommended to obtain a reliable picture of antimicrobial activity of the olive oil towards pathogen-causing periodontal tissue breakdown. Further exploration of the antibacterial mechanisms of the phenolic compounds in olive oil should be made to identify the active components in the compound that exhibit antimicrobial activity.

CONCLUSION

The phenolic compound in olive oil extract possesses antibacterial activity towards *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*. Its phenolic compounds exhibit a bactericidal effect on *A. actinomycetemcomitans* and *F. nucleatum* but act bacteriostatically on *P. gingivalis*. The adhesion activities and bacterial cell surfaces of all tested bacteria are disrupted after exposure to the phenolic compounds.

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