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The antibacterial effect of *Plectranthus scutellarioides* (L.) R.Br. leaves extract against bacteria associated with peri-implantitis

Nor Amiyah Bismelah [a], Rohana Ahmad [a, b, *], Zethy Hanum Mohamed Kassim [a], Nor Hadiani Ismail [c], Nurulfazlina Edayah Rasol [c]

[a] *Centre of Restorative Dentistry Studies, Faculty of Dentistry, Universiti Teknologi MARA Sg. Buloh Campus, Jalan Hospital, 47000, Sungai Buloh, Selangor, Malaysia*
[b] *Integrative Pharmacogenomics Institute (iPromise), Universiti Teknologi MARA, Puncak Alam Campus, 42300, Bandar Puncak Alam, Selangor, Malaysia*
[c] *Atta-ur-Rahman Institute for Natural Product Discovery (AuRins) Universiti Teknologi MARA, Puncak Alam Campus, 43600 Bandar Puncak Alam, Selangor, Malaysia*

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ABSTRACT

Background and aim: The present study investigates *Plectranthus scutellarioides* (L.) R.Br. as potential antibacterial oral rinse against bacteria associated with peri-implantitis to prevent the initial infection as well as disease progression.
Experimental procedure: Phytochemical screening was done on *P. scutellarioides* lyophilized extract to identify the presence of chemical constituent by using mass-based identification. The extract was screened for its antibacterial activity against 4 Gram-positive aerobes (early colonizer) and 5 Gram-negative facultative anaerobes as well as obligate anaerobes (late colonizer) using disc diffusion method. The extract was tested for minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), its cytotoxicity effects on human gingival fibroblast cell (HnGF) as well as bacteria morphological changes by scanning electron microscopy (SEM).
Results and conclusion: Four flavonoid compounds were identified namely quercetin-3-glucoside, quercitrin, quercetin 3-(6″-acetylglucoside) and quercetin 3-*O*-acetyl-rhamnoside. The sensitivity test revealed that *P. scutellarioides* extract was effective against all the bacteria tested. MIC concentrations for the Gram-positive aerobes were in the range of 1.56–12.50 mg/mL, and the MBC concentrations were within 3.13–12.50 mg/mL. For Gram-negative obligate anaerobes, the MIC concentration were within 3.13–12.50 mg/mL and MBC within 6.25–200.00 mg/mL. The ethanolic extract did not have any cytotoxic effect on HnGF cells at the tested concentrations. SEM images showed bacterial cell wall disruption for all the bacteria tested. The results showed that *P. scutellarioides* extract exerts its antibacterial property by disrupting the cell wall of all the bacteria tested. Hence, *P. scutellarioides* may benefit from further investigations on its safety for oral use as an adjunctive treatment for peri-implantitis.

1. Introduction

Dental implants have been widely used to replace missing teeth due to their high success rates of above 95% over 10 years.[1,2] Despite the remarkable success rates, biological complications such as peri-implantitis occur at an incidence rate of 10–40%.[3] Peri-implantitis is characterized by the destruction of supporting mucosa and alveolar bone as an adverse outcome of bacterial propagation around the implants. The biofilm formation is similar to that of natural teeth, involving both early and late bacterial colonizers.[4] The early colonizers of dental plaque in the first few days are essentially composed of Gram-positive bacteria that are mostly in cocci form. The population then becomes more complex, shifting progressively to a large Gram-negative community with the presence of rods, filamentous organisms, vibrio and spirochetes, and

* Corresponding author. Centre of Restorative Dentistry Studies, Faculty of Dentistry, Universiti Teknologi MARA Sg. Buloh Campus, Jalan Hospital, 47000, Sungai Buloh, Selangor, Malaysia.
E-mail address: drrohana@uitm.edu.my (R. Ahmad).
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List of abbreviations

MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
HnGF	Human Gingival Fibroblast
SEM	Scanning Electron Microscopy
UPM	Universiti Putra Malaysia
UHPLC	Ultra High Performance Liquid Chromatography
RRHD	Rapid Resolution High Definition
ESI	Electrospray Ionization
BHI	Brain Heart Infusion
HCL-	Hydrochloric acid
CLSI	Clinical and Laboratory Standard Institute
NC	Negative Control
OD	Optical Density
MIC_{50}	Minimum Inhibitory Concentration of 50%
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
PBS	Phosphate Buffer Saline
SDS	Sodium Dodecyl Sulphate
IC_{50}	Inhibitory Concentration of 50%
HMDS	Hexamethyldisilane
TIC	Total Ion Chromatogram
ANOVA	Analysis of Variance
LD_{50}	Median Lethal Dose of 50%

organizing into a biofilm and thus increasing the virulence of the microbes.[5]

The established biofilm is often removed through a combination of scaling and polishing and oral rinsing with chlorhexidine digluconate. However, both methods carry some risks.[6–10] Scaling may scratch the implant surfaces, which promotes further adherence of bacteria, and chlorhexidine produces unpleasant taste and brown staining of the teeth, which can only be removed by scaling.[11] An anti-discoloration system was developed to overcome the staining problem, but it reduces the chlorhexidine's bactericidal effects.[12] Chlorhexidine has been reported to cause cell death through apoptosis at lower concentrations (0.002%) and induced fibroblast necrosis at higher concentrations.[13] Chlorhexidine digluconate has also been found to promote apoptosis by disrupting mitochondrial function, increasing intracellular calcium ions and inducing oxidative stress on the viability of cell lines of osteoblasts, fibroblasts and endothelial cells.[14] Hence, an alternative antibacterial oral rinse without these damaging side effects is required.

The present study investigates *Plectranthus scutellarioides* (L.) R.Br. (syn. *Coleus blumei*) as a potential antibacterial oral rinse against both the early and late colonizers in peri-implantitis. *P. scutellarioides* belongs to the Lamiaceae family with the genus plectranthus and species *P. scutellarioides*. It is known as 'ati-ati' in Malay or as 'painted nettle' in English *P. scutellarioides* has often been referred to as a decorative plant due to its variety of bright colours[15] and shapes of the leaves. However, the plant has also been used as a traditional medicine. Particularly in Indonesia, *P. scutellarioides* has been used either in a fresh, boiled or infused form to treat ailments like blain, abscess, ulcers and inflammation in the ear or eye.[16] The root of the plant has been used to treat diarrhea and stomach pain as well as for diabetes mellitus, constipation, fever, and dysmenorrhea.[17] The Toraja ethnic group in Indonesia uses the leaves to treat pulmonary tuberculosis.[18] This exemplifies the importance of this plant for its medicinal values.

Hence, the objective of this study was to determine the chemical contents of the purple variant of *P. scutellarioides* extract and to test the crude extract for its antibacterial activity against early colonizers, which include *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus salivarius*, and *Streptococcus oralis* as well as the late colonizers, that include *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia* and *Treponema denticola*. The extract was also tested for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) as well as for its cytotoxicity effects on human gingival fibroblast cell. The morphological changes of the bacteria were subsequently examined under scanning electron microscope to the surface structure of the bacteria post-exposure to the *P. scutellarioides* extract. The plant was chosen as the subject for this study due to the fact that it is commonly used as a local traditional medicine although the practice lacks scientific evidence to support its usage, especially as an antibacterial agent that if present can further be tested for application in Dentistry.

