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Original Research Article

Biological Activities of Extracts and Secondary Metabolites from *Millettia phuwuaensis*

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ABSTRACT

A study of the phytochemicals constituents of the stems of, *Millettia phuwuaensis* has led to the isolation of 7-methoxy-5′,6′-methylenedioxyisoflavone (**1**), and 12-deoxo-12α-hydroxyelliptone (**2**. Their structures were confirmed using NMR spectroscopy. Both extracts and the purified compounds were evaluated for their antibacterial, anti-HIV, and cancer activity. The antibacterial test results of the extracts and pure compound were found to be valuable, MIC is in the range of 12.5-200 mg/mL, in the range of 0.188-6 mg/mL, respectively. Mechanistic anti-HIV affect RT and MC99 found that ethyl acetate extract inhibited the very high level with IC_{50} with 75.93%. It was also found that all extracts were effective in inhibiting AIDs by mechanism MC99 at EC_{50} at 1.35 µM (TI>2.41). Further, the ethyl acetate extract showed marked cytotoxicity (ED_{50} = 17.58 µg/ml against the SH-SY5Y cancer cell line. Additionally, compound **1** also exhibited RT, moderately active with IC_{50} 55.19 % inhibition. More than that, compounds **1** and **2** also exhibited MC99 at 50% (EC_{50}) values of > 3.01 (TI >1.70) and 1.78 (TI >1.70), respectively.

Keywords: *Millettia phuwuaensis*, Fabaceae, Isoflavonoids, Rotenoids, Biological activity .

Introduction

The genus *Millettia* belongs to the family Fabaceae. It consists of about 150 species, which are distributed in the tropical and subtropical regions of the world. The genus was formerly known by the name *Pongamia*, but that name species has been reclassified[1]. Traditional medicine used this genus to treat various diseases including gynecological, rheumatic arthritis, cardiovascular, and skin diseases. Previous phytochemical investigations have shown that plants in this genus contain diverse phytochemical constituents[2]. This genus *Millettia* is well recognized for its medicinal properties due to the presence of a number of secondary metabolites. An extensive and depth investigation of different *Millettia* species has led to the isolation and characterization of various secondary metabolites belonging to alkaloids, triterpenoids, coumarins, flavonoids, isoflavonoids, phenols, and phytosterols.[3] *M. phuwuaensis* was also known in Thai as "Panarai Phu Wua", It is a vine with. leaves composed of feathers, and arranged in a spiral, with 5-7 leaflets, inflorescences in the axillary or branches, reddish-purple pink flowers, flat pods, and round seeds.[4]

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As part of an ongoing program to search for bioactive compounds, various biological assays on the isolated compounds from the hexane, ethyl acetate, methanol extracts of the stems of *M. phuwuaenis* were carried out. The isolated compounds and extracts were evaluated for their antimicrobial activity in nine strains, anti-HIV in HIVs-1RT together with MC99, and cytotoxicity activity against eight human cancer cell lines. In the present study, the chemical constituents and isolated bioactive compounds from this plant. As well as the structure elucidation of the two described compounds, and their biological activities are reported.

Material and Methods

General experimental procedures

^{1}H (500, 400 MHz), ^{13}C (125, 100 MHz), and 2D NMR spectra were recorded on a Bruker AV-500 spectrometer in deuterated chloroform ($CDCl_3$). Melting points were determined using a Büchi 322 micro melting point apparatus and were uncorrected. Optical rotations were acquired using a Rudolph Research Analytical Autopol, Automatic Polarimeter. UV-visible absorption spectra were acquired using a UV-2550 (SHIMADZU) UV-Vis spectrometer (Shimadzu). Infrared spectra (IR) were obtained as KBr discs using a Shimadzu 8900 FT-IR spectrophotometer and major bands were recorded in wavenumbers (cm^{-1}). The mass spectra were recorded on a Thermo Finnigan Polaris Q mass spectrometer at 70 eV (probe) and EIMS were run on a Bruker Esquire apparatus Column chromatography (CC) were carried out using silica gel 60 H from E. Merck. 70-230 mesh ASTM, cat. No. 7734 and No.7736 and thin-layer chromatography (TLC) were carried out on silica gel 60 PF_{254} precoated on aluminum sheets.

Plant materials

The stems of *M. phuwuaensis* (Mattapha, Suddee & BKF staff 1127 holotype BKF) were collected at, Phu Wua Wildlife Sanctuary, Bung Khla District, Bueng Kan Province, Thailand, 18°15'N 103°54'E Alt.300m., in November 2020. The plant materials were identified by Dr. Narong Nantasean, a botanist at the Forest Herbarium, Ministry of Natural Resources and Environment. Bangkok.

