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Antibacterial and Antiplasmodial Properties of Chemical Compounds Isolated from Bark of *Phyllanthus acidus* (L.) Skeels

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Phyllanthus acidus (L.) Skeels (Phyllanthaceae family) is a plant that is widely distributed in Asian countries, which is traditionally used as a medicinal plant to treat various ailments. Inspired by the uses of the plant, the aim of this study was to extract and isolate bioactive compounds from *P. acidus* as potential antimicrobials. The isolation and identification of phyllanthol **(1)** and *meso*-hydrobenzoin **(2)** from the dichloromethane bark extract of *P. acidus* are presented. The structures of these compounds were elucidated by extensive spectroscopic analyses. To the best of our knowledge, compound **1** is a known compound and compound **2** is the first report presented on the isolation and structural elucidation as a natural compound. The bark extract and the isolated compounds were evaluated for antibacterial activities against *Escherichia coli*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* by disc diffusion assay, minimal inhibitory concentration (MIC), and half-maximal inhibitory concentration (IC_{50}). Compounds **1** and **2** showed significant activities against *Escherichia coli* (IC_{50} value = 0.42 and 0.47 μg/mL, respectively), *Enterococcus faecium* (IC_{50} value = 0.86 and 0.43 μg/mL, respectively), *Pseudomonas aeruginosa* (IC_{50} value = 0.45 and 0.44 μg/mL, respectively), and *Staphylococcus aureus* (IC_{50} value = 12.87 and 0.44 μg/mL, respectively). These compounds also showed significant antiplasmodial activities towards the 3D7 strain with IC_{50} value of 0.218 μM for compound **1** and 0.228 μM for compound **2**, *in vitro*. All the isolated compounds were not active against MRC-5 cells with an IC_{50} value of more than 60 μg/mL. From the results obtained, *P. acidus* has been proven as a source of molecules with therapeutic potentials.

Key words: Antibacterial activity; antiplasmodial activity; phyllanthol; meso-hydrobenzoin; *Phyllanthus acidus*

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There is a global resurgence in the use of natural plant for drug discovery. In some developing countries it is being gradually integrated into the primary and secondary health care systems [1]. Almost all societies have used plant materials as sources of medicines and the development of these plant medicines depended on local botanical flora. The extensive use of natural plants as primary health medicines is because their pharmacological properties are quite common, and many countries rely mainly on plant drugs [2]. Thus, the investigation of the efficacy of plant-based drugs has been paid great attention due to their cheap and easy availability properties. Misuse of antibiotics and drugs leading to drug resistance is now pushing a considerable proportion of people to use herbal medicines [3].

Phyllanthus acidus (L.) Skeels (*P. acidus*) is a tree that varies in height from 3-10 m, depending on the habitat. The bark is rough, grey, and greyish brown with prominent lenticels. The leaves are in pinnate shape, with around 30 cm in length. The leaflets are alternately arranged, and green and smooth on the upper surface. The berries are seasonal and pale yellow in color [4]. This plant is traditionally used as a medicinal plant to treat various ailments [5]. The roots of the plant are boiled for steam inhalation to relieve coughs and headaches, even though it is regarded as toxic [6]. The leaves of *P. acidus* are commonly used in Thailand as one of the ingredients to control fever, blood pressure, and relieve headaches [7]. The fruits contain high contents of vitamin C, which are used to make vinegar and cold drinks [8]. The bark of *P. acidus* is mixed and heated with coconut oil, then spread on the skin to relieve itching in Indonesia [9]. Jagessar *et al.* [10] reported some antibacterial and

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antiplasmodial properties of the extracts of *P. acidus* in 2008. However, there is no reported antiplasmodial work on isolated chemical constituents of *P. acidus*. Furthermore, resistance to almost all the known antibiotics has developed. Thus, we suggest the continuation of the search for newer antimicrobial from natural products. In this study, we report the *in vitro* antibacterial and antiplasmodial activities on the 3D7 strain on the partitioned extracts and chemical constituents **1** and **2** of the bark of *P. acidus*.