2. Materials and methods

2.1. Plants materials and extraction of P. scutellarioides leaves

One kilogram of purple variant *P. scutellarioides* leaves were freshly collected from Taiping, Perak in the early year of 2015. The identification of the plant was conducted at the Institute of Bioscience, Universiti Putra Malaysia (UPM), following which a voucher specimen (MFI 0063/19) was deposited at the herbarium. The dried leaves were grounded into fine powder by using a mechanical blender (Panasonic, MX-377). One hundred grams of the powdered plant material was then soaked in 200 mL of 70% ethanol. The plant material was extracted using ultrasonic extraction in a sonicator for 30 min, and subsequently concentrated in a rotary evaporator at 45 °C temperature under reduced pressure. The extract was later lyophilized by using a freeze dryer to yield 15 g of crystallized extract.

2.2. Phytochemicals screening

Phytochemical screening was done on the crude extract to identify the presence of chemical constituents by using mass-based identification. The mass spectra of the extract were recorded using Q Exactive Focus Orbitrap Mass Spectrometer, equipped with Thermo Scientific Ultimate 3000 UHPLC system (Thermo Scientific, United States). A total of 5 μL of the sample was eluted onto Zorbax RRHD Eclipse Plus-C18 (2.1 × 150 mm, 1.8 μm) (Agilent, United States) column under a gradient elution system of deionized water (A) and acetonitrile (B) with the addition of 0.2% formic acid. The solvent was eluted at a flow rate 0.30 mL/min under a gradient system of 20–60% of B in 8 min, followed by 60–100% of B in 2 min and 100% of B in 5 min. The sample was ionized in the positive mode in the range of *m/z* 120–1000 with dual electrospray ionization (ESI) operated at a sheath gas flow rate 45 arb (capillary temperature of 320 °C). The data acquisition and processing were performed using Thermo Xcalibur Qualitative software (Waltham, Massachusetts). Xcalibur is a complete quantitative and qualitative analysis software package that enables users to acquire data specifically for analytes of interest, to perform confirmatory library searches, and to determine the concentration of analytes in the samples. However, in this study, only qualitative analysis was done to identify the compounds based on mass. The chemical

constituents were identified by comparing the mass data of the crude sample with the available mass data of the compounds from similar genus. The chemical structures of the 4 compounds were illustrated using ChemDraw Ultra 12.0 (PerkinElmer, Waltham, Massachusetts).

2.3. Microbial culture

A total of 9 oral microorganisms were employed in the study including 4 Gram-positive bacteria (early colonizer) and 5 Gram-negative, facultative or obligate anaerobes (late colonizer) which have been associated with peri-implantitis. Gram-positive bacteria included *Streptococcus mitis* (NCIMB 13770), *Streptococcus salivarius* (ATCC® 13419™), *Streptococcus oralis* (ATCC® 6249™) and *Streptococcus sanguinis* (ATCC® 10556™). The Gram-negative, facultative or obligate anaerobes included *Aggregatibacter actinomycetemcomitants* (ATCC® 700685™), *Porphyromonas gingivalis* (ATCC® 33277™), *Treponema denticola* (ATCC® 35405™), *Prevotella intermedia* (ATCC® 25611™) and *Tanerella forsynthia* (ATCC® 43037™).

Streptococcus mitis, *Streptococcus salivarius*, *Streptococcus oralis* and *Streptococcus sanguinis* were cultured and maintain on Brain Heart Infusion (BHI) agar (BD BBL™). *Streptococcus mitis* and *Streptococcus salivarius* were incubated in aerobic condition for 24 h, whereas *Streptococcus oralis* and *Streptococcus sanguinis* were incubated at 37 °C under anaerobic conditions for 72 h (3 days) by using an anaerobic gas jar with anaerobic gas pack (Oxoid™ AnaeroGen™ 2.5 L). *Aggregatibacter actinomycetemcomitants*, *Porphyromonas gingivalis*, *Treponema denticola*, *Prevotella intermedia*, and *Tanerella forsynthia* were cultured on BHI agar enriched with supplements and nutrients such as hemin (Sigma Aldrich, United States), menadione (Vitamin K1) (Sigma Aldrich, United States) and L-cysteine- HCL (Sigma Aldrich, United States). The obligate anaerobes were handled in a glove box chamber in an anaerobic environment (85% N_2: 10% CO_2: 5% H_2), and incubated at 37 °C under anaerobic conditions for 3–7 days.

2.4. Bacterial suspension

The bacterial suspension was prepared in two different manner.[19] For antibacterial screening, the bacterial suspension was adjusted to approximately 1×10^8 cells/mL (McFarland 0.5 standard). Briefly, one or two colonies of the bacteria from the agar plate were transferred into a test tube containing Brain Heart Infusion (BHI) broth (BD BBL™) using a sterile wire loop. The turbidity of the bacteria was then adjusted using a spectrophotometer (Manufacturer's info) at a wavelength of 625 nm at 1-cm path, with absorbance in the range of 0.08–0.1. For Minimum Inhibitory Concentration (MIC), bacterial suspension was standardized to approximately 1×10^8 cells/mL. Later, a serial of tenfold dilution was done to achieve 1×10^6 cells/mL, by adding diluted bacteria suspension (0.1 mL) into 0.9 mL of fresh BHI broth.[19] For obligate anaerobes, the bacterial suspension was prepared using enriched BHI broth with supplements and nutrients such as hemin, menadione (Vitamin K1) and L-cysteine-HCL under anaerobic condition.

2.5. Anti-bacterial test

2.5.1. Anti-bacterial screenings

The antibacterial screening was carried out using the disc diffusion method. Empty discs (Oxoid) were soaked in 10 μL of *P. scutellarioides* extract at two different concentrations, of 100.00 mg/mL and 200.00 mg/mL. Antibiotic discs, penicillin (10 μg), doxycycline (30 μg), erythromycin (15 μg), tetracycline (30 μg) and amoxicillin (30 μg), as well as discs impregnated with 10 μL of 0.12% chlorhexidine digluconate mouth rinse (Oradex, Cavico (M) Sdn. Bhd, Malaysia) acted as positive controls, while discs impregnated with 10 μL of distilled water acted as the negative control. All the discs were then placed on pre-inoculated BHI agar containing respective bacteria cultures and left for incubation at 37 °C under aerobic and anaerobic conditions with appropriate incubation time for the respective bacteria. The diameter of the inhibition zones around all the discs was subsequently measured and recorded.[20]

2.6. Determination of minimum inhibitory concentration (MIC)

2.6.1. Broth dilution method

Minimum inhibitory concentration (MIC) was determined by the microdilution method using 96 well microplates, as described by the Clinical and Laboratory Standards Institute (CLSI), with slight modifications.[21] A 100 mg/mL of the extract solution was diluted in the Brain Heart Infusion (BHI) broth in two-fold serial dilutions to obtain concentrations from 0.1 mg/mL-100 mg/mL, at a total volume of 100 μL per well in 96 well microtiter plates. For facultative or obligate anaerobes, 200 mg/mL of the extract solution was diluted, with final concentrations ranging from 0.2 mg/mL-200 mg/mL. Each tested strain (50 μL) at a final concentration of 1×10^6 cells/mL was added to each well and incubated at 37 °C in appropriate conditions and time. A negative control (NC) consisted of wells that contained only the broth and the bacteria suspension (non-treated).