Extraction and isolation

The air-dried powdered of the stems from *M. phuwuaensis* (4.9 kg) was extracted with hexane (23 L× 3 days× 5 times), ethyl acetate (21 L× 3 days× 5 times), and methanol (21 L× 3 days× 4 times) to give crude hexane extract (9.77 g), crude ethyl acetate extract (46.38 g) and crude methanol extract (205.07 g), respectively.

The hexane extract (9.77 g) was separated by column chromatography on silica gel Merck No.7734 mesh 70-230 ASTM. Unfortunately, the hexane extract was not found in some compounds.

The ethyl acetate extract (46.38 g) was purified by column chromatography on silica gel and eluted with a gradient system of hexane, ethyl acetate, and methanol to give four fractions (A_1-A_4). Fraction A_3 (3.44 g) was further purified by flash CC with hexane: ethyl acetate (100:0-0:100) then with ethyl acetate: methanol (100:0-0:100) to yield two subfractions (B_1-B_2). Subfractions B_2 were further separated by flash CC with hexane: ethyl acetate (100:0-0:100) to afford D_1-D_3. The subfraction D_2 was recrystallized in ethanol to yield white needles of 7-methoxy-5′,6′-methylenedioxyisoflavone 0.68 mg (**1**).

The methanol extract (205.07 g) was further divided by column chromatography on silica gel eluted with a gradient system of hexane, ethyl acetate, and methanol to give eight fractions (E_1-E_8). Fraction E_7 (2.6 g) was separated by flash CC with hexane: ethyl acetate (100:0-0:100) then with ethyl acetate: methanol (100:0-0:100) to yield three subfractions (F_1-F_3). Subfraction F_2 was further separated by flash CC with hexane: ethyl acetate (100:0-0:100) to afford G_1-G_3. The subfraction G_3 was recrystallized in ethanol to give white needles of 12-deoxo-12α-hydroxyelliptone 0.54 mg (**2**).

Anti-bacterial Activity

Bacterial Strains

The study on *in vitro* antibacterial activity was implemented against nine strains (*S. aureus* ATCC 25923 DMST 8840, *E. aerogenes* ATCC13048 DMST 8841, *E. coli* O157: H7 DMST 12743, *E. coli* Entertoxigenic, ETEC DMST 30543, *E. coli* Enteropathogenic, EPEC DMST 30546, *S. typhimurium* ATCC 13311 DMST 562, *S. flexneri* DMST 4423, *P. mirabilis* DMST 8212, *V. cholera* nonO1/nonO139 DMST 2873).

Minimum Inhibitory Concentration (MIC)

The samples were dissolved in 10% DMSO to the concentrations of 200 mg/mL(extracts),100 mg/mL (extracts), 2 mg/mL (pure compounds) and 1 mg/mL (chloramphenicol). The extracts were diluted to final concentrations of 6.25 mg/mL, 3.125 mg/mL, 0.065 mg/mL and 0.0325 mg/mL. The final concentration of *S. aureus*, *E. aerogenes*, *E. coli* 0157: H7, *E. coli* (ETEC), *E. coli* (EPEC), *S. typhimuriam*, *S. flexneri*, *P. mirabilis*, and *V. cholera* in Mueller Hinton Broth (MHB) was 1×106 cfu/mL, 50 µL/well (Mcfarland standard No. 0.5) in a 96-well plate, and they were then mixed into the samples (50 µL/well). The plates were matured at 37 °C for 24 hours, and the growth of the organisms was detected by the color change of resazurin (1 mg/mL, 20 µL/well). No color change indicated the prevention of microbial growth.[5]

Minimum Bactericidal Concentration (MBC)

The MBC assay was operated for samples that did not show any visible growth and were subsequently sub-cultured onto nutrient agar plates. These plates were matured at 37 °C for 24 hours. MBC was only employed for the lowest concentration of the bacteria that did not retrieve 2 single colonies.[5]

HIVs assay

Anti-HIV1-RT (Reverse Transcriptase) Assay

Anti-HIV1-RT and cytotoxicity assay of the extracts of *M. phuwuaensis* were conducted at the Service Centre of Department of Physiology and Microbiology, Mahidol University, Thailand. The anti-HIV1-RT activities were decided by testing RT inhibition.[7, 8] The extracts were diluted to give 20 mg/mL of 100% dimethyl sulfoxide (DMSO) after the removal of tannin by polyvinylpyrrolidone (PVP). The final volume was 200 µg/mL in 10% DMSO, and Nevirapine, 2 µg/mL was worked as a positive control. The HIV1-RT (Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong) kit was used. The 96-well plate (100 U/µL, 4 µL/well) was filled with samples (2 µL/well), and then 2.5 µg/µL of poly-A and 0.125 µg/mL of oligo dT16 primer were added to 4 µL/well and incubated at 37°C for 20 mins. The reaction was affixed by 0.2 M EDTA (2 µL/well) and incubated at 4°C for 15 mins. The signal of fluorescence was measured at an emission wavelength of 535 nm and excitation wavelength of 480 nm after Pico green dissolved in TE buffer (1:2000) was put in (volume 200 µL/well). The results were evaluated as a percentage of inhibition. [6]

