ORIGINAL ARTICLE

The Effect of *Senna alata* (Daun Gelenggang) Extract on *Propionibacterium acnes.*

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Abstract

Propionibacterium acnes is widely recognized as one of the agents that implicated in inflammatory acne. In order to treat acne, antibiotics have been extensively used for more than 30 years and are still widely prescribed. However, frequent use of antibiotics may increase the risk of antibiotic resistance. Hence, many countries use medicinal plants as complementary to modern medicine. *Senna alata* is one of the medicinal plants that have been used as traditional medicine due to various antimicrobial properties. Therefore, the aim of this study is to determine the effectiveness in antimicrobial properties of *S. alata* extract against *P. acnes* (ATCC 6919). *S. alata* leaves were extracted with three different solvents which are n-Hexane (non-polar), DCM (semi-polar) and ethanol (polar) before antibacterial sensitivity was tested. These were carried out by performed MIC and MBC, and the bacteria were observed under SEM and TEM for microscopic analysis. Phytochemical analysis was done using Gas chromatography-mass spectrometry (GC-MS) to determine the active compounds. Results showed *S. alata* crude extract exhibited antibacterial activities against *P. acnes. S. alata* extracted with DCM showed the most potent antibacterial against *P. acnes* with MIC and MBC were 31.25 µg/ml for both. In addition, microscopic observation revealed that the treated *P. acnes* with leaf extracts were disrupted and lost normal cell wall structure. Phytochemical analysis showed 37 active compounds were isolated. The present study suggests the crude extract of *S. alata* possess antimicrobial activity against *P. acnes.*

Keywords: *antibacterial, natural medicine, natural product, Propionibacterium acnes, Senna alata.*

Introduction

Acne, often involve in inflammation affecting the sebaceous gland, is a common skin disease that promote the inflammation at the skin surface of face, neck, chest or back.[1][2]. According to the data from Global Border of Disease study in 2013, acne has contributed approximately 10% of the global population, ranking acne as the eighth most prevalent disease worldwide [3] and second among the most prevalent dermatological conditions after dermatitis [4]. Factors that contribute to acne are such as hormonal imbalance, stress, food, genetic factors and cosmetic application [2].

Propionibacterium acnes is a Gram-positive, non-spore forming, obligate anaerobic organism, and pleomorphic rod-shaped bacterium [5][6]. These bacteria can be commonly found in skin sites with high numbers of sebum excreting sebaceous follicles, including the face, chest, and thorax [7]. Furthermore, this organism has the ability to metabolize sebaceous triglycerides into fatty acids, which inducing neutrophils. *P. acnes* are implicated in the acne development by secreting inflammation-inducing factors [8].

Various methods have been used in order to treat acne; inhibition of excessive sebum production by using hormonal agents, inhibition of proliferation of *P. acnes* by lasers, antibacterial agents or antibiotics and prevention of pore blockages through chemical peels [9]. Among these methods, topical agents are most widely used and it is recommended to use antibacterial materials such as benzoyl peroxide rather than antibiotics (i.e. erythromycin, clindamycin or tetracycline) due to tolerance issue [10][11]. However, long term and frequent use of antibiotics may run a risk of antibiotic resistance [4].

Hence, medicinal plants are widely used in several countries to prevent bacterial resistance. As a natural medicine, *Senna alata*, commonly known as Candle Brush, or Daun Gelenggang in Malaysia, is known as a medicinal plant that has minimal side effects on the human body and shows various activities against bacteria [12]. It is reported that *S. alata* has therapeutic properties such as antibacterial, antifungal, and analgesic for different parts of the plant. In addition, it has been used for the treatment of constipation, haemorrhoids, intestinal parasitosis, inguinal hernia, blennorrhagia, diabetes, syphilis, and proven to cure ringworm [13]. *S. alata* is one of the excellent plants that exhibit antimicrobial properties due to the presence of phenolic compounds such as anthraquinones and flavonoids [14]. The present study is done to evaluate the antimicrobial activities of the *S. alata* crude leaf extract on *P. acnes.*

Materials and Method

Plant Material and Plant Extraction

S. alata was collected from Terengganu, Malaysia and the sample was identified at the Institute of Bioscience in Universiti Putra Malaysia with voucher specimen number MFI 0099/18. The leaves were washed to remove foreign substances and oven dried at 40°C for 24 hours to remove the water content.