MATERIALS AND METHODS

1. Plant Material

The bark of *P. acidus* was collected from Gurun, Kedah in 2018. It was taxonomically authenticated by the botanist of University Pendidikan Sultan Idris (UPSI). The Voucher specimen (TM 1046) was deposited in the herbarium of UPSI. The bark was air-dried at room temperature and sealed properly, kept under herbarium conditions before analyses.

2. Materials and Instrumentations

All the chemicals, such as ethanol, dichloromethane (DCM), hexane, and methanol (MeOH), were of analytical grade. Dimethyl sulfoxide (DMSO) Hybri-Max™ cell culture grade was used for antibacterial and antiplasmodial assays. Silica gel 60 for the column chromatography (70-230 mesh and 230-400 mesh) and thin layer chromatography aluminium sheets with silica gel 60 F_{254} were purchased from Merck, Germany. Agilent 5975C GC-MS was used to record the mass spectra, Shimadzu IR tracer-100 was used to obtain the IR spectra, Hitachi UH530 spectrometer was used to get the UV spectra, and JASCO P-2000 polarimeter was used to obtain the angle of rotation of compound. The NMR spectra were obtained by using JEOL ECX (500MHz) in deuterated chloroform with TMS as an internal standard.

3. Microbial Strains

Four bacterial strains, namely *Staphylococcus aureus* (ATCC9144), *Escherichia coli* (ATCC11775), *Pseudomonas aeruginosa* (ATCC10145), and *Enterococcus faecium* (ATCC6569) were obtained from ATCC, Manassas VA, USA. The bacteria were grown in nutrient broth and maintained on nutrient agar. The malaria parasite *Plasmodium falciparum* 3D7 used was obtained from the Faculty of Medicine of University of Malaya. Giemsa-stained blood smears were studied for parasite identification and quantification to obtain the selected phase of the isolate. The parasite density was determined from the Giemsa-stained blood smear after the antiplasmodial activity by counting the number of infected erythrocytes among 200 erythrocytes in each range under a microscope. Human cell MRC-5 fibroblasts (ATCC@CCL-171) were obtained from ATCC, Manassas VA, USA. The cells were cultured in EMEM medium and 10% fetal bovine serum (FBS).

4. Compound Extraction and Purification

The air-dried bark of the plant (2.0 kg) was soaked in 20% ethanol and heated up to 60°C for two hours with continuous stirring with a glass rod and then filtered. This extraction was partitioned with hexane, dichloromethane, and ethyl acetate to get various crude extracts. The solvent was evaporated using a rotary evaporator. Two grams of the dichloromethane extract was column chromatographed over silica gel 60 (70-230 mesh) and eluted with different ratios of hexane/DCM/MeOH (100:0:0 - 0:0:100, v/v). The elution resulted in 74 subfractions (D1-D74).

Subfractions D14 to D19 were combined and dried at room temperature to give **2** (8 mg). The position of the compound was detected under a UV lamp at 254 nm. Subfractions D37 to D44 were combined and subjected to silica gel 60 (230-400 mesh) and eluted with hexane/DCM/MeOH (100:0:0 - 0:0:100, v/v) to obtain 40 subfractions (E_1 - E_{40}). Subfractions E_3 and E_4 were combined and dried at room temperature to obtain **1** (5.7 mg). The position of the compound was detected under a UV lamp at 254 nm. Both compounds were subjected to spectral analyses by UV-Vis, IR, ass and NMR spectroscopies.

5. Disc Diffusion Assay for Extracts

The *in vitro* susceptibility tests were performed on the partitioned extracts using the disc diffusion method on agar plates [11]. The test microorganisms grown in nutrient broth at 37°C for 18 hours were diluted with sterile nutrient broth and adjusted to a turbidity of 0.5 McFarland standard. The suspensions were swabbed on the surface of agar plates for confluent growth by using a sterile swab. 24 mg of extract was dissolved in 1.2 mL of 0.1% DMSO to make up to the concentration of 40 mg/mL and then diluted to the concentrations of 20, 10, and 5 mg/mL. 6 mm paper discs (Whatman No. 4) were soaked in different concentrations of extracts and placed on the agar plates containing the bacterial cultures. Tetracycline (10 mg/mL/disc) was used as the positive control. The agar plates were incubated at 37°C for 18 hours. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of Inhibition Zone Diameters (IZD) produced. The processes were performed in triplicate to get more accurate results.