Cell-based assay for anti-HIV-1

The syncytium assay was performed in triplicate using $^{\Delta Tat/rev}$MC99 virus and 1A2 cell system[9, 10] starting at the final concentrations of 3.9–125 µg/mL or higher. Virus control and cell control wells contained neither the extracts nor the virus; cytotoxicity control wells containing cells with the extracts and positive control, i.e., azidothymidine, AZT, were included. The result was expressed as 50% effective concentration (EC_{50}). Cytotoxicity of the extracts was also carried out, in parallel and in duplicate, using a colorimetric XTT assay. The result was indicated as the concentration that inhibited 50% formazan formation in uninfected cells (IC_{50}). The therapeutic index (TI) was calculated using the equation: $TI=IC_{50}/EC_{50}$.

Cytotoxicity Assay

Cytotoxicity activity of the extracts of *M. phuwuaensis* was also investigated using the standard Sulforhodamine B (SRB) assay. Ellipticine used operated as a positive control.[11, 12] The concentrations of the samples were 20 - 0.16 µg/mL in 0.5% DMSO. The cancer cell lines were employed, including human intrahepatic cholangiocarcinoma (KKU-M213), human pharyngeal squamous carcinoma (FaDu), human colorectal adenocarcinoma (HT-29), human mammary gland/breast adenocarcinoma (MDA-MB-231), human neuroblastoma (SH-SY5Y), human lung carcinoma (A 549), and highly differentiated immortalized human cholangiocyte cell line (MMNK-1). MEM (minimum essential medium with Earles salt and L-glutamine) in 10% FBS was for culturing the cell lines. The cell lines were kept at a temperature of 37 °C for 72 hours 5% CO_2 in the air, and 100% relative humidity, followed by stabilizing with 20% trichloroacetic acid at 4 °C for 60 minutes and then stained for 30 minutes by 0.4% SRB in 1% acetic acid at room temperature. The unbound dye was cleaned with 1% acetic acid, while the already-dried stain was mixed with 10 mM Tris base with pH = 10. The absorbance was gauged at 510 nm on a microplate reader, and the 50% effective dose (ED_{50}) was calculated.

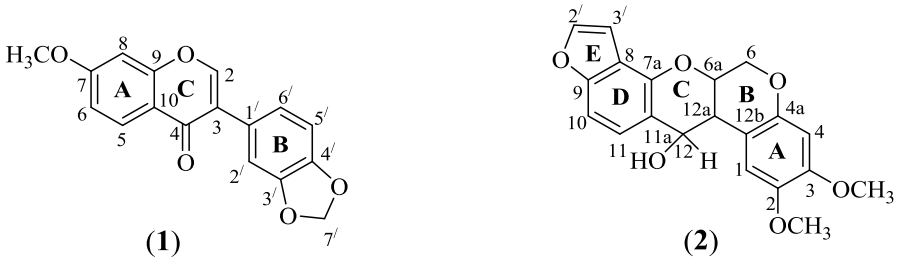
Results and Discussion

M. phuwuaensis is a new type of tree that has been researched before. The report draws on additional research from previously reported data, such as optical rotation, melting point, ultraviolet, and more definitive structural proof using 2D NMR techniques including COSY, HSQC, HMBC, NOESY, Karplus equation calculator as well as biological activity assays, including antibacterial, anti-HIV, and anticancer tests.

Compound **1** was isolated as white needles. The molecular formula was determined to be $C_{17}H_{12}O_5$ on the basis of the pseudo molecular ion $[M+H]^+$ peak in EIMS at *m/z* 297. The UV spectrum displayed the absorption maxima at 240 (1.38), 291 (1.16) and 350 (0.82) nm with melting points (245.1-245.9)°C. The IR spectrum showed characteristic absorptions for a conjugated ketone (1637 cm^{-1}), two aromatics (1620, 1597, 1570, 1514), C-O-C stretch ether, aralkyl (1276, 1250 cm^{-1}), and C-H deformation out of plane aromatics (854, 887, 916, 954). The mass spectrum showed a characteristic fragment ion, base peak at *m/z* 268, produced by the molecular ion losing a CO group, also supporting the structure of a methylenedioxy unit in the structure. The fragment ion at *m/z* 146 in the mass spectrum is due to a retro Diels-Alder fragmentation. Additionally, the fragment ions at *m/z* 282 in the mass

