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Antibacterial Activity of Bioactive Compound in *Salvadora persica* (Chewing Stick) Against *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*

Haslinda Ramli[1], Zalina Baharudin[2] and Rohazila Mohamad Hanafiah[3]

[1]*Department of Periodontology and Community Oral Health, Faculty of Dentistry, Universiti Sains Islam Malaysia, Pandan Indah, 55100 Kuala Lumpur, Malaysia*

[2]*Polyclinic 17, Faculty of Dentistry, Universiti Sains Islam Malaysia, Pandan Indah, 55100 Kuala Lumpur, Malaysia*

[3]*Department of Basic Sciences and Oral Biology, Faculty of Dentistry, Universiti Sains Islam Malaysia Pandan Indah 55100 Kuala Lumpur, Malaysia*

This study aims to determine the phytochemical properties of *Salvadora persica* extracts and their antibacterial activities against Gram-negative oral anaerobes which are responsible of periodontitis such as *Porphorymonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Aqueous and ethanolic extracts were prepared using the root powdered stem of *S. persica*. The phytochemical compounds were determined with Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS). The antibacterial activities were assessed according to the levels of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The *S. persica* ethanolic and aqueous extracts contained antibacterial compounds such as Octadecenoic acid, methyl ester, n-Hexadecanoic acid and Pentadecanoic acid, 2,6,10,14-tetramethyl-, methylester, 1-(2,2-Dimethylpropanoyl)-L-prolyl-L-prolyl-N,2-dimethylalaninamide and 2-({4-[(Difluoromethyl)sulfanyl]phenyl} amino) benzoic acid, respectively. Both *P. gingivalis* (0.025 mg/mL) and *A. actinomycetemcomitans* (0.125 mg/mL) were susceptible to *S. persica* ethanolic extract. Ethanolic extract preparation has equal antibacterial activity with 0.2% chlorhexidine gluconate solution ($p<0.05$). Ethanolic extract (0.025-0.125 mg/mL) preparation has comparable require biocompatible testing.

Keywords: *Aggregatibacter actinomycetemcomitans*; periodontitis; *Porphyromonas gingivalis*; *Salvadora persica*

I. INTRODUCTION

The chewing stick has been used as an alternative tooth cleaning tool for many years in various Middle Eastern, African, Asian, and Latin American countries (Aboul-Enein 2014). *Salvadora persica* (*S. persica*) tree is the commonest source of the chewing stick. The stems or roots of the *S. Persica* tree contain many widely spaced phloem. With these features, they become spongy and highly chewable. The fraying fibres produced from the chewing are highly effective for mechanical plaque removed (Niazi *et al.,* 2016). Recent research has shown that freshly cut chewing stick contains a higher level of the active chemical compound than dry stick (Khan *et al.*, 2020).

Based on previous research, *S. persica* contains various bioactive phytochemical compounds such as flavanoids, sterols, saponins, tannins, basic alkaloids, reducing component, and volatile oils (Al Sadhan & Almas, 1999). The strong antibacterial effect of the volatile active compound has been clinically and experimentally proven to be effective in

*Corresponding author's e-mail: rohazila@usim.edu.my

warding off gram-positive and gram-negative oral bacteria (Sukarwalla *et al.*, 2013).

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* are two of the commonest pathogens that cause periodontitis (Wakabayashi *et al.*, 2010). *P. gingivalis* is the most prevalent microorganism associated with periodontal abscess while *A. actinomycetemcomitans* is closely related to advanced and aggressive periodontal disease (How *et al.,* 2016). Higher amounts of *A. actinomycetemcomitans* and *P. gingivalis* was identified from subgingival plaque of patients diagnosed of generalised aggressive periodontitis, than chronic periodontitis (Casarin *et al.,* 2010). In a recent study, both organisms were detected in the subgingival plaque that triggered the subsequent immuno-inflammatory reaction that initiated the periodontal destruction (Cekici *et al.,* 2014). In the worst-case scenario, periodontitis can result in tooth loss.