Another series of similar dilution of the plant extract with the same concentration was done separately to avoid the interference of the plant extract colour in the results. The plates were also incubated in a similar condition as the MIC plates. After incubation, the absorbance of each of the plate were then measured at an optical density of 600 nm (OD_{600}). MIC was defined as the lowest concentration of the extract that inhibit the growth of the bacteria in comparison with the non-treated control. However, in this study, MIC was determined in the form of percentage of inhibition, notably known as MIC_{50}. MIC_{50} was defined as the required concentration to inhibit 50% of growth of the organisms. The MIC_{50} can be calculated by using the formula below:

$$OD600\,of\,NC\,wells - OD600\,of\,the\,test\,wells / OD600\,of\,the\,NC\,wells \times 100$$

Hence, the wells that showed a 50% reduction in the number of colonies was determined as MIC_{50}. Minimum bactericidal concentration (MBC) was defined as the lowest concentration at which bacterial growth does not occur. A 10 μL of the well contents that showed MIC were plated on agar and grown at 37 °C in appropriate conditions. The plant extract was considered to have bactericidal effect if there was no growth of the bacteria. The extract was considered bacteriostatic if there was bacterial growth recorded.[20]

2.7. MTT cytotoxicity assay

Cells with a concentration of 1×10^5 were seeded into 96 microplates and incubated for 24 h in 5% CO^2 at 37 °C. After 24 h, each well was added with 100 μL of *P. scutellarioides* extract with a concentration ranging from 0.20 to 200.00 mg/mL and incubated again for the next 72 h. Maximum incubation time of 72 h was chosen because beyond this duration, the viability of HGnF decreases (<100%) as the cells enter stationary phase. At the end of incubation, the culture medium was removed, and cells were washed with 100 μL of phosphate-buffered saline (PBS). MTT assay

[3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was performed with incubation of 4 h in 5% CO2 at 37 °C. The reaction was stopped by using the MTT stopper reagent that consists of 10% of sodium dodecyl sulphate (SDS) in 0.1 N hydrochloric acid (HCl) and following this, the plate was incubated overnight at 37 °C. Cell viability was determined using a microplate reader (Tecan, Infinite® M1000 PRO, Männedorf, Switzerland) at an absorbance of $\lambda = 595$ nm. Then, data were then converted into a percentage of cell viability[22] using the following formula:

$$\textit{Cell viability}\ (\%) = 1 - \textit{OD of test wells}/\textit{OD of control wells} \times 100$$

From the cell viability results, IC_{50} were determine. Inhibitory concentration of 50% or also known as IC_{50}, is define as the concentration of drug which exhibited 50% of the cell viability and is used to determine the toxicology of a given drug *in vitro*. The concentration of the drugs tested will be considered as toxic if the IC_{50} value is lower than 50% (<50%) and non-toxic if the IC_{50} is higher than 50% (>50%).

2.8. Scanning electron microscopy (SEM) evaluation

The SEM was performed as reported by Yenugu et al. (2004) and Agizzio et al. (2006) with slight modifications.[23,24] All the bacteria tested were grown in BHI agar at 37 °C under aerobic and anaerobic conditions in the respective period of time for each bacterium to grow. After the bacteria have grown, one or two colonies were then used to make a bacterial suspension in BHI broth and were then incubated again for 24–48 h. MIC concentration for each of the bacteria was prepared accordingly and added into the suspension and was further incubated for 24–48 h. The bacteria suspensions were then centrifuged at 10000×*g* for 10 min and the supernatant removed to obtain the pellet. The bacteria pellet was then fixed with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for a minimum of 1 h or overnight. The centrifugation was repeated twice. The pellet was then fixed with 1% osmium tetroxide prepared in phosphate buffer for 1 h at room temperature. After centrifugation, the pellet was suspended in distilled water before undergoing the dehydration process for 10 min using two rounds of 50%, 75%, 95%, and 100% ethanol and followed by two rounds of hexamethyldisilane (HMDS). The cells were then allowed to dry at room temperature before being mounted onto SEM stub and sputter-coated with gold. The samples were then examined under the SEM (Quanta 450 FEG, United States).

2.9. Statistical analysis

All experiments were done in triplicate and repeated three times. The results were expressed in means ± standard deviations. A repeated measures ANOVA Model was used to determine the mean difference between the variables involved in the antibacterial screening, with significance value accepted at $p < 0.05$. Repeated measures ANOVA Model was used for the statistical analysis as the present study design involve multiple measures of the same variable taken over two or more time periods. The statistical analysis was done by using SPSS Statistics Version 26 (IBM).

3. Results

3.1. Phytochemical screenings

The phytochemicals screening showed that a total of 4 flavonoids compounds have been identified with the mass error below 5 ppm as tabulated in Table 1. Total ion chromatogram (TIC) was shown in Fig. 1, which represents the summed intensity across the entire range of masses being detected at every point. The chemical compound structure of each of the compound have been illustrated in Fig. 2(A) and (B), (C) and (D).

3.2. Antibacterial sensitivity test

The sensitivity test revealed that *P. scutellarioides* possessed antibacterial activity at both concentrations tested (Table 2). The largest zone inhibition was seen in *S. sanguinis*, followed by *S. mitis*, *S. oralis* and lastly *S. salivarius*. *P. scutellarioides* extract was most effective against *S. sanguinis* and *S. oralis* at 200 mg/mL concentrations compared to Oradex. However, both antibiotics showed the largest zone of inhibition against all the tested bacteria.

For the facultative anaerobic bacteria, *P. scutellarioides* extract was most effective against *A. actinomycetemcomitans*, where both concentration tested showed inhibition zones. However, the rest of the anaerobes does not show any zone of inhibition at 100.00 mg/mL concentration instead the zone of inhibition can only be seen at the concentration of 200.00 mg/mL. Hence, higher concentration of the extract is required to exhibit its antibacterial activity on obligate anaerobes. *P. scutellarioides* extract was most effective against *T. forsythia*, followed by *P. gingivalis*, and *P. intermedia*. The extract was less effective against *T. denticola*. Oradex and antibiotics were seen to be more effective with higher zone of inhibition.

For aerobes, there was a statistically significance difference between the groups as determined by the repeated measures ANOVA Model. However, *S. mitis* and *S. oralis* showed no significance difference between *P. scutellarioides* extract at concentration of 200.00 mg/mL and Oradex. *S. sanguinis* also did not showed any significance difference between extract at concentration of 100.00 mg/mL and Oradex. As for obligates anaerobes, the results showed statistically significance difference between the groups except for *T. forsythia* and *P. intermedia* that exhibit no significant difference between *P. scutellarioides* extract at concentration of 200.00 mg/mL and Oradex as shown in Table 3.