6. Broth Microdilution Assay for Extracts and Compounds

A microdilution broth susceptibility assay was used for the determination of the Minimum Inhibitory Concentration (MIC) and IC_{50} [12]. The tests were performed in 96-well plates. The extracts and compounds **1** and **2** were dissolved in 0.1% DMSO and transferred into microplates to obtain a serial dilution ranging from 0.0195 to 20 mg/mL (for extracts) and 0.000488 to 0.5 mg/mL (for pure compounds). The microbial suspensions were prepared according to the 0.5 McFarland standard to

adjust the turbidity. Then, the microplates were inoculated with 100 µL of diluted microbial suspension in each well. The final volume in each well was 200 µL. After 24 hours of incubation, bacterial growth was indicated by the presence of turbidity in the wells. The results were then compared with tetracycline, as the positive control, and 0.1% DMSO, which is the solvent used to dissolve the extract as the negative control. The microplates were read at 620 nm by a microplate reader and each of the processes was performed in three independent replicates.

7. *In Vitro* Antiplasmodial Assay for Extracts and Compounds

The malaria parasite *P. falciparum* 3D7 was grown *in vitro* in a 96-well plate according to the method described [13]. Blood samples were washed three times with RPMI 1640 medium. Then, the medium supplemented with 4, 2 mM L-glutamine, 10% bovine serum, 25 mM HEPES, and 100 IU mL^{-1} streptomycin/ penicillin was used to suspend washed erythrocytes. 5% hematocrit was used in the analysis. The extracts and compounds **1** and **2** were dissolved in 0.1% DMSO and a serial dilution was made in the wells to have a final concentration of 0.5% DMSO in the first well [14]. Chloroquine phosphate was dissolved in distilled water as the positive control. The extracts and compounds were prepared in concentrations ranged from 0.3125 to 10 mg/mL and 0.0078 to 0.25 mg/mL in the wells, respectively. Each concentration was made in duplicate. The microplates were placed in a CO_2 and nitrogen incubator at 37°C with 5% CO_2 for a period of 24-30 hours. The antimalarial activity was determined by the light microscopic method using Giemsa-stained smears as described and the maturation was determined by counting mature schizonts among all asexual parasites for 200 erythrocytes under each range. The concentrations causing 50% inhibition of the maturation (IC_{50} values) were determined from parasite's growth percentages using regression equations.

8. *In Vitro* Cytotoxicity Assay for Compounds

In vitro cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and for drug screening. Human lung fibroblast MRC-5 cell line was cultured in 25 mL flat-bottom tissue culture flasks in EMEM medium and 10% fetal bovine serum (FBS). The cell count was adjusted to 5×10^4 cells/mL/well using the medium and 10% FBS and transferred to a 96-well plate. To each well of the 96-well plate, 0.1 mL of the diluted cell suspension was added. After 24 hours, partial monolayers were formed. The supernatant was discarded and the monolayers were washed once with the medium. The isolated compounds **1** and **2** were dissolved in 0.1% DMSO and transferred into microplates to obtain a serial dilution ranging from 60 µg/mL to 0.875 µg/mL. The compounds were tested for their *in vitro* cytotoxicity towards MRC-5 via MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay developed by Mosmann (1983) [15] with slight modifications. The microplates were incubated at 37°C with 5% CO_2 for a total period of 24 hours. After 24 hours of incubation, the drug solutions in the wells were discarded and 50 µg of 3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide in phosphate buffer solution was then added to each well. The microplates were gently shaken and incubated for 3 hours at 37°C with 5% CO_2. The supernatant was removed and 100 µL of propanol was added to solubilize the formed formazan. 0.1% DMSO solution was used as the negative control. IC_{50} values were determined at 540 nm by a microplate reader and each of the processes was performed in triplicate to get more accurate results.

