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Antibacterial Activity and Morphological Changes of *Peperomia Pellucida* Extract against *Staphylococcus epidermidis*

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Abstract

Peperomia pellucida has been used as traditional medicine to treat various diseases. This study aimed to evaluate antibacterial activity of *P. pellucida* against *Staphylococcus epidermidis*. *Peperomia pellucida* was extracted using three different solvents: n-Hexane, dichloromethane and ethanol. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using microbroth dilution method. Phytochemical analysis was conducted on the extract that exhibited the lowest MIC value using gas chromatography-mass spectrometry (GC-MS). The morphological changes of *S. epidermidis* with the lowest MIC value after treatment with the plant extract were observed under the scanning electron microscope (SEM) and transmission electron microscope (TEM). All extracts inhibited the growth of *S. epidermidis*, with ethanol extract demonstrated the most potent antibacterial activity (MIC value= 3.91 mg/mL) as compared to n-Hexane and DCM (MIC value=15.63 mg/ml). Phytochemical analysis of *P. pellucida* extract revealed 21 bioactive compounds that can be explored further for antibacterial property. The morphological analysis of SEM and TEM demonstrated that *P. pellucida* extract caused membrane disruption and organelle damage to *S. epidermidis*. This study showed the therapeutic efficacies of *P. pellucida* extract against *S. epidermidis* and further research that include elucidating other antibacterial mechanisms and testing to multidrug-resistant strain of this plant against *S. epidermidis* should be conducted.

Keywords

Peperomia pellucida, *Staphylococcus epidermidis*, antibacterial activity, electron microscope, natural product

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Introduction

Peperomia pellucida is a herbaceous plant under the genus of *Peperomia* and family of *Piperaceae*. It is small, herbaceous, and edible plant. Other than *P. pellucida*, there are nearly 1500 to 1700 other species under the *Piperaceae* family. *Peperomia pellucida* can be found in different part across the world including in the North and South America, Africa and Asia and in Malaysia, its local name is Ketumpang Air[1,2]. *Peperomia pellucida* plant parts that include leaf and stem have been widely consumed by locals in those regions for various alternative treatments that include conjunctivitis, gout, rheumatic pains, skin diseases and high blood cholesterol level[3].

Peperomia pellucida extracts using different solvents have been tested on many microorganisms. Leave extracts of different solvents of *P. pellucida* were shown to possess antibacterial activities on both Gram positive and Gram negative bacteria and fungi[4]. Another study described that antibacterial activity of the plant were more effective towards Gram negative than Gram positive bacteria[5]. Later on, another research revealed that n-Hexane extract of *P. pellucida* had the most potent antibacterial activity to both Gram-positive and Gram-negative bacteria compared to methanol and ethyl acetate extract[1].

Staphylococcus genus consists of many species of Gram-positive bacteria that colonise the skin and mucous membranes of humans and most mammals[6,7]. This genus includes different types of coagulase negative *Staphylococcus* and one of them is *S. epidermidis*. *S. epidermidis* is an opportunistic pathogen that is frequently isolated from human epithelia and resistant to multiple antibiotics[8,9]. This pathogen causes mainly skin and soft tissue infection and life-threatening bacteremia and sepsis if it gets into human's bloodstream. The ability of this bacterium to form biofilm on medical devices such as indwelling catheter poses significant health threat to immuncompromised patients[10]. The emergence of multi-drug resistant bacteria has led to urgency in discovery of new antibiotics using natural products from plants that can be used to treat bacterial infections[11,12]. Recent study conducted in Malaysia reveals that *S. epidermidis* is resistant to multiple essential antibiotics that are commonly prescribed to treat bacterial infection[8]. Given the alarming rate of antibiotic-resistant *S. epidermidis*, it is important to find alternative therapy that includes natural products as new therapy against this bacterium.

Peperomia pellucida has unexplored antibacterial activity against multiple bacteria. The plant possesses antibacterial activity as evaluated using standard antibacterial activity evaluation in culture, namely MIC and MBC. Nevertheless, research and discovery of this plant as new alternative therapy against *S. epidermidis* is lacking. Furthermore, the mechanism of the inhibitory action of *P. pellucida* against *S. epidermidis* is lacking and has not been firmly established. Thus, examining bacterial morphological changes after treatment with *P. pellucida* extract is crucial for understanding its antibacterial mechanism [13,14]. The objective of this study was to evaluate the antibacterial activity of *P. pellucida* extracts against *S. epidermidis*. We report the antibacterial activity, phytochemical analysis, and morphological changes of *S. epidermidis* after treatment with *P. pellucida* extract.