3.3. Determination of MIC and MBC

Based on the antibacterial sensitivity results, MIC was tested with an initial concentration of 100.00 mg/mL for aerobes and *A. actinomycetemcomitans*, whereas for the rest of the obligate anaerobes, the initial concentration for MIC was at 200.00 mg/mL. *P. scutellarioides* (Table 4). Looking at the aerobic bacteria, the extract was most effective against *S. oralis* (MIC = 1.56 mg/mL, MIC_{50} = 61% of inhibition), followed by *S. mitis* (MIC = 6.25 mg/mL, MIC_{50}= 79% of inhibition), *S. sanguinis* (MIC = 6.25 mg/mL, MIC_{50}= 66% of inhibition), and *S. salivarius* (MIC = 12.50 mg/mL, MIC_{50}= 60% of inhibition). *P. scutellarioides* extract was most bactericidal against *S. oralis* at 3.13 mg/mL concentration, followed by *S. sanguinis* at 6.25 mg/mL, *S. salivarius* at 12.50 mg/mL and *S. mitis* at 25.00 mg/mL.

For facultative or obligate anaerobes, *P. scutellarioides* extract was most effective against *A. actinomycetemcomitants*, (MIC of 3.12 mg/mL with 54% of inhibition), followed by *P. intermedia* (MIC = 6.25 mg/mL, MIC_{50}= 61% of inhibition), *T. forsythia* (MIC = 6.25 mg/mL, MIC_{50}= 53% of inhibition), *P. gingivalis* (MIC = 12.50 mg/mL, MIC_{50}= 60% inhibition) and *T. denticola* (MIC = 12.50 mg/mL, MIC_{50}= 62% of inhibition). *P. scutellarioides* extract was most bactericidal against *A. actinomycetemcomitants* at 6.25 mg/mL, followed by *P. intermedia* at 12.50 mg/mL, *P. gingivalis* at 25.00 mg/mL, *T. forsythia* at 100.00 mg/mL and *T. denticola* at 200.00 mg/mL.

Table 1
Chemical composition of the ethanol extracts of *P. scutellarioides* leaves.

No	Retention time (RT)	Compound Identification (ID)	Experimental mass $[M+H]^+$	Mass error (ppm)
1.	2.05	Quercetin-3-glucoside	463.0877	−0.43
2.	2.61	Quercitrin	447.0927	−1.39
3.	2.89	Quercetin 3-(6″-acetylglucoside)	505.0985	0.98
4.	3.20	Quercetin 3-*O*-acetyl-rhamnoside	489.1035	−1.22

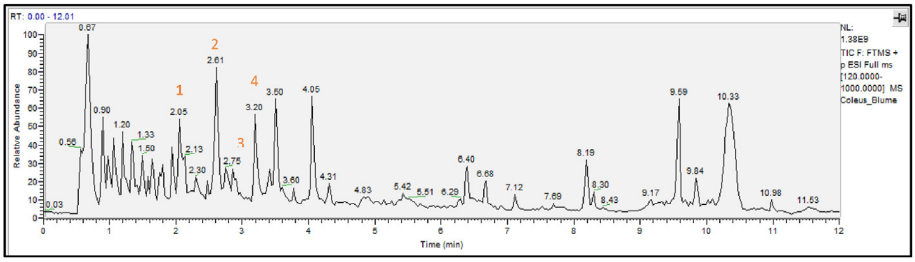

Fig. 1. Total ion chromatogram (TIC), ionized at the positive mode in the range of *m/z* 120–1000. A total of 4 compounds were detected at the respective retention time (RT).

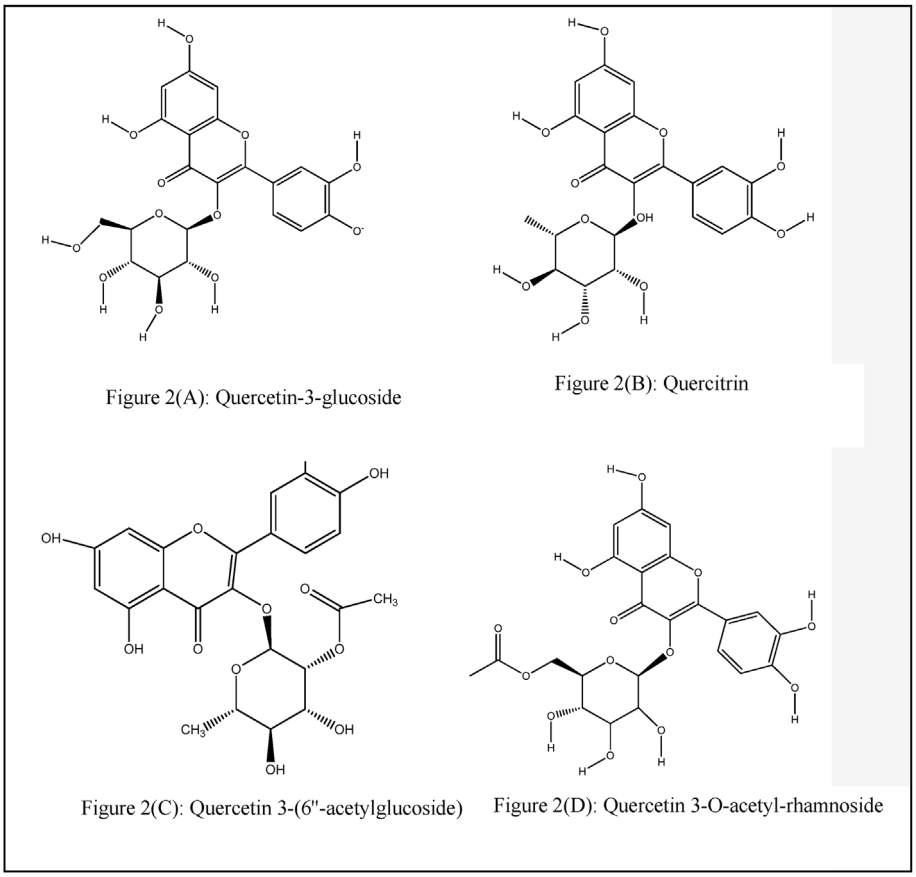

Fig. 2. Chemical compounds of the 4 derivatives of quercetin identified in P. scutellarioides extract namely 2(A) Quercetin-3-glucoside, 2(B) Quercitrin, 2(C) Quercetin 3-(6″-acetylglucoside) and 2(D) Quercetin 3-*O*-acetyl-rhamnoside.

3.4. Cytotoxicity assay

Fig. 3 shows the percentage of cell viability of human gingival fibroblast cells after 72 h of exposure to *P. scutellarioides* extract at various concentrations of between 1.56 and 200.00 mg/mL. The concentrations chosen for the cytotoxicity study is based on the MIC results. Based on Fig. 3A, the highest extract concentration of 200 mg/mL produces the lowest cell viability of 57% whereas the lowest extract concentration of 1.56 mg/mL produces the highest cell viability of 69%. Fig. 3B shows that at all concentration, the plant extract does not result in a drop of cellular viability up to a threshold value of 50% (IC_{50}).