RESULTS AND DISCUSSION

1. Yield of Extracts

The aqueous extract (with 20% ethanol) was partitioned by using the solvent-solvent partitioning method with hexane, DCM, and ethyl acetate. The dichloromethane extract was isolated and purified by using extensive column chromatography. In this study, we used various combinations of solvents for the isolation process. The yields of hexane, dichloromethane, and ethyl acetate extracts are tabulated in Table 1.

2. Structural Elucidation of Compounds

Two compounds, phyllanthol **(1)** and *meso*-hydrobenzoin **(2)**, were isolated from the dichloromethane extract of the bark of *P.acidus* using column chromatography with silica gel. Compound elucidation was done by spectroscopic methods and compared with the previously reported data. Compound **1** (5.7 mg) with the R_f value of 0.89 (hexane:DCM 3:7) was isolated in white crystalline powder form with a melting point of 233-234°C. The GC-MS analysis of **(1)** resulted in a molecular ion peak at *m/z* 426.4, which confirmed the molecular formula of $C_{30}H_{50}O$. UV spectroscopy using methanol as solvent gave a peak at wavelength, λ_{max} = 208 nm. The IR spectrum obtained showed absorption bands at 3235 cm^{-1} (O-H stretching), 2924 cm^{-1} (C-H

Table 1. The yields of extracts of *P. acidus*

Partition solvent	The yield of plant extract (g)	Percentage of yield (%)
Hexane	12.6	0.63
Dichloromethane	2.73	0.14
Ethyl acetate	1.37	0.07

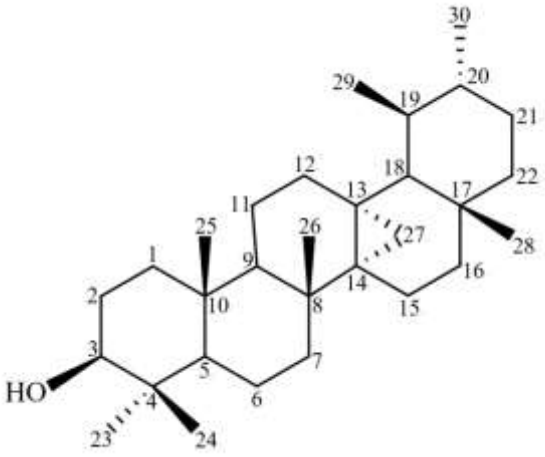
stretching), and 1449 cm^{-1} (cyclopropyl ring stretching), which suggests that the compound consists of C-H, O-H, and cyclopropyl ring.

The data of the NMR analysis of the compound are tabulated in Table 2. The ^{1}H-NMR spectrum showed a proton resonance at δ_H 3.18 (dd, *J* = 4.5, *J* = 5.5 Hz), which suggested the presence of the hydroxyl group, and the equivalent ^{13}C-NMR resonance occurred at δ_C 79.2. The singlets at δ_H 0.95, 0.75, 0.83, 1.12, and 0.87 indicated the presence of five methyl groups, which were assigned as H-23, H-24, H-25, H-26, and H-28, respectively. In the ^{1}H-^{1}H COSY spectrum, the H-3 resonance showed coupling with H-2 (δ_H 1.58) methylene proton, which in turn was observed to be coupled with the H-1 (δ_H 0.85, δ_H 1.55) methylene proton. The signal of H-18 (δ_H 0.98) methine proton was coupled with H-19 (δ_H 0.84), methine proton of H-20 (δ_H 0.85), and 29-CH_3 (δ_H 0.91, d, J = 6.5 Hz). The coupling of methine proton of H-20 with H-30 (δ_H 0.86) and the protons of H-21 (δ_H 0.95, δ_H 1.29), as well as with H-22 protons (δ_H 1.22, δ_H 1.31) further supported the positions of these protons. In the HMBC spectrum, the 23-CH_3 (δ_H 0.95), 24-CH_3 (δ_H 0.75), and a methine proton of H-5 (δ_H 0.70) were correlated with C-3. The C-1 (δ_C 38.5) was correlated with the methine proton of H-9 (δ_H 0.75) and the methyl proton H-25 (δ_H 0.83). The H-27 methylene protons (δ_H 0.64) were seen to correlate with the previously assigned C-12, C-14, and C-15. The HBMC spectrum obtained further verified the structure of the compound by the correlations of C-12 (δ_C 35.2) with the methine proton H-18 (δ_H 0.98) and the cyclopropyl ring protons H-27 (δ_H 0.00, d, J = 5.5 Hz and δ_H 0.64, d, J = 5.5 Hz). Based on the thorough analysis the spectral data, compound **1** can be assigned as phyllanthol.