Materials and Methods

Plant Material and Plant Extraction

Peperomia pellucida was collected from the wild in Kuala Nerus, Terengganu, Malaysia. The plant collected was stored at 4 °C in less than 24 hours after collection. The plant was authenticated at the Institute of Bioscience at Universiti Putra Malaysia in comparison with the specimen voucher MFI 0100/18. The whole plant was oven dried and then grounded into powder. The powdered samples collected were stored in a Schott bottle and kept at 4 °C until further used.

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Three different solvents; n-Hexane, dichloromethane (DCM) and ethanol were used for the sequential extraction. As *P. pellucida* is a type of plant that is easy to get extracted, the ratio of mass:plant used in this study was 1:1. A total of 500 g powdered sample was soaked with 500 mL of each of the solvents, mixed thoroughly and stored in air tight jars for 72 hours under strict observation. The temperature was set at 25°C and monitored every 12 hours to ensure it remained constant throughout the experimentThe extracts were filtered using Whatman No. 1 filter paper and the filtrate was evaporated using rotary evaporator at 26 °C boiling point, 46 °C bath temperature and 203 mbar pressures. The settings for rotary evaporator were chosen based on our optimization experiment for each solvent. Each crude extract was in a viscous liquid form, kept in a universal bottle and stored at 4 °C until further use.

Culture of *S. epidermidis*

S. epidermidis (accession no: ATCC 12028) was purchased from American Type Culture Collection (ATCC). The strain was grown and maintained on nutrient agar at 37°C for 24h. Prior to broth micro-dilution assay, suspension of bacteria was prepared in a nutrient broth and the concentration was adjusted 0.5 McFarland Standard (~1.5×10^8 CFU/mL). Bacterial growth was determined using spectrophotometer at optical density (OD) of 600 nm and compared to bacterial standard curve to obtain concentration of bacteria in CFU/mL

Determination of MIC and MBC

The procedures for determination of MIC and MBC values were according to the standard antibacterial susceptibility method as described by Clinical and Laboratory Standards Institute [15]. MIC of the *P. pellucida* extract was determined by two-fold serial broth micro-dilution method. The concentration of plant extract started with 1000 mg/mL and were serially diluted using two-fold dilution series until 3.91 mg/ml. Serial dilutions of *P. pellucida* extract were performed in 96-well microtiter plate using 10% dimethyl sulfoxide as diluents. The bacterial suspensions in nutrient broth were then inoculated into the microtiter plate which contained the serial dilutions. The microtiter plate was then incubated at 37°C for 24h before MIC values were taken. Negative control used in this experiment was nutrient broth without bacterial culture while positive control was fusidic acid. Experiment was performed in duplicates.

The suspension which showed no apparent growth was sub-cultured onto nutrient agar to determine MBC values. The lowest concentration which showed no visible growth on sub-cultured agar after an overnight incubation at 37 °C was considered as the MBC value.

Phytochemical Analysis of *P. pellucida* Extract

Ethanol extract of *P. pellucida* was analysed for its phytochemical compounds using gas-chromatography-mass spectrometry (GC-MS) 6890 series (Agilent GC System, USA)Extracted sample was injected, followed by screening of the bioactive compounds from the extract . The analysis was carried out using an HP5-ms capillary column (30 m × 0.25 mm, 0.5 µm film thickness) with helium as the carrier gas at a flow rate of 0.57 mL/min. The injector and detector temperatures were both set at 250 ºC, and ethanol was used as the diluent. The oven temperature was programmed to increase from 80 ºC to 250 ºC at a rate of 10 ºC/min, under a pressure of 19.5 psi, using the splitless injection method. Blank control was included to ensure no contamination. Using the database of National Institute of Standard and Technology version 20 (NIST20), the spectrum of the unknown compounds detected were compared with the spectrum of known compounds in the NIST20 library. The names together with CAS registry number of the components of the test materials were determined.

Preparation of Cells for SEM and TEM

Bacteria for SEM and TEM samples were treated using the lowest MIC value (3.91 mg/mL) for 24h before fixation with 4% glutaraldehyde. For SEM, the cell suspension was then placed on a membrane filter (pore size 0.22 µm). The samples were washed and post-fixed with 1% OsO4 for 1 hour. Subsequently, the samples were washed and dehydrated using a series of graded ethanol (30–100%) and acetone mixture series (3:1, 1:1, 1:3) on membrane filters, followed by pure acetone. Critical point drying process was conducted for 3 hours and coated with gold and carbon in sputter coater. Microscopy analysis was performed with scanning electron microscopy (SEM) Quanta FEG 650.