Mechanical plaque controls such as scaling, polishing, and root debridement are commonly used to disrupt the dental plaque biofilm. However, in medically compromised patients with severe periodontal destruction and, physical and mentally handicapped patient, adjunctive localised antibacterial agents have been associated with an improved outcome of periodontal therapy (Trombelli & Tatakis, 2003). Chlorhexidine gluconate 0.2% solution is considered as the gold standard for this purpose as it is effective in fighting off infections by aerobic and anaerobic oral bacteria (Balagopal & Arjunkumar, 2013). However, it can produce several side effects such as brown discolouration of tongue and teeth, the formation of supragingival calculus, taste alteration, and oral desquamation in children (Tartaglia *et al.,* 2019).

Although rarely reported, the most serious problem related to usage of chlorhexidine was lethal allergic reaction. Consequently, clinicians are reluctant to prescribe for patient use, especially to pregnant mother. In contrast, *S. persica* is extracted from natural products and it is not associated with any severe side effect when applied properly (Nordin *et al.,* 2012). Therefore, this study set out to determine antibacterial activity of *S. persica* ethanol and aqueous extracts against *A. actinomycetemcomitans* and *P. Gingivalis* and to investigate phytochemical compound of *S. persica*.

II. MATERIALS AND METHOD

A. Aqueous Extraction

A total of 600g root powdered stems were mixed with one litre deionised distilled water in sterile beaker. The extract was soaked for 48 hours at 4° C. About 50 mL of mixture was centrifuged at 2000 rpm for 20 minutes (Almas, 2001). The step was repeated on remain mixture. The supernatant was filtered through Whatman No. 1 filter paper. The prepared extract was then stored in labelled sterile bottles and then kept in a freezer at 20°C (Bhat *et al.,* 2012). The filtrate was then dried using a freeze drier before being kept in desiccator. Following that, 200mg of the extracted powder was transferred into a sterile universal bottle to which 1ml of sterile deionised distilled water was added. Lastly, the mixture was turbinated and filtered to remove any residue.

B. Alcoholic Extraction

A total of 600g root powdered stems from *S. Persica* was exhaustively extracted with one litre of ethanol (100%) and concentrated under reduced pressure using a rotary evaporator at 40°C to produce a concentrated extract. Concentrated extract is transferred into vial and kept in desiccator at room temperature. It is weighed to get final weight.

C. Bacterial Culture

Two strains were used for the antimicrobial test, namely *P. gingivalis* (ATCC 33277) and *A. actinomycetemcomitans* (ATCC 29522). They were aseptically inoculated on Columbia blood agar plates and incubated at 37°C for 48 hours in an anaerobic atmosphere of 10% H_2, 10% CO_2, and 80% N_2.

D. Antibacterial Activity

Tryptic soy broth and Columbia blood agar were used to grow and dilute the suspensions containing the microorganism. They were cultured to grow exponentially in nutrient broth at 37 °C for 18 hours. Lastly, to adjust to a final density of 1×10^8 cfu/mL, the fresh cultures were diluted by comparing to the McFarland density (Al-Bayati & Sulaiman, 2008). To assess the optimum antimicrobial concentrations of *S. Persica* extracts against *P. gingivalis* (ATCC 33277) and *A.*

actinomycetemcomitans (ATCC 29522), the minimal inhibitory concentration (MIC) applied. The microdilution method in 96 multi-well microtiter plates (Al-Bayati & Sulaiman, 2008) was applied with minor modification and adjusted to a final density 1 x 10^8 CFU/ml McFarland density. The dissolved extracts were diluted to the highest concentration (200 mg/mL) for aqueous extract in 1ml sterile deionised distilled water and 0.1 mg/mL for ethanol extract in 1mL Dimethyl sulfoxide-and incubated at 37°C for 18-24 hours. Any colour change was then visually inspected and recorded. Chlorhexidine was used as positive control for this study. The MIC value was equivalent to the lowest concentration at which the colour change occurred. Following that, spot samples from the microtiter plate wells with visible growth were cultivated on Columbia blood agar for the analysis of Minimum Bactericidal Concentration.

E. Statistical Analysis

The data was analysed using two-way ANOVA (IBM SPSS Statistic Base), to find the statistical significance of parameters between three or more groups of subjects where the *p* value is less than 0.05 ($p<0.05$).