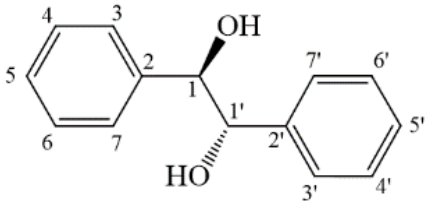
Compound **2** (8 mg) with the R_f value of 0.34 (DCM:MeOH 98:2) was isolated as a colorless solid with melting point of 137-139ºC. The GC-MS analysis of **(2)** resulted in a molecular ion peak at *m/z* 107.0, which confirmed the molecular formula of $C_{14}H_{14}O_2$.

Besides, this molecule was a *meso* compound, it is considered an achiral molecule and showed optically inactive in the polarimeter with $[\alpha_D^{25}] = 0°$ (Dichloromethane; $c = 8 \times 10^{-4}$ g/mL). UV spectroscopy using DCM as solvent gave peaks at wavelength, λ_{max} = 228 nm, 251 nm, and 258 nm. The IR spectrum obtained showed the absorption bands at 3378.38 cm^{-1} (O-H stretching), 3310.87 cm^{-1} (O-H stretching), 2918.35 cm^{-1} (C-H stretching), 1711.85 cm^{-1} (C-H bending for aromatic), 1453.39 cm^{-1} (C-H bending), and 1032.90 cm^{-1} (C-O stretching), which suggests that the compound consists of C-H, C=C, C-O, and C-C bonds.

In ^{1}H-NMR spectrum (Table 3) showed a proton resonance at δ_H 4.84, which suggested the presence of the hydroxyl group, and the equivalent ^{13}C-NMR resonance occurred at δ_C 78.2. The presence of an aromatic ring was indicated by five aromatic protons with H-3 (δ_H 7.27, d, J = 2.3 Hz), H-4 (δ_H 7.31, t, J = 7.45 Hz), and H-5 (δ_H 7.37). H-6 and H-7 were reflected from H-3 and H-4. In the ^{1}H-^{1}H COSY spectrum, the correlations between H and H in the structure indicated the compound was a *meso* compound. This is because the data showed the H-1 (δ_H 4.84) has a relationship with H-1' (δ_H 4.84). From the previously reported data by Ji-Tai Li in 2006 [16], resonance δ_H 4.84 is referring to *meso*-proton in the structure which can form *meso* stereoisomers. The DEPT spectrum obtained further verified the presence of seven carbons in each side of the meso compound. The whole structure on this compound consists of two quaternary carbons; δ_C 139.8 at C-2 and C-2', which specifies it has no proton attached to it and two secondary carbons; δ_C 78.2 at C-1 and C-1'. The HMBC spectrum also further proved that the structure of the compound was a *meso* compound. This is because the HMBC spectrum showed that H-1 (δ_H 4.84) correlated with C-1' (δ_C 78.2). From the above analyses, compound **2** is assigned as meso-hydrobenzoin based on its spectral data. This compound is first time reported from the *P. acidus*.