Table 2
Inhibition zone (mm) of ethanol extracts of *P. scutellarioides*, Oradex, antibiotics and distilled water against bacteria associated with peri-implantitis.

	Extract (100 mg/mL)	Extract (200 mg/mL)	Oradex	Penicillin	Doxycycline	Amoxicillin	Tetracycline	Erythromycin	Distilled water
				Zone of Inhibition (mm)					
S. mitis	16.0 ± 2.8	19.0 ± 2.2	19.0 ± 1.7	40.0 ± 6.4	N/A	N/A	N/A	N/A	0.0
S. oralis	16.0 ± 1.7	20.0 ± 2.7	19.0 ± 1.7	40.0 ± 0.0	N/A	N/A	N/A	N/A	0.0
S. salivarius	11.0 ± 1.6	14.0 ± 1.1	27.0 ± 2.5	N/A	N/A	N/A	N/A	40.0 ± 0.0	0.0
S. sanguinis	19.0 ± 1.7	22.0 ± 2.6	20.0 ± 0.0	41.0 ± 2.2	N/A	N/A	N/A	N/A	0.0
A. actinomycetemcomitans	13.0 ± 2.3	22.0 ± 3.2	28.0 ± 3.6	N/A	44.0 ± 6.8	N/A	N/A	N/A	0.0
P. gingivalis	0.0	13.0 ± 2.4	19.0 ± 2.0	N/A	N/A	N/A	N/A	28.0 ± 2.5	0.0
T. forsythia	0.0	14.0 ± 4.2	16.0 ± 1.7	N/A	N/A	N/A	33.0 ± 2.6	N/A	0.0
T. denticola	0.0	10.0 ± 8.7	16.0 ± 1.7	N/A	N/A	N/A	N/A	28.0 ± 3.5	0.0
P. intermedia	0.0	12.0 ± 2.6	15.0 ± 2.5	N/A	N/A	23.0 ± 3.0	N/A	N/A	0.0

*N/A – Not Applicable.

Table 3
Repeated Measures ANOVA of the mean difference of the size of the zone of inhibition between the groups.

	S. mitis	S. oralis	S. salivarius	S. sanguinis	A. actinomycetemcomitans	P. gingivalis	T. forsythia	T. denticola	P. intermedia
Comparison between groups					**p- value (<0.05)**				
Extract 100 mg/mL vs Extract 200 mg/mL	0.003[a]	0.004[a]	0.000[a]	0.020[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Extract 100 mg/mL vs Oradex	0.002[a]	0.001[a]	0.000[a]	0.122	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Extract 100 mg/mL vs Antibiotic	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Extract 200 mg/mL vs Oradex	0.154	0.920	0.000[a]	0.035[a]	0.014[a]	0.000[a]	0.359	0.000[a]	0.051
Extract 200 mg/mL vs Antibiotic	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]

[a] The mean difference is significant at the .05 level ($p < 0.05$).

Table 4
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. scutellarioides* extract.

	MIC (mg/mL)	MIC50	MBC (mg/mL)
S. mitis	6.25	79%	25.00
S. oralis	1.56	61%	3.13
S. salivarius	12.50	60%	12.50
S. sanguinis	6.25	66%	6.25
A. actinomycetemcomitans	3.12	54%	6.25
P. gingivalis	12.50	60%	25.00
T. forsythia	6.25	53%	100.00
T. denticola	12.50	62%	200.00
P. intermedia	6.25	61%	12.50

3.5. Evaluation of the bacteria morphologic changes via SEM

It was observed that for the aerobic bacteria, each of them showed an intact cell wall and well-defined membrane and cocci formation before exposure to the *P. scutellarioides* plant extract. When *S. mitis* was expose to 6.25 mg/mL of the plant extract, the bacteria cells of *S. mitis* showed morphological destruction with blisters and deep craters on the surface of the cells. At a magnification of 20000 ×, the bacteria cells were seen to be ruptured to form deep craters in their cell walls. The presence of numerous lysed cells and cellular debris was also evident (Fig. 4A). *S. oralis* have shown that, at the concentration of 1.56 mg/mL, the bacteria cells have been inhibited in the region with fewer bacteria appeared (Fig. 4B) compared to the untreated region. Besides that, the bacteria cells can also be seen to have multiple blisters on their surface before the ruptured state.

At a concentration of 6.25 mg/mL, *S. sanguinis* appeared as highly aggregated coccoid lenticular cells with constriction at the junction between the cells (Fig. 4C). Some cells appeared as distressed revealing cell membrane indentations. The extract reduced the cells' surface area that resulted in the cells becoming more elongated and appearing as a mixture of cocci and bacillus. *S. salivarius* did not exhibit any significant morphological changes and this agrees with the MIC and MBC results. However, the treated *S. salivarius* with 12.50 mg/mL MIC revealed the presence of blisters

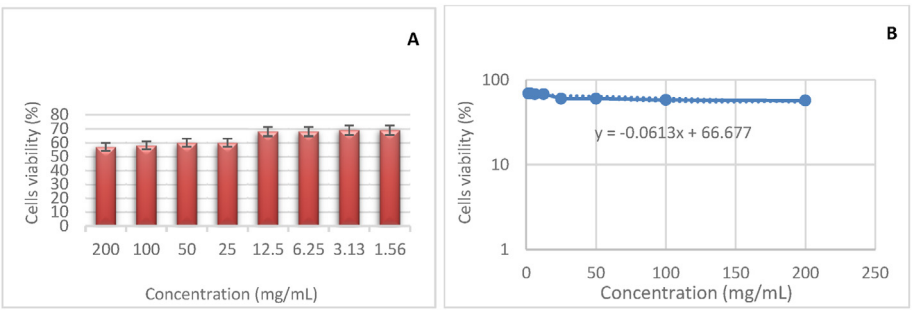

Fig. 3. (A) Cell viability of human gingival fibroblast cells after treatment with *P. scutellarioides* extract of different concentrations after 72 h. (A). The graph of the cell viability and (B) the scatter plot of the cell viability. The scatter plot shows no IC_{50}, thus the extract does not exhibit toxicity effects towards human gingival fibroblast cells.