The powders of *S. alata* were extracted in a round bottom flask in the respective solvents [n-hexane (non-polar), dichloromethane (semi-polar), and ethanol (polar)] at room temperature. After two days, the extracts were filtered through filter paper No. 2 and dried using rotary evaporator. The final extractions were kept in a refrigerator at 4°C for further use.

Microorganism Collection and Preparation

The microorganism used in this study was the standard strain of *P. acnes* from American Type Culture Collection (ATCC 6919). The bacteria were cultivated on brain and heart infusion agar (BHIA) at 37°C, anaerobic conditions for 72 hours and maintained at 4°C for the growth of *P. acnes*.

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) value was carried out in microdilution assay based on method recommended by Clinical & Laboratory Standards Institute (CLSI). The procedure was done by using dimethyl sulfoxide (DMSO) as a diluent, microorganisms that are subculture in BHI broth was incubated at 37°C overnight with *S. alata* using 96-well microtiter plate containing the *P. acnes*. For the first step, serial two-fold dilutions were performed. For each well, the mixtures were mixed using different micropipette tips to avoid cross-contamination. Then, the microtiter plate was incubated at 37°C for 18-20 hours and one drop of MTT (3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) reagent was dropped into each well after the incubation period. A colour change from yellow to purple indicated active metabolism, and bacterial growth has occurred as well. The lowest concentration of extract that showed no colour change and exhibited inhibition was taken as the MIC level. All samples were measured in triplicate.

The minimum bactericidal concentration (MBC) was determined by taking a loopful of inoculum from each well of the microtiter plate with clear content and streaked it on BHI agar plates. Then, the plates were incubated for 24 h at 37°C in anaerobic condition. The MBC values were determined as the lowest concentration of the extract that permits no growth of bacteria.

Morphological study of P. acnes under SEM

Three samples were prepared for morphological analysis under scanning electron microscope (SEM), which are untreated *P. acnes, P. acnes* treated with extract of *S. alata* and *P. acnes* treated with Doxycycline antibiotic. The MIC value was used for SEM procedure. *P. acnes* was treated with *S. alata* and Doxycycline before proceed with SEM procedure. The specimens were fixed with McDowell-Trump fixative prepared in 0.1M phosphate buffer (pH 7.2) for at least 2 h. The fixed specimens were washed with the same buffer (each for 10 minutes), and post-fixed with 1% osmium tetroxide prepared in the same buffer for an hour. Afterwards, the post-fixed specimens were washed briefly with distilled water twice (each for 5 minutes). Then, dehydration process performed in a graded ethanol series (from 50% to 95%) each for 15 minutes and dehydrated in absolute ethanol (twice for 30 minutes). The dehydrated specimens were dried with Hexamethyldisilazane (HMDS) overnight in room temperature coated with sputter coat. Observations were carried out with a scanning electron microscope [8][15].

Morphology Changes Analysis of P. acnes under TEM

For the purpose of transmission electron microscopy (TEM) examination, the specimens underwent a series of preparation steps as previously described [15]. These steps involved fixation and post-fixation followed by a dehydration process. Dehydration included immersions in 50% ethanol for 15 minutes, 75% ethanol overnight, two rounds of 95% ethanol for15 minutes each, and two rounds of absolute ethanol for30 minutes each. Afterwards, the specimens were double-washed with propylene oxide for 30 min. The dehydrated samples were infiltrated with resin and acetone (1:1 ratio) combinations for 30 minutes, then were embedded with 100% resin and kept overnight. Eventually, the specimens were polymerized in mould at 60 - 70ºC oven for a day. The resulting polymerized samples were then cut into thin sections. The samples were mounted on carbon-coated copper grids and stained with 2% uranyl acetate followed by 0.4% lead citrate for 15 min. The stained sections were observed using a transmission electron microscope [8][15].

GC-MS Analysis and Identification of Components

Sample of DCM extracts of *S. alata* underwent further analysis by gas chromatography-mass spectrometry (GC-MS) to screen and identify various groups of plant phytochemicals. Interpretation on mass spectrum of GC-MS was

done using the database of National Institute of Standard and Technology (NIST), which possesses more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known component stored in the NIST data system library. This comparison allowed for the determination of the names, molecular weights, and structural characteristics of the components present in the test materials [16].

Results

The MIC and MBC Values

The results as shown by MIC and MBC values and the morphological changes from observed through SEM and TEM , indicate the potential of *S. alata* extracts against *P. acnes.* The solvent extracts that possessed anti-bacterial effect were determined for their values of MIC and MBC by serial two-fold dilutions and presented in Table 1. It is worth noting that *P. acnes* displayed resistance to all of the solvent extracts derived from *S. alata*. However, among the tested extracts, the DCM extract of *S. alata* exhibited the highest level of antibacterial activity against this organism, surpassing n-Hexane and ethanol extracts.