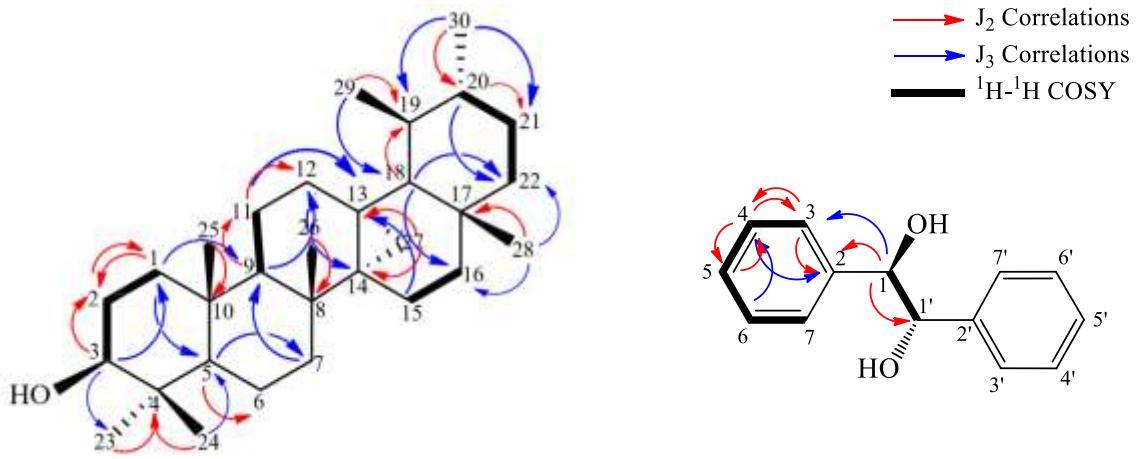


(1)



(2)

Figure 1. Chemical structures of **(1)** and **(2)**



(1) **(2)**

Figure 2. Selected COSY and HMBC correlations for **(1)** and **(2)**

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **1**.

Positions	δ_H (*J* in Hz)	δ_C	COSY	HMBC	
				J_2	J_3
1	0.86 m 1.55 m	38.5	H-3, H-25	C-2	C-4, C5, C-9
2	1.58 m 1.58 m	27.4		C-1	C-3
3	3.18 (dd, 4.5, 5.5)	79.2	H-1	C-2	C-24, C-23, C-1
4	-	38.9			
5	0.70 m	55.8		C-6	C-7
6	1.37 m 1.59 m	18.3			
7	1.73 m 1.17 m	38.5	H-26	C-8	C-9
8	-	37.1			
9	0.75 m	54.1	H-11	C-11	C-5, C-7, C-12
10	-	37.4			
11	1.26 m 1.26 m	17.8	H-9	C-12	C-13
12	1.73 m 1.85 m	35.2		C-11, C-13	C-27, C-14, C-18
13	-	26.7			
14	-	32.2			
15	1.38 m 1.42 m	21.4	H-16	C-14	C-13
16	0.80 m 0.71 m	28.4	H-22, H-15		
17	-	32.1			
18	0.98 m	50.2		C-19	C-16, C-22
19	0.84 m	40.9			
20	0.95 m	38.7	H-22	C-21	C-22
21	0.95 m 1.29 m	31.2	H-22, H-30		
22	1.22 m 1.31m	42.2	H-20, H-21 H-16		

23	0.95 s	28.1		C-4	C-3, C-5
24	0.75 s	15.5		C-4	C-3, C-5
25	0.83 s	16.2	H-1	C-10	C-5
26	1.12 s	18.1		C-8	C-13, C-14
27	0.64 (d, 5.5)	13.4	H-7	C-13, C-14	C-15, C-12
28	0.87 s	28.3		C-17	C-16, C-22
29	0.91 s	18.0		C-19	C-18, C-20
30	0.86 m	20.9	H-21	C-20	C-19, C-21

Table 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **2**.