F. Identification of Phytochemical Compound

To identify the phytochemical compound, Varian Chrompack CP-3800 ion-trap GC-MS 2000 equipped with DB-5.625 GC column (30m x 0.25mm) (Sigma-Aldrich) was used. The injector temperature was set at 220°C for 5 minutes with a split ratio of 10. The GC oven was programmed to be started at 50 °C and hold for 2 minutes, raised to 150°C at a rate of 10 °C/min hold 5 minutes, raised to 220 °C at a rate of 20 °C/min, and finally hold for 10 min. The total run time was 30.5 minutes the mass detector was set to scan between 40 and 400 *m/z* using the fixed mode EI.

The bioactive compounds were determined by matching their recorded spectra with the mass spectra of data bank (Saturn and NIST library databases) delivered by the instrument software and by comparing their retention indices values with those in the literature, measured on columns with identical polarity. RI retention index was calculated on DB-5 capillary column material. The databases were compiled using electron impact (EI) of mass spectra. Concentration (% content) of the oil components were calculated by integrating their peak areas, in the total ion current (TIC) chromatograms.

Another equipment used was Waters Aquity UPLC with Acquity UPLC CORTECS HILIC column (100 mm x 2.1 mm x 1.6µm) (Sigma-Aldrich). The mobile phase involved Solution A: Water + 0.1% Formic acid (LCMS Grade) and solution B: Acetonitrile + 0.1% Formic acid (LCMS Grade). The total run time was 10 minutes at 30°C with positive and negative ionisation modes. The injection volume was 1 µL. Waters Single Quadrupole Detector (SQD) was used for MS. The bioactive compounds were determined by matching the mass spectra of compounds into metlin scripps applications (Smith *et al.,* 2005). The identifications will be confirmed by the analysis of pure materials. The following parameters had been used: cent-Wave peak picking algorithm (ppm =15 and minimum and maximum peak width of 2s and 25 s respectively), obiwarp retention time correction (profStep = 0.1) and alignment (mzwid = 0.01, min-frac = 0.5 and bw = 2). CAMERA parameters were: error = 5 ppm and m/z absolute error = 0.015 Da.

III. RESULT AND DISCUSSION

Table 1 showed the results of Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The most significant compounds in the ethanol extract were Octadecenoic acid, methyl ester, n-Hexadecanoic acid and Pentadecanoic acid, 2,6,10,14-tetramethyl-,methylester (Figure 1). Those compounds are fatty acid compounds which exhibit antibacterial activity against pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella spp* (Pu *et al.,* 2010).

Table 2 showed the results of Liquid chromatography–mass spectrometry (LCMS) analysis, two major compounds were identified in the aqueous extract, namely 1-(2,2-Dimethylpropanoyl) -L- prolyl-L-prolyl-N, and 2-({4-[(Difluoromethyl)sulfanyl] phenyl} amino) benzoic acid (Figure 2). According to Boakye *et al.* (2019), those compounds from natural product exhibited antibacterial activities against pathogenic bacteria.

Identification of phytochemical compounds in an extract is based on liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS)

for aqueous extract and alcoholic extract, respectively. From this study, the ethanolic extract of S. *persica* was found to contain volatile compounds (Octadecenoic acid, n-Hexadecanoic acid, Pentadecanoic acid,2,6,10,14-tetramethyl-, methyl ester, and 9-Hexadecanoic acid) that confer the antibacterial properties. Fatty acids from plant extracts altered the membrane permeability of bacteria cells due to hydrophobic interaction. The alteration will disrupt pressure of osmosis and caused cells lysis (Nazzaro *et al.,* 2013).

Table 3 showed that *P. gingivalis* was resistant towards aqueous extract of *S. persica* up to 200 mg/mL concentration. There was a significant difference between the results of chlorhexidine and aqueous extract in which chlorhexidine was more superior against *P. gingivalis* than aqueous extract. In contrast, ethanolic extract (0.025 and 0.125 mg/mL) showed a non-statistically significant difference when compared with 0.2 % chlorhexidine. In other words, the ethanolic extract has a similar level of effectiveness with 0.2 % chlorhexidine in inhibiting the growth of *P. gingivalis* and *A. actinomycetemcomitans*. The ethanolic extract displayed bactericidal effects on both *P. gingivalis* and *A. actinomycetemcomitans* while aqueous extract only showed bactericidal effects on *A. Actinomycetemcomitans* (Table 4).