Morphology Study on P. acnes Treated with S. alata Extract

The killing mechanism was visualized by SEM analysis. The SEM photographs of untreated *P. acne*s in Figure 1a illustrated normal pleomorphic structure as long-rod cells shape with two rounded end, smooth, intact cell wall around the organism. While *P. acnes* treated with *S. alata* extract and Doxycycline antibiotic displayed irregularly deformed bacterial cell surfaces of *P. acne* (Figure 1b and Figure 1c).

Observation under higher magnification of TEM images in untreated *P. acne*s in Figure 2a revealed normal morphological characters with rigid and complete cell structure, distinct cell wall which was long, spindle shaped, smooth and line with cell membrane. Also, *P. acnes* structure showed well demarcated outer and inner dark lipophilic layer, with a lighter hydrophilic peptidoglycan layer present at the center. However, morphology of *P. acnes* changed when the bacteria were treated with extract and Doxycycline antibiotic. Figure 2b and Figure 2c showed the cell wall of *P. acnes* was found lysed and cell debris was observed. In addition, cell wall and cell membrane of *P. acnes* found ruptured as well.

Phytochemical Analysis

The GC-MS analysis of DCM extract of *S. alata* extract revealed the presence of 37 compounds (phytochemical constituents) in the plant (Table 2). The active principles in the DCM extract of *S. alata* were affirmed based on peak area, retention time and molecular formula. The active principles with their Peak Area and Retention Time (RT) were presented in Table 2.

Discussion

This study was conducted to investigate the effect of the crude extract of *S. alata*, primarily from the leaves of the plant against *P. acnes.* Initial tests were performed to determine the minimum inhibitory and minimum bactericidal concentrations (MIC and MBC) of the extract. The crude extract of *S. alata* was partitioned into the non-polar n-Hexane extract, semi-polar DCM and polar ethanol extract. Based on the values of MIC and MBC in Table 1, the result demonstrated that the DCM extract of *S. alata* exhibited higher potency compared to ethanol and n-Hexane extracts, with the MIC values of 31.25 µg/ml, respectively. These findings align with previous research where *S. alata* showed strong inhibitory effects (MIC = 0.625 mg/ml) on *P. acnes* [8]. Additionally, the result was in conformity when the dichloromethane fraction of the flower extracts was also found to be highly effective against bacteria in a study by Khan et al.in 2001.[17] These results provide support for the traditional medicinal use of these herbs in treating skin diseases. However, it is important to

acknowledge that various studies have reported different trends for different solvent extracts of the *S. alata* plant. This contrast with a study by Somchit et al. in 2003, which found that water extracts of *S. alata* exhibited higher antibacterial activity against *Staphylococcus aereus* and *Escherichia coli* [18]. The variations in antibacterial activity of *S. alata* across different solvents may be attributed to the differences in geographical environments, cultivar types, seasonality and age of the plant aging [12].

The plant extracts were further analysed with electron microscopy (SEM and TEM). Untreated *P. acnes* in SEM images showed normal bacterial structure with a smooth and intact cell wall. Moreover, in the higher magnification of TEM images, *P. acnes* exhibited a typical cell wall structure with a lipophilic layer. However when exposed to the crude extract of *S. alata* and antibiotic, *P. acnes* demonstrated a disruption of the bacterial cell wall membrane.This observation suggests a potential mechanism for the antimicrobial activity of *S. alata*. These microscopic observations align with prior research by Friedman et al in 2013, which investigated the effects of chitosan-alginate nanoparticles against *P. acnes.* Friedman et al proposed that the observed alterations in the cell structure could be due to osmotic disturbances that caused changes in cell wall structure andand increased space within the cell wall [19]. The significant activity of the plant extract on the *P. acnes* show that it is effective as the commercial antibiotics (Doxycycline) and can be used as the alternative medication to treat acne. The destruction of bacterial cells may be attributed to the to the antibacterial activity of *S. alata* plant extracts.

Typically, Gram positive bacteria tend to exhibit higher sensitivity to plant extracts when compared to Gram negative bacteria due to differences in their cell wall structure and the composition [20]. This research demonstrates that the crude plant extract effectively disrupted the bacterial cell wall membrane due to the susceptibility of Gram-positive bacteria, which possess only an outer peptidoglycan layer and lack an efficient permeability barrier. This disruption resulted in the leakage of the cell contents or alterations in membrane permeability, resulting in the loss of cytoplasm [21]. This study showed that *S. alata* leaf extract can be a potential antibacterial because of its ability to affect the growth of the Gram-positive organism at the lowest concentration (31.25 µg/ml).