While for TEM procedure, the bacteria samples were prepared and centrifuged to form the pellet. Pellets were re-suspended in McDowell-Trump fixative. The pellets were washed and post-fixed with 1% OsO_4. Samples were then washed and dehydrated using a series of graded ethanol prior to infiltration and embedment with resin. Ultrathin sections measuring 75-85 nm g was prepared and stained using positive staining. Samples were viewed using Ziess Libra 120 Electron Microscope.

Results and Discussion

MIC and MBC values of *P. pellucida* Extract

The MIC and MBC values of *P. pellucida* extract against *S. epidermidis* are shown in Table 1. The ethanol extract inhibited the *S. epidermidis* at a lower concentration compared to n-Hexane and DCM extract. In addition, the *P. pellucida* extract exhibited MBC/MIC ratio of ≤ 4 against the tested strain which was considered as bactericidal. A ratio ≤ 4 is indicative of bactericidal activity [16].

Table 1: The MIC and MBC values of *P. pellucida* extract against *S. epidermidis*

Microorganism	Extract solvents	MIC value (mg/mL)	MBC value (mg/mL)
S. epidermidis	n-Hexane	15.63	62.5
	DCM	15.63	31.25
	Ethanol	3.91	3.91

MIC and MBC were conducted to determine the antibacterial effects of the *P. pellucida* extract. The antibacterial activity can either be bacteriostatic or bactericidal. It is critical to determine the MIC and MBC values at the start of this investigation in order to obtain the appropriate concentration for electron microscopic studies[17].

In this study, the growth of *S. epidermidis* was inhibited by all three extracts of *P. pellucida* at concentrations below 500 mg/ml. Among the three, ethanol extract inhibited the growth of *S. epidermidis* at a lower concentration than n-Hexane and DCM extracts. MBC results also showed that the ethanol extract showed the highest antibacterial activity than the other extracts by killing 99.9% of *S. epidermidis* at lower concentration. Based on the results, ethanol extract exhibits the most promising potential as antibacterial agent against *S. epidermidis* compared to that n-Hexane and DCM extract. This study showed that ethanolic extract at 3.91 mg/mL was the lowest concentration that inhibited growth of *S. epidermidis*. In comparison, study conducted previously showed that *P. pellucida* ethanolic extract at concentration of 75 mg/mL demonstrated strong antibacterial activity against *S. epidermidis.* This discrepancy can be explained by different method used to assess antibacterial activity, in which this study employed microbroth dilution while previous study used agar well diffusion assay[18]. This finding also suggests that ethanol extract consists of more potent antibacterial bioactive compounds, namely flavonoids and phenols, against *S. epidermidis* that that of n-hexane and DCM extracts.

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Phytochemical Analysis

The results obtained from the GC-MS analysis of ethanol extract of *P. pellucida* led to the identification of a number of various phytochemicals. Table 2 shows the identification of 21 compounds by GC-MS (Table 2).

Table 2: Bioactive compounds detected in *P. pellucida* extract

Peak no	RT	Area Pct	Volatile compound	Composition (%)
1	12.9983	0.0836	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	96
2	13.1579	0.165	Epizonarene	93
3	13.8262	0.1757	3-Heptadecene, (Z)-	95
4	13.9105	4.6715	Apiol	98
5	14.2999	0.4077	Benzaldehyde, 2,4,5-trimethoxy-	96
6	14.381	0.2364	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-	91
7	15.7322	0.7947	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	91
8	15.813	1.0062	Dibenzo[b,f][1,4]oxazepin-11(10H)-one, 8-methyl-	92
9	15.9254	1.1774	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	90
10	16.349	0.3891	Hexadecanoic acid, methyl ester	98
11	16.7197	0.7507	n-Hexadecanoic acid	99
12	16.9597	0.1071	2-Dodecen-1-yl(-)succinic anhydride	94
13	17.0206	1.4517	Hexadecanoic acid, ethyl ester	98
14	17.4504	2.1159	trans-Geranylgeraniol	93
15	18.0183	0.444	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	99
16	18.0845	0.2869	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	99
17	18.2019	5.4018	Phytol	96
18	18.4581	0.4937	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	98
19	18.6327	1.9165	Linoleic acid ethyl ester	99
20	18.6984	2.305	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	99
21	18.8834	0.4409	Octadecanoic acid, ethyl ester	98