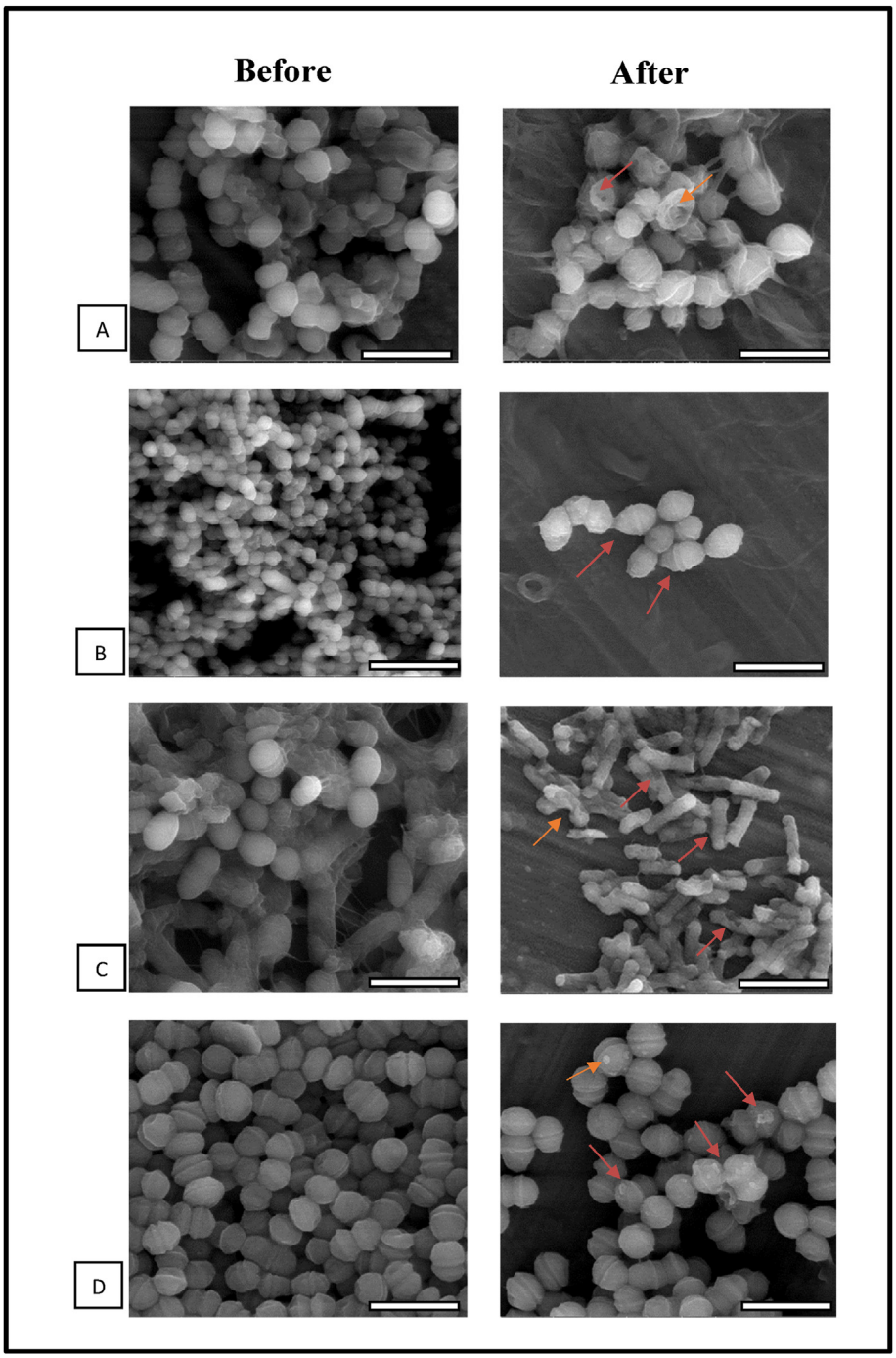

Fig. 4. SEM micrograph of before and after treatment with *P. scutellarioides* extract in (A) *S. mitis*, (B) *S. oralis*, (C), *S. sanguinis*, (D), *S. salivarius* at scale bar of 2 μm. Under 20000 × magnification, untreated bacterial cells remained intact and evenly distributed with no sign of morphological depression, whereas bacterial cells treated with *P. scutellarioides* extract depicted morphological disruption with blisters and deep craters on their surface (orange arrow).

(Fig. 4D) on the cells surfaces.

Before exposure of *P. scutellarioides* plant extract, *P. gingivalis*, *A. actinomycetemcomitans*, and *P. intermedia* appeared as small grape like clusters with bacillus and coccobacillus morphology revealing smooth cell surface as well as intact cell wall. *T. forsythia* appeared as larger fusiform rod and *T. denticola* was seen as the long thin spirochete. At the concentration of 12.50 mg/mL, treated *P. gingivalis* (Fig. 5A) underwent cell lysis that caused deformation of the cell's morphology. Treated *A. actinomycetemcomitans* at the concentration of 3.12 mg/mL appeared flat with complete loss of cellular structure indicating the presence of severe stress. The extract also induced cell elongation from its coccobacillus shape that can clearly be seen in Fig. 5B.

Treatment of *P. intermedia* (Fig. 5C) with the extract at the concentration of 6.25 mg/mL showed cell shrinkage and cell lysis with deformed cell walls. At similar concentration of 6.25 mg/mL, *T. forsythia* (Fig. 5D) appeared to have undergone morphological changes within the peripheral cell surfaces and cell disintegration. The cell surfaces exhibited precipitations resulting in deformed cell structures. *T. denticola* with MIC concentration of 12.50 mg/mL, also