Many researchers report that fatty acid exhibit antibacterial activities against pathogenic bacteria. For example, hexadecanoic acid has been proven to display antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Streptococcus mutans* (Pu *et. al.,* 2010; Nalina & Rahim, 2007). Apart from that, Octadecanoic acid was found to have antibacterial activity against *P. gingivalis and* several other bacteria including *Streptococcus mutans, Peptostroptococcus anerobius, Prevotella oralis, Lactobacillus acidophilus*, and *Fusobacterium nucleatum* (Koru *et al.,* 2007). In addition, Pentadecanoic acid was proven to have antibacterial activity against *Enterococcus faecalis* (Ligia *et al.,* 2014).

Furthermore, another study on the phytochemical analysis of *S. persica* extracts also revealed volatile compound (essential oils) as being responsible for the antibacterial activity against *Staphylococcus aureus* and *Pseudomonoas aeruginosa* (Alali *et al.,* 2005). Several types of volatile compound can be found in the *S. persica* extract, including Dodecanoic acid, 2-Hexadecen-1-ol,3,7,11,15-tetramethyl, Octadecanoic acid, Nonadecane, Tetra dodecanoic acid, and 9-Octadecanoic acid (Ahmed *et al.,* 2008). Besides that, Khan *et al.* (2020) was reported that essential oil (benzyl isothiocynate) from *S. persica* exhibited antibiofilm activity against *Streptococcus mutans* at concentration 5-20 µg/mL.

The results from this study showed the bactericidal effect of 100% (0.025 and 0.125 mg/mL) ethanolic extract on *P. gingivalis and A. actinomycetemcomitans*, respectively. On the contrary, the water extract of *S. persica* did not display any antibacterial effect against *P. gingivalis*. *S. persica* methanol (100%) extract exhibited antibacterial activity against several pathogen such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* at 200-12.5 mg/mL (Al-Bayati & Sulaiman, 2008). This finding showed that alcoholic extract exhibited antibacterial activity more than water extract. Another study by Fouad Hussein *et al.* (2010) reported that 0.2% chlorhexidine gluconate solution exerted higher antibacterial activity than the 60% ethanolic extract of *S. persica*.

However, in-vitro study by Siddeeqh *et al.* (2016) supported the effectiveness of alcoholic extract of *S. persica* can significantly inhibit the periodontal pathogen. *P. gingivalis* and *A. actinomycetemcomitans* were significantly inhibited at lower concentration (200 and 400 µg/mL) of chewing stick essential oil than with chlorhexidine, being *P. gingivalis* was the most susceptible. By comparison, our study showed that 100% ethanolic (0.025 and 0.125 mg/mL) extract has an equal antibacterial activity with 0.2% chlorhexidine gluconate solution. In other words, it has similar effectiveness with 0.2 % chlorhexidine in reducing the growth of *P. gingivalis* and *A. actinomycetemcomitans*. Although number of studies reported on fatty acid obtained from *S. persica* extract have antibacterial activity against *P. gingivalis*.

Table 1. Representative of GCMS analysis result in *S. persica* ethanol extract

Retention time	Compounds name	Formula	Molecular Weight	R. Index	% content
24.733	Octadecenoic acid, methyl ester	$C_{16}H_{32}O_2$	256.4241	940	45.90
24.425	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4241	990	26.30
26.000	Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester	$C_{20}H_{40}O_2$	312.5304	1036	1.32
26.017	Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester	$C_{20}H_{40}O_2$	312.5304	1043	1.33
28.650	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.4879	1089	12.85
28.792	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.4879	1099	11.15
30.675	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	268.4348	1145	1.03
30.792	10-Undecenoic acid, octyl ester	$C_{19}H_{36}O_2$	296.4879	1178	1.65
30.800	9-Octadecenoic acid (Z)-, hexadecyl ester	$C_{34}H_{66}O_2$	506.8866	1189	14.58