Type of solvent used in extraction process is pertinent in the phytochemical analysis to determine the composition of bioactive compounds in the extract[19,20]. In this research, ethanol extract was subjected to phytochemical analysis by GC-MS to determine its bioactive compounds. Ethanol is a polar solvent and it is known as a good solvent in recovering polyphenols from plant[19]. Following GC-MS analysis, 21 bioactive compounds were identified from the extracts. The compounds obtained are mostly terpenoids, phenol, aldehyde, palmitic acid and lineloic acid. Among the identified components were Phenol, 2,6-dimethoxy-4-(2-propenyl)- and 3,7,11,15-Tetramethyl-2-hexadecen-1-OL (Table 2). Both compounds have been previously detected in this plant[21]. Phenol, 2,6-dimethoxy-4-(2-propenyl)- is a phenolic compound with antioxidant properties that protects plant, fruits and vegetables from oxidative stress[17]Study on the application of this compound against pathogenic bacteria is lacking, but it is structurally related to eugenol, which has been widely known as antibacterial compound[22]. 3,7,11,15-Tetramethyl-2-hexadecen-1-OL or phytol is a diterpene with antibacterial, anti-inflammatory, anticancer and diuretic properties[21]. Previous study demonstrated that antibiotics supplemented with phytol exhibited strong antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*[23].

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Morphological Study of *S. epidermidis* Treated with *P. pellucida* Extract

Under the SEM, *S. epidermidis* without treatment with plant appeared round, intact and undamaged (Figure 1a). After treatment of *S. epidermidis* with *P. pellucida* extract, the bacteria cell had burst with deep craters in their cell wall (Figure 1b), while after treatment with fusidic acid, numerous lysed cells with irregular and roughened cell membrane shape were observed (Figure 1c). Fusidic acid is an antibiotic that functions to inhibit protein synthesis of Gram-positive bacteria[24].

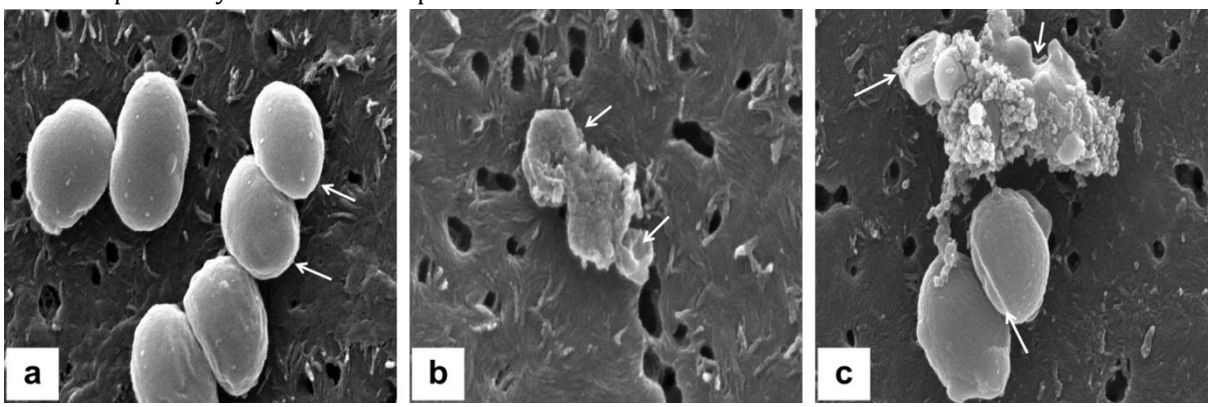

Figure 1: (a) Scanning electron microscope of *S. epidermidis*. Untreated *S. epidermidis* showed a round, intact bacterial cell with smooth cell membrane, (b) Scanning electron microscope of *S. epidermidis* treated with *P. pellucida* (3.91 mg/mL) showed lysed bacterial cell and deep craters, (c) Scanning electron microscope of *S. epidermidis* treated with fusidic acid showed lysed cells and irregular bacterial cell membrane as indicated by arrow in figure (c). Arrow indicates bacterial cell membrane.