Several studies have substantiated the effectiveness of medicinal plants employed in herbal medicine. It has been revealed that antimicrobial activity and the inhibitory effect of plant extract might be associated from the activity of phytochemicals present in the extract. Through GC-MS analysis, we identified 37 compounds. The first compound identified in GC-MS chromatogram with a shorter retention time (8.5018) was Phenol,4-(2-propenyl)- making up 91% of the composition, whereas Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester was the last compound, accounting for 97% of the composition,and exhibited the longest retention time (22.7628).. Almost all the compounds identified have been reported to possess various pharmacological or biological activity. It has been reported that almost all the compounds identified exhibited antibacterial, antifungal, antioxidant and antiviral activities against several pathogenic bacteria, fungal and viral species. For instance, Phytol, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- and Stigmasterol exerted antimicrobial, anti-inflammatory anticancer; antiacne, and antioxidant properties. [22][23][24]. n-Hexadecanoic acid, Hexadecanoic acid, ethyl ester and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- were found to act as antioxidant, hypocholesterolemic agents, nematicide, 5-Alpha reductase inhibitor, antiarthritic, anticoronary agents, antieczemic and antiacne. [22][23]. A prior study reported an excellent antioxidant activity of ethanol extract of *S. alata* leaves, primarily due to the abundance of Hexadecanoic acid (RT=18.09) in the leaves [25]. Additionally, n-hexadecanoic acid, Hexadecanoic acid, and Phytol have also been identified in the ethanol

leaf extract of *Aloe vera*, which demonstrated inhibitory effects on the growth of the two Gram-positive bacteria *Shingella flexneri* and *Streptococcus pyogenes.* [26].

Conclusion

This study was conducted to assess the antimicrobial efficacy of *S. alata* extract on *P. acnes*. The DCM extract of *S. alata* demonstrated remarkable effectiveness in modifying the morphological structure of *P. acnes* and effectively eliminating the organism**.** The promising results from this study suggest that the extract of *S. alata* holds the potential to serve as an alternative to conventional medicine for treating *P. acnes* infections. Further research can be done to study the gene expression and protein molecular analysis of *P. acnes* strains. These plants could prove invaluable as sources for the development of future antimicrobial agents.

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Table 1. Values of MIC and MBC (µg/ml)

Extraction method	MIC	MBC
n -Hexane	62.5	125
DCM	31.25	31.25
Ethanol	250	500

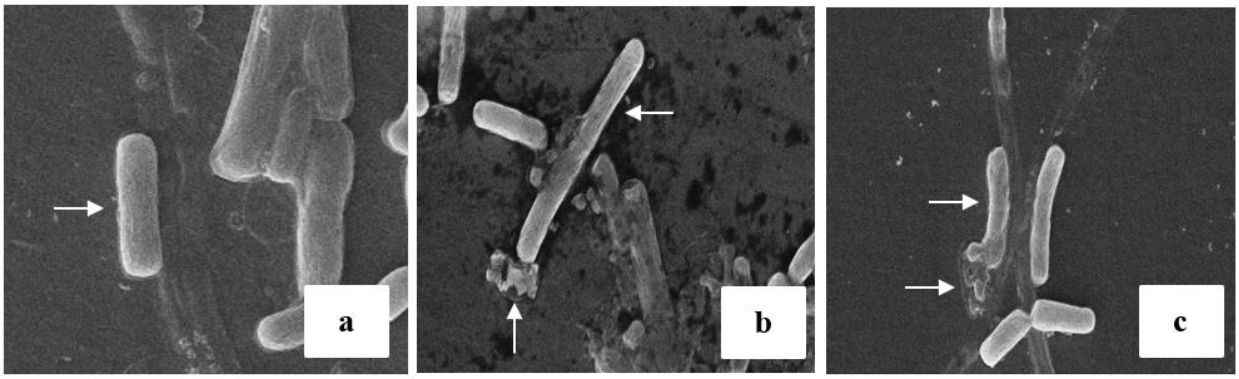

Figure 1. SEM studies of *P. acne.* (a) Untreated *P. acnes* showed a normal pleomorphic structure with two rounded end, (b) *P. acnes* treated with *S. alata* extract (31.25 µg/ml) showing irregularly deformed bacterial cell surfaces and fragments and (c) *P. acnes* treated with antibiotic (Doxycycline) showing abnormally shaped of the bacteria.