Positions	δ_H (*J* in Hz)	δ_C	COSY	HMBC	
				J_2	J_3
1	4.84 s	78.2	H-1'	C-2, C-1'	C-3
2		139.8			
3	7.27 (d, 2.3)	127.2	H-4	C-2, C-4	
4	7.31 (t, 7.45)	128.2	H-3, H-5	C-3, C-5	C-2
5	7.37 m	128.4	H-4, H-6	C-4	
6	7.31 (t, 7.45)	128.2	H-5, H-7	C-3, C-5	C-2
7	7.27 (d, 2.3)	127.2	H-6	C-2, C-4	

3. Disc Diffusion Assay

The zone of inhibition above 7 mm in diameter was taken as a positive result. All the crude extracts were tested and showed positive results for antibacterial activity. The ethyl acetate extract showed the highest activity against all the bacteria when compared to the other extracts. At 10 mg/mL, the hexane extract was effective against *Staphylococcus aureus*, which resulted in an inhibition zone of 8.33±1.16 mm. At 5 mg/mL, the dichloromethane extract was effective against *Staphylococcus aureus* with an inhibition zone of 12.33±0.58 mm and the ethyl acetate extract was the most effective against *Pseudomonas aeruginosa* with an inhibition zone of 9.00±0.00 mm. From the results of the disc diffusion assay, it was impossible to identify whether the materials exhibited bactericidal or bacteriostatic effects, as the production of inhibition zones on the agar plates only showed that the bacterial growth was inhibited. To investigate the natural antibacterial activity of the extracts, MIC values were determined. The results of the *in vitro* antibacterial activity of the extracts against different types of bacteria are tabulated in Table 4.

Table 4. *In vitro* antibacterial activity of the extracts from the bark of *P. acidus*

Concentration of extracts (mg/mL)	Type of bacteria	Hexane	Dichloromethane	Ethyl acetate	Tetracycline
		Inhibition zone diameter (mm) ± RSD			
40	*E. coli*	10.67±0.58	11.33±0.58	15.67±0.68	29.67±0.58
	E. faecium	10.00±0.00	10.33±0.58	10.00±0.00	36.33±0.58
	P. aeruginosa	15.00±0.00	12.67±0.58	20.00±0.00	17.67±0.58
	S. aureus	10.67±0.58	14.33±0.58	13.00±1.00	28.67±0.58
20	*E. coli*	9.33±1.16	10.33±0.58	11.33±0.68	29.67±0.58
	E. faecium	8.33±0.58	8.33±0.58	8.33±0.58	36.33±0.58
	P. aeruginosa	NA*	10.33±0.58	12.00±0.00	17.67±0.58
	S. aureus	9.33±1.16	13.00±1.00	10.33±0.58	28.67±0.58
10	*E. coli*	NA*	9.33±0.58	10.00±0.00	29.67±0.58
	E. faecium	NA*	NA*	NA*	36.33±0.58
	P. aeruginosa	NA*	NA*	10.33±0.58	17.67±0.58
	S. aureus	8.33±1.16	12.00±0.00	9.00±0.00	28.67±0.58
5	*E. coli*	NA*	NA*	8.67±0.57	29.67±0.58
	E. faecium	NA*	NA*	NA*	36.33±0.58
	P. aeruginosa	NA*	NA*	9.00±0.00	17.67±0.58
	S. aureus	NA*	12.33±0.58	8.00±0.00	28.67±0.58

*NA: not active

Table 5. *In vitro* antibacterial activity of the extracts from *P. acidus*

Type of bacteria	MIC (µg/mL) ± RSD			IC_{50} (µg/mL) ± RSD		
	Hexane	DCM	Ethyl acetate	Hexane	DCM	Ethyl acetate
E. coli	625.00±0.00	625.00±0.00	8.77±0.00	50.12±0.02	31.62±0.01	6.31±0.01
E. faecium	2500.00±0.00	1250.00±0.00	312.50±0.00	1318.25±0.01	562.34±0.02	158.49±0.01
P. aeruginosa	312.50±0.00	312.50±0.00	39.06±0.00	165.96±0.01	263.03±0.01	21.38±0.01
S. aureus	39.06±0.00	78.13±0.00	8.77±0.00	25.12±0.01	35.48±0.02	6.61±0.06