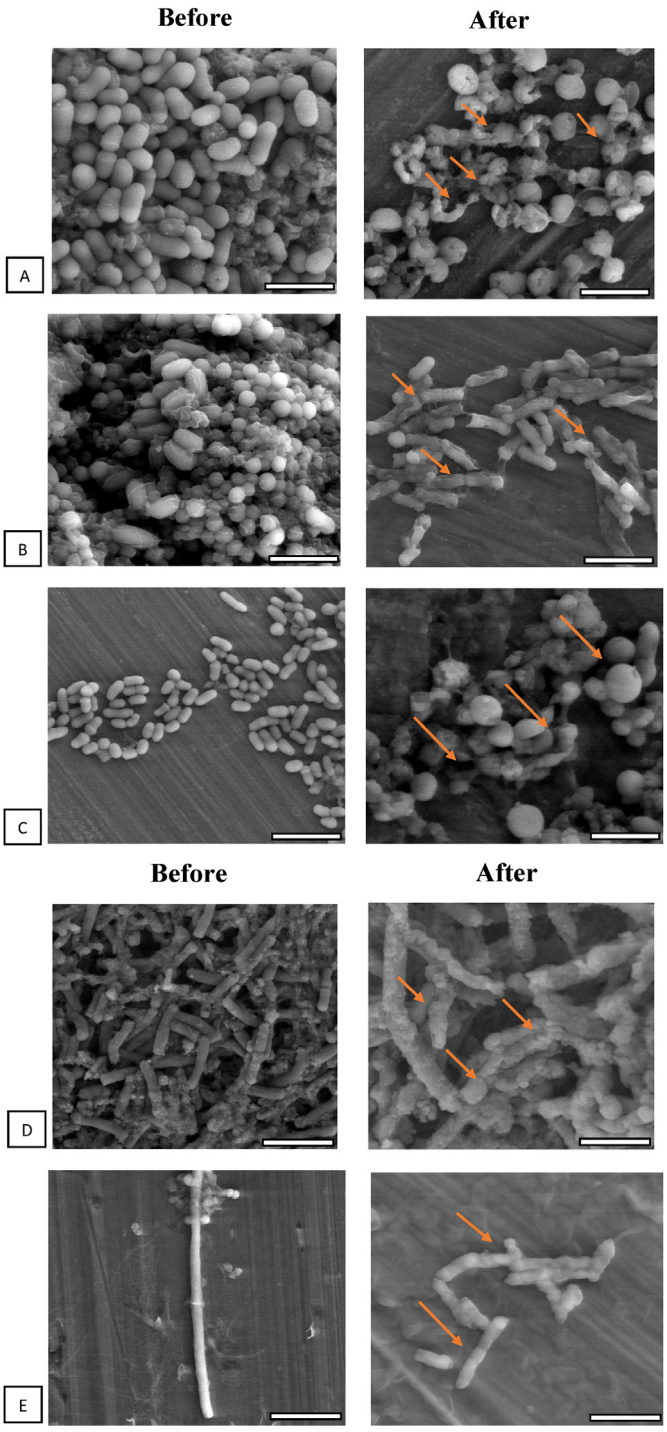

Fig. 5. SEM micrograph of before and after treatment with *P. scutellarioides* extract in obligate anerobes of (A) *P. gingivalis*, (B) *A. actinomycetemcomitans*, (C) *P. intermedia*, (D) *T. forsythia* and (E) *T. denticola* with scale bar of 3 μm. Under 20000 × magnification, untreated bacterial cells remained intact and evenly distributed with no sign of morphological depression. Cells treated with *P. scutellarioides* extract revealed deformed and shrunken cells (orange arrow).

exerted its effects on the cell morphology (Fig. 5E). After treatment, the cells appeared to have lost its spirochete shape by becoming shorter, disintegrated and hollow. Deformation of the outer cell walls can also be observed.

4. Discussions

P. scutellarioides that has been used as traditional medicine[16–18] has now been investigated in the present study for its potential application in Dentistry as an adjunctive treatment for peri-implantitis. The plant can be found in many colours and varieties and according to Osman, 2013 each of the plant variety have different genetic mutability that distinguish is from one another.[15] There were not much study conducted on its application as anti-bacterial agent and only a small fraction of studies focusses on the anti-inflammatory activity. Hence, the present study elaborate more on the antibacterial activity of *P. scutellarioides* ethanolic leaf extract from the purple variant.

Four compounds have been identified from the ethanolic extract of *P. scutellarioides*. The compounds detected were from the quercetin group, a kind of flavonoid that has been widely explored for its bioactivity to inhibit the Gram-positive and Gram-negative bacteria through the inactivation of extracellular proteins. The chemical constituents were not isolated and tested separately due to time and financial limitations. However, the isolation and characterisation of the compounds will be our main focus in future studies as the phytochemical screenings revealed interesting results in which these chemical constituents can be further isolated by adapting to the present methods.

Quercetin has been successfully detected in *P. scutellarioides* by Moektiwardoyo et al. (2010)[25] and Moektiwardoyo et al. (2011).[26] Moektiwardoyo et al. (2011)[26] have detected 0.05% of quercetin in methanol extract of *P. scutellarioides* by comparing with the quercetin reference standard. The differences between the results obtained by Moektiwardoyo et al. (2011)[26] and the present study is that to the best of our knowledge, we are the first to determine these derivatives of quercetin. Derivative can be defined as a compound that is formed from a similar compound or that can be conceptualize to formed from another compound when one atom is replaced with another atom or group of atoms.[27] Besides that, no optimal mass-based identification protocol has ever been developed for the crude extract using the mixture of ethanol and water as extraction solvent. Therefore, the protocol that we have developed in this study are reproducible and repeatable which can be used for future work.

The present study showed that the ethanolic extract of *P. scutellarioides* possessed antibacterial activity against the bacteria associated with peri-implantitis including both the early and late colonizers. For the early colonizers, the bacteria involved were from the Gram-positive viridan *Streptococci* groups that were aerobes and facultative anaerobes. The antibacterial activity test demonstrated that the extract showed the presence of the zone of inhibition at a starting concentration of 100.00 mg/mL, and the zone increased when the concentration was increased to 200.00 mg/mL. The extract also showed encouraging results for MIC and MBC. Overall, the extract inhibited the growth of bacteria at a low concentration of 1.56 mg/mL and was able to completely kill some of the bacteria at a minimal concentration of 3.13 mg/mL.

The late colonizer consisted of Gram-negative facultative and obligate anaerobes that are implicated in peri-implantitis. The obligate anaerobes can be categorized into red-complex (*P. gingivalis*, *T. forsythia* and *T. denticola*), orange-complex (*P. intermedia*) as well as aa complex (*A. actinomycetemcomitans*). Based on the antibacterial results, obligate anaerobes required higher concentration of plant extract to produce zone of inhibition compared to the aerobes except for facultative anaerobe, *A. actinomycetemcomitans*. The MIC and MBC were also higher for the anaerobic bacteria.

The major difference between the two groups of bacteria lie in the thickness of the cell wall and the presence of an outer

membrane that only exist in the Gram-negative bacteria. The bacteria cell wall ranges from 20 to 80 nm thick for gram-positive bacteria and between 1.5 and 10 nm thick for Gram-negative bacteria. The main element of the cell wall is peptidoglycan which can be found in almost all bacteria and is responsible for preserving the integrity of the cell. Cell lysis has often been associated with the impairment of peptidoglycan either through mutations or external stresses induced by foreign substances such as antibiotics.[28,29] Despite the thick cellular wall of the Gram-positive bacteria, they can still absorb antibiotics and cleaning products, making them easier to be eradicated. This explains the effectiveness of the plant extract against the Gram-positive aerobes.

The presence of the outer membrane that consists of phospholipids and lipopolysaccharides[28] affects the efficacy of certain antibiotic treatment for the Gram-negative bacteria. This extra membrane layer acts as an effective permeation barrier and retards the influx of antibiotic molecules into the bacterial cell. Besides that, for most of the periodontophatic bacteria, the presence of their virulence factors may contribute to the difficulties in treating the infection. *T. denticola*, *P. gingivalis* and *T. forsythia* were strongly characterized by their high proteolytic and peptidolytic activities[30–32] that cause antibiotic hydrolysis which led to the inactivation of their antimicrobial activity. This might have contributed to moderate effectiveness of the plant extract against the obligate anaerobes that can be observed in the susceptibility test for which high concentration of the extract was needed to produce antibacterial effects. *P. scutellarioides* extract was seen to be less sensitive towards *T. denticola* compared to other anaerobes, whereby bactericidal effect was seen at 200.00 mg/mL. In summary, *P. scutellarioides* can be used as an adjunctive treatment for peri-implantitis, due to its effectiveness against early colonizers which may preventearly infection from happening. The plant extract was also useful in inhibiting the late colonizers, thus may also be useful to prevent progression of infection.