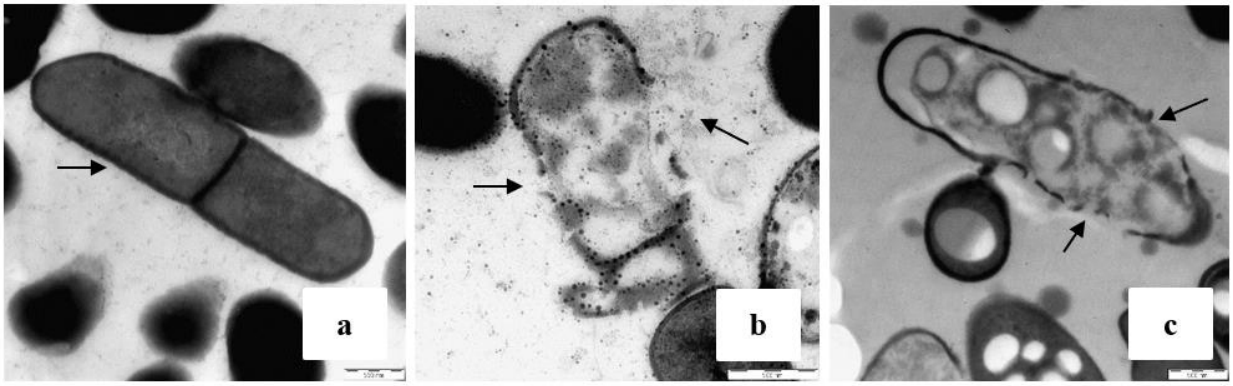

Figure 2. TEM studies of *P. acnes* (a) Untreated *P. acnes* showed distinct cell wall, (b) *P. acnes* treated with *S. alata* extract (31.25 µg/ml) displayed lysed of the cell wall and cell debris was observed and, (c) *P. acnes* treated with antibiotic (Doxycycline) exhibited the rupture of the cell wall and cell membrane.

Table 2. Activity of the components identified in the crude extract of *S. alata*

Peak no	RT	Area PCT	Volatile Compound	Composition (%)
1	8.5018	0.564	Phenol, 4-(2-propenyl)-	91
2	9.817	0.5535	.alpha.-Cubebene	97
3	10.0785	10.9095	3-Allyl-6-methoxyphenol	90
4	10.2094	1.6722	.alfa.-Copaene	98
5	10.3883	0.8579	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	98
6	10.6802	1.3804	1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.,4.alpha.,4a.beta.,7b.alpha.)]-	99
7	10.8319	1.9472	Caryophyllene	99
8	10.9267	0.5237	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [S-(E,E)]-	95
9	11.2712	0.5051	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	98
10	11.5057	1.2401	.gamma.-Muurolene	98
11	11.5944	1.4475	Pentadecane	97
12	11.7924	1.3236	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	95
13	12.0281	1.2248	1,3-Benzodioxole, 4-methoxy-6-(2-propenyl)-	97
14	12.0664	1.2862	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	98
15	12.2707	1.2603	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	97
16	12.5647	1.3941	Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-	95
17	12.8213	0.7261	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	90
18	12.9076	0.7777	Caryophyllene oxide	91
19	13.0636	2.0708	Carotol	90
20	13.8117	0.4266	Asarone	96
21	16.351	0.862	Pentadecanoic acid, 14-methyl-, methyl ester	95
22	16.7211	3.4397	n-Hexadecanoic acid	99
23	16.8157	1.0605	Dibutyl phthalate	89
24	17.0169	0.8473	Hexadecanoic acid, ethyl ester	97
25	17.5207	0.443	Hexadecanoic acid, trimethylsilyl ester	98
26	18.0162	0.5562	Methyl 10-trans,12-cis-octadecadienoate	99
27	18.0836	1.2635	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	99
28	18.1943	4.8025	Phytol	98
29	18.462	3.9489	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	99
30	18.541	0.6635	Methyl 8,11,14-heptadecatrienoate	91
31	18.6256	1.6594	Linoleic acid ethyl ester	95
32	18.6954	2.8353	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	99
33	18.8148	0.6804	Stigmasterol	96
34	18.8806	0.4368	Stigmasterol	97
35	18.9616	0.2771	Stigmasterol	99
36	20.6162	0.6708	4,8,12,16-Tetramethylheptadecan-4-olide	93
37	22.7628	1.761	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	97

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