Table 6. *In vitro* antibacterial activity of the isolated compounds from *P. acidus*

Type of bacteria	MIC (µg/mL) ± RSD		IC_{50} (µg/mL) ± RSD		
	Compound 1	Compound 2	Compound 1	Compound 2	Tetracycline
E. coli	0.98±0.00	0.98±0.00	0.42±0.01	0.47±0.01	0.50±0.01
E. faecium	0.49±0.00	0.98±0.00	0.86±0.08	0.43±0.01	2.94±0.12
P. aeruginosa	0.49±0.00	0.98±0.00	0.46±0.02	0.44±0.01	5.18±0.14
S. aureus	0.49±0.00	0.49±0.00	12.87±0.02	0.44±0.01	0.50±0.01

4. Broth Microdilution

In the disc diffusion method, the extracts gradually leached out from the discs and a certain period was needed for the extracts to disturb the bacterial cells. Contrarily, in MIC determination using suspended bacteria, a sufficient amount of extract can quickly inhibit or stop the growth of bacteria. [17]. In this study, the ethyl acetate extract showed the best results, then follow by the dichloromethane and hexane extracts. Besides, compounds **1** and **2** showed significant results on *E. coli*, *E. faecium*, *P. aeruginosa*, and *S. aureus*. The average and RSD values of the *in vitro* antibacterial analyses of the extracts and compounds are tabulated in Tables 5 and 6.

5. *In Vitro* Antiplasmodial Assay

The extracts and compounds **1** and **2** were also tested for their antiplasmodial activity via *in vitro* antiplasmodial assay on the 3D7 strain. The results showed that the hexane, dichloromethane, and ethyl acetate extracts possessed moderate inhibition properties against the 3D7 strain at IC_{50} = 0.50±0.09 mg/mL, 0.43±0.01 mg/mL and 0.58±003 mg/mL, respectively. The lower the IC_{50} value, the lower the concentration of extract needed to inhibit the *Plasmodium* 3D7 strain. All the extracts tested were considered active against the 3D7 strain culture with IC_{50} values < 0.60 mg/mL. The dichloromethane extract showed the best result compared to the others. Although *P. acidus* is used in traditional medicine in different countries, the *in vitro* antiplasmodial activity of chemical constituents of *P. acidus* has not previously been reported.

For the *in vitro* antiplasmodial assay, compounds **1** and **2** showed moderate activity with IC_{50} values of 92.9±1.7 µg/mL and 46.5±1.2 µg/mL, respectively, against the 3D7 strain. Chloroquine phosphate (with IC_{50} value of 0.00048±0.01 µg/mL) was used as the positive control. Also, there are several *Phyllanthus* species showing antiplasmodial activities which had been reported by previous researchers [18]. However, the chemical composition of *P. acidus* was not identified and the clinical study was not performed. Investigations to identify the active antimalarial compounds of *P. acidus* by bioassay-guided fractionation are now in progress.

6. *In Vitro* Cytotoxicity Assay

The prediction of drug cytotoxicity remains a major goal in drug development. Treating cells with cytotoxic compounds can result in a variety of cell fates. Therefore, the lower the IC_{50} values obtained, the higher the cytotoxicity of compounds. Compounds **1** and **2** were also tested for their cytotoxicity activity via the MTT assay against the human lung MRC-5 cell line. The results showed that the IC_{50} values for compound **1** and **2** were more than 0.60 µg/mL. Therefore, the compounds were not cytotoxic to the human lung MRC-5 cell line.

CONCLUSION

The present study was aimed to study the chemical constituents of *P. acidus*. Elucidation and identification of the isolated compounds were done via extensive spectroscopic methods. Two known compounds were obtained, namely phyllanthol (**1**) and meso-hydrobenzoin (**2**). These compounds were found to be potential bioactive natural compounds which showed strong *in vitro* antibacterial and antiplasmodial activities. Therefore, *P. acidus* can be considered a valuable medicinal source for future research.

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