The antibacterial activity of *P. scutellarioides* crude extract can be related to the presents of quarcetin, since this compound was detected in the crude extract itself. Quercetin has been reported to have antibacterial activity against caries-related bacteria as well as periodontitis-associated bacteria[33,34] such as *A. actinomycetemcomitans* and *P. gingivalis*. Shu et al. (2011) also studied the antibacterial effects of quercetin on 11 oral pathogens that includes *S. mutans*, *S. sanguinis*, *S. sobrinus*, *A. viscosus*, *A. naeslundii*, *L. acidophilus*, *P. gingivalis*, *F. nucleatum*, *A. actinomycetemocomitans*, *P. intermedia* and *C. albicans*. The antibacterial activity were expressed in MIC and MBC assay in which quercetin inhibited each of the bacteria tested at the following concentrations for *S. mutans* (MIC of 2 mg/mL and MBC of 8 mg/mL), *S. sobrinus* (MIC of 1 mg/mL and MBC of 8 mg/mL), *L. acidophilus* (MIC of 2 mg/mL and MBC of 16 mg/mL), *S. sanguinis* (MIC of 2 mg/mL and MBC of 16 mg/mL), *A. actinomycetemocomitans* (MIC of 1 mg/mL and MBC of 8 mg/mL), *P. intermedia* (MIC of 4 mg/mL and MBC of 16 mg/mL), respectively.[35,36]

Besides quercetin, there were also few compounds that have been isolated, identified and characterized from *P. scutellarioides* and reported to have antimicrobial properties. Ito et al. (2018) isolated, identified and characterized three new abietane type diterpenoids from the leaves of Indonesian *P. scutellarioides* namely Spiroscutelone A, spiroscutelone B, and spiroscutelone C.[37] A known abietane type diterpene, was also identified in the study by comparison of its mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) data with those reported in the literature.[38] The isolated diterpenes by Ragasa et al. (2001) were active against *B. subtilis*, *P. aeruginosa* and *C. albicans*.[38] Another two new uncharacterised abietane diterpenes were also successfully isolated from a methanol extract of *P. scutellarioides* alongside two known compounds.[39] The two new compounds were named as sincoetsin C and 3-hydroxyspirocoleon 7-*O*-β-D-glucoside, whereas the known compounds were scutellarioidone A and spirocoleon 7-*O*-β-D-glucoside. All of the compounds were tested against methicillin-resistant *Staphylococcus aureus* subsp. *aureus* CCM 4750 (MRSA) and the results showed that the compounds were found effective in treating MRSA 4750 activity at the concentration of 512 μg/mL. One of the new compounds isolated, Sincoetsin C showed the highest *anti*-MRSA 4750 activity with a minimum inhibitory concentration of 128 μg/mL.

The present findings on the effectiveness of *P. scutellarioides* extract against oral bacteria, agrees with previous studies reporting its antibacterial activity against oral pathogens namely *S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa* and *C. albicans*.[38,40,41] However, the lack of studies conducted on the antibacterial activity of the ethanolic leaf extract of *P. scutellarioides* on oral bacteria specifically on the early and the late colonizers of peri-implantitis makes it quite challenging to compare the present results with those in the existing literature. However, there were few researches that used the similar genus which is plectranthus to test its antibacterial efficacy against oral diseases. Arumugam et al. (2017)[42] investigated *P. vettiveroides* for anti-caries activity. It was shown to be most effective against *S. oralis, followed* by *S. mutans* and *L. acidophilus*. Susanth et al. (2014)[43] reported that *P. aromaticus* was effective against *S. aureus*, *E. coli*, *Pseudomonas* sp. and *Klebsiella* sp. The phytochemicals of plectranthus species have also been shown to exhibit antiviral, wound healing, anti-cancer, anti-hyperglycemic, anti-inflammatory, anti-parasitic and antioxidant properties.[44–48] Furthermore, the findings from previous studies were in company with the present results in which the plant extracted with alcohol-based solvent showed promising antibacterial effects and the plectranthus species were more effective against viridans streptococci. Similarly, the ethanolic extract of *P. scutellarioides* also were observed to have more promising effects on the early colonizers bacteria that consisted of the viridans streptococcus species.

In the present study, the *in vitro* cytotoxicity test determined the extract toxicity level on human gingival fibroblast cells (HGnF). The extract did not demonstrate cytotoxicity towards the HGnF cells after 72 h of incubation period. The time period of 72 h was considered a suitable time for the gingival fibroblast to have 70–80% of confluency.[49] This finding is in line with an *in vivo* toxicity study which showed no mortality in mice that received 1000–5000 mg/kg body weight of *P. scutellarioides* leaves extract after 24 h. Hence, the median lethal dose (LD_{50}) value of *P. scutellarioides* leaves extract was estimated to be above 5000 mg/kg body weight and that *P. scutellarioides* plant extract has no significant toxic effect at least in mice. Furthermore, there was neither death nor discernible gross pathological lesion seen in animals dosed with 1000–5000 mg/kg of the extract.[40]

The SEM results showed that all the bacteria treated with *P. scutellarioides* extract was morphologically disrupted resulting in the formation of blisters and deep craters in their cell walls. The morphological impairment might be due to the presence of active phytochemicals such as flavonoid, terpenoid, tannins and saponin. These phytochemicals have been detected in *P. scutellarioides* extract in a previous study by Bismelah et al. (2019).[41] Flavonoid has been reported to exert effects such as cytoplasmic membrane damage,[50,51] inhibition of nucleic acid synthesis and energy metabolism.[52,53] Tannins inhibit bacterial growth and protease activity by damaging the cell wall and cytoplasm, causing rapid structural destruction.[54] Terpenoids also have a similar mechanism of action as tannins by initiating membrane disruption.[55] Saponin, on the other hand, has been reported to inhibit bacterial adhesion[56] and increasing the permeability of bacterial cell walls.[57] The extract

was seen to exhibit a similar mechanism of action as chlorhexidine, whereby at a low concentration, chlorhexidine affects the integrity of the cell wall, eventually causing damage. Once the cell wall is damaged, chlorhexidine crosses into the cell itself and causes cytoplasm leakage that leads to cell death.[8–10] Chlorhexidine in high concentration causes the cytoplasm to congeal or solidify.[13,14]

5. Conclusion

Summing-up, the ethanolic extract of *P. scutellarioides* leaves contains 4 flavonoid compounds derived from quercetin groups, namely quercetin-3-glucoside, quercitrin, quercetin 3-(6″-acetyl-glucoside) and quercetin 3-*O*-acetyl-rhamnoside. This study is the first to identify these compounds in this variety. The ethanolic extract of *P. scutellarioides* leaf demonstrated antibacterial effects against the bacteria associated with peri-implantitis. The plant extract was most effective against the early colonizing bacteria, exhibiting bactericidal effects at low. However, higher concentration of the plant extract was needed for the bactericidal effects against the late colonizing bacteria. The extract was non-toxic towards HnGF cells suggesting its potential to be formulated into oral rinse as an adjunctive treatment for peri-implantitis. The SEM results suggested that the extract is capable of distorting the morphological structure of bacterial cells by causing cell wall damage.

Authors' contributions

Nor Amiyah Bismelah: Carried out the experiments, analysed the data and wrote the report. Rohana Ahmad: designed and supervised the experiments, preparing the projects funding as well as corrected the report. Nor Hadiani Ismail and Nurulfazlina Edayah Rasol: Supervised the mass-identification method, analysis and corrected the report. Zethy Hanum Mohamed Kassim: Read and corrected the report. All authors have read and approved the final manuscript.

Declaration of competing interest

All authors report no conflict of interest.

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