TEM micrographs of normal and untreated *S. epidermidis* (Figure 2a) showed round, proliferating cells with intact cell walls and well-defined membranes. After treatment with *P. pellucida* extract, the morphology of *S. epidermidis* was observed with deformed cell wall, disintegrated cell membrane and membrane inclusion with dense cytoplasmic (Figure 2b). TEM micrographs of *S. epidermidis* treated with fusidic acid is characterised by a deformed and disintegrated peptidoglycan layer with dense cytoplasm, similar to the effects observed with *P. pellucida* extract, suggesting a comparable mechanism of bacterial cell wall disruption (Figure 2c). Phytochemical composition of *P. pellucida* ethanolic extract that include phenols and diterpenes have been demonstrated to disrupt bacterial membrane in previous studies[25,26].

This study provides insights into the antibacterial activity of Peperomia pellucida against *Staphylococcus epidermidis* by integrating phytochemical analysis, solvent-specific extraction, and ultrastructural evaluation using SEM and TEM. With morphological study on the ultra-cellular level using SEM and TEM, analysis of bacterial single-cell will be available [27]. By using different extraction solvents, the study was able to identify distinct phytochemical compounds such as phenolic compounds and diterpenes—that are known for their antimicrobial properties. These active compounds are likely responsible for the antibacterial effects observed. After administration of *P. pellucida* extract to *S. epidermidis*, several distinct indications of damages to the cells were visibly observed in the SEM and TEM micrographs that included roughened and irregular surface membranes, lysed and burst cells and deep craters as compared to the normal one that retained the intact and round cell structure. This finding indicates that one of the antibacterial mechanisms of the plant is through damage to the cell wall of the bacteria. These alterations contrast with the intact, round structure of untreated cells and suggest that one of the mechanisms of antibacterial action is the disruption of bacterial cell walls and membranes. This finding suggests that the presence of bioactive compounds, namely phytol and phenolic compounds, in ethanolic extract of this plant

have mechanism that disrupts integrity of bacterial cell wall. By correlating solvent-specific phytochemical composition with observed cellular damage, this study provides a more comprehensive understanding of the antibacterial effects of *P. pellucida*, offering new insights on mechanism of the plant ethanolic extract against *S. epidermidis*.

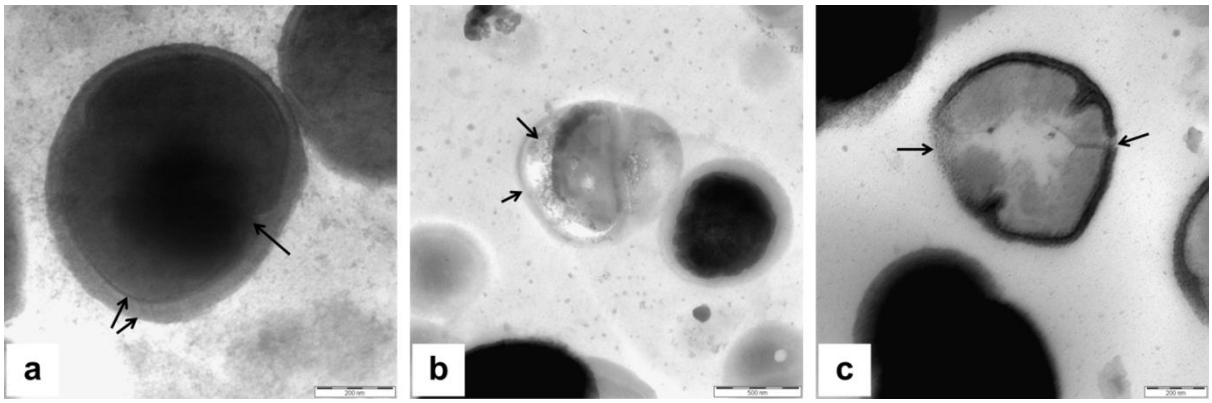

Figure 2: TEM studies of *S. epidermidis*. (a) normal untreated *S. epidermidis* exhibited round and complete cell structure with peptidoglycan layer, (b) *S. epidermidis* treated with *P. pellucida* extract (3.91 mg/mL) demonstrated membrane inclusion with dense cytoplasmic, (c) *S. epidermidis* treated with fusidic acid displayed disintegrated peptidoglycan layer as depicted by arrow in figure (c). Arrow indicates peptidoglycan layer.

Conclusion

In conclusion, *P. pellucida* has potential to be a source for antibacterial drug against *S. epidermidis*. However, further research on the toxicological effects of the extractcytotoxic study, and antimicrobial mechanism against multidrug-resistant *S. epidermidis* are recommended.

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