Table 2. Representative of LC-MS analysis results in *S. persica* aqueous extract

Retention time	Formula	ION	MS/MS	Identification
14.88	$C_{12}H_{20}$	$[M+H]^+$	196.286, 197.12	(2Z,8Z)-2,8-Dodecadienoic acid
17.425	$C_{13}H_{26}$	$[M+H]^+$	182.346, 184.00	Cyclotridecane
18.98	$C_{20}H_{34}$	$[M+H]^+$	3365.25, 94.508,	1-(2,2-Dimethylpropanoyl)-L-prolyl-L-prolyl-N,2-dimethylalaninamide
25.31	$C_{14}H_{11}F$	$[M+H]^+$	295.304, 296.90, 365.2960	2-({4-[(Difluoromethyl)sulfanyl]phenyl}amino)benzoic acid

Table 3. MIC and MBC values of *S. persica* extract against *P. gingivalis*

Samples	MIC	MBC
Ethanol extract	0.025 ± 7.22x10^{-3} mg/mL	0.025 mg/mL
Aqueous extract	> 200 mg/mL	>200 mg/mL
Chlorohexidine	7.8125x10^{-4} ± 4.5x10^{-4} %	7.8125x10^{-4} ± 4.5x10^{-4} %

Table 4. MIC and MBC values of *S. persica* extract against *A. actinomycetemcomitans*

Samples	MIC	MBC
Ethanol extract	0.125 ± 7.22x10^{-3} mg/mL	0.125 mg/mL
Aqueous extract	12.5 ± 3.61 mg/mL	12.5 mg/mL
Chlorohexidine	1.5625x10^{-3} ± 4.5x10^{-4} %	1.5625x10^{-3} ± 4.5x10^{-4} %

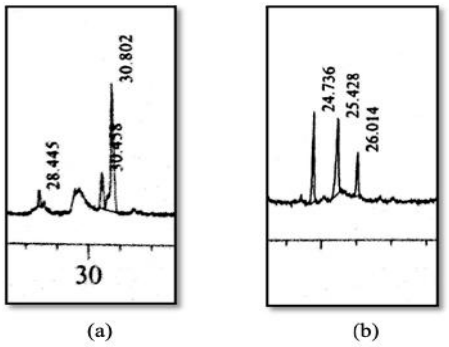

Figure 1. A representative of GC-MS chromatogram analysis showed (a) 9-Hexadecenoic acid, methyl ester, (Z)- (Time peak = 30.675) and(b) Octadecenoic acid, methyl ester (Time peak = 24.733) in *S. persica* ethanol extract.

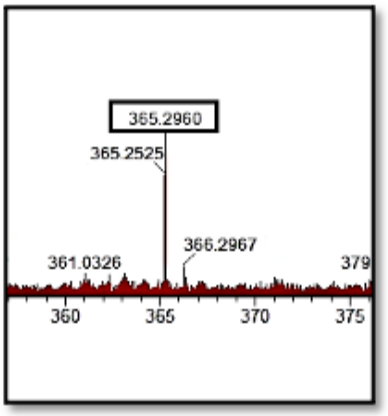

Figure 2. A representative of LC-MS analysis chromatogram showed the compound of 1-(2,2-Dimethylpropanoyl) -L-prolyl-L-prolyl-N, and 2-({4-[(Difluoromethyl) sulfanyl] phenyl} amino) benzoic acid in *S. persica* aqueous extract.

III. CONCLUSION

The major phytochemical compound found from ethanolic extract of *S. persica* was octadecanoic acid (fatty acid) and Hexadecenoic acid. *S. persica* ethanol extract has bactericidal properties against *P. gingivalis* and *A. actinomycetemcomitans* at 0.025 and 0.125 mg/mL, respectively. *S. persica* ethanol extract also found to have an equal antibacterial activity as a 0.2% chlorhexidine gluconate solution in vitro. Therefore, this extract can be potentially suggested as an alternative antibacterial agent to chlorhexidine gluconate solution.

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