spectrum indicated the presence of the methoxy group in the structure. The ^{1}H and ^{13}C NMR signals of **1** at δ_H 8.16 (d, J = 2.7) and δ_C were attributed to H-2 and C-2, respectively, typical of the isoflavone nucleus (Table 1). Furthermore, the ^{1}H NMR spectrum showed a signal from a methylenedioxy group (δ_H 5.99, s). Three aromatic proton signals at δ_H 7.01 (1H, dd, J = 1.4, 2.8 Hz, H-2′), δ_H 6.98 (1H, dd, J = 1.4, 6.4 Hz, H-5′) and δ_H 7.47 (1H, dd, J = 2.8, 6.4 Hz, H-6′) indicated that a 1,4,5-trisubstituted aromatic ring was present. Together with, there were also three aromatic protons (Ring A) with three signals at δ_H 8.06 (1H, dd, J = 2.0, 8.9 Hz, H-5), δ_H 6.95 (1H, dd, J = 2.3, 8.9 Hz, H-6) and δ_H 6.87 (1H, d, J = 2.0 Hz, H-8). Proton signal at δ_H 5.99 (2H, s, H-7′) from methylene bound to aromatic ring B and methoxy proton signal δ_H 3.83 (s) at 7-position of ring A. HMBC correlations revealed the location of these substituents on the isoflavone nucleus, Further, the NMR coupling information in the COSY spectrum of **1** enabled the determination of the protons between H-5′ with H-6′ (Figure 2). The H-2 proton signal at δ_H 8.16 showed correlation to C-4 (δ_C 176.7) and C-1′ (δ_C 124.1).

The correlation between H-7′ (δ_H 5.99) and C-5′ (δ_C 113.5) and between C-7-OMe (δ_H 3.83) and C-7 (δ_C 159.7) revealed the positions of the methylenedioxy and methoxy groups, respectively. In addition, H-5 (δ_H 8.06) with C-7 (δ_C 159.7) suggested that the methoxy group was substituted at C-7 on Ring A of the isoflavone moiety. In the NOESY experiment, there were cross-peaks between H-2/H-6′, H-6′/H-5′, H-7′/H-5′, H-5/H-6 (Figure 2). A NOESY experiment revealed a strong interaction between this proton and the aromatic proton (H-2′) resonance at 7.01, thus requiring the placement of the aromatic ring B. The presence of methylenedioxy at position C-3′, and , C-4′ was confirmed by a NOESY correlation between the methylene protons at (δ_H 5.99) and the aromatic proton at the δ_H 6.98 (H-5′). These data are in agreement with structure **1** (Figure 1) for the isoflavone compound of *M. phuwuaensis*, for which the trivial name 7-methoxy-3′,4′-methylenedioxyisoflavone is suggested.[13]



(**1**) (**2**)

Figure 1: Structures of compounds 1-2

Table 1: ^{1}H and ^{13}C NMR spectra data of compounds **1** and **2**

Compound 1(CD_3OD)[a]			Compound 2 ($CDCl_3$)[b]		
Position	**δ_C**	**δ_H (Int., Mult., *J* in Hz)[c]**	**Position**		**δ_H (Int., Mult., *J* in Hz)[c]**
1	-		1	111.1, CH	7.71 (s)
2	153.4, CH	8.16 (d, J = 2.7)	2	143.9, C	-
3	125.7, C	-	3	149.2, C	-
4	176.7, C	-	4	100.2, CH	6.42 (s)
5	127.1, CH	8.06 (dd, J = 2.0, 8.9)	4a	148.0, C	-
6	115.1, CH	6.95 (dd, J = 2.3, 8.9)	6	67.1, CH_2	a) 4.56 (dd, J = 4.2, 9.8)
					b) 4.12 (t, J = 9.8)
7	159.7, C	-	6a	71.8, CH	4.32 (dt, J = 4.2, 10.7)
8	101.9, CH	6.87 (d, J = 2.0)	7a	155.9, C	-
9	163.3, C	-	8	116.8, C	-
10	116.8, C	-	9	147.4, C	-
1′	124.1, C	-	10	105.7, CH	7.2 (dd, J = 1.0, 8.6)
2′	122.3, CH	7.01 (dd, J = 1.4, 2.8)	11	123.7, CH	7.46 (d, J = 8.6)
3′	147.7, C	-	11a	112.5, C	-
4′	124.3, C	-	12	70.7, CH	4.97 (t, J = 9.7)
5′	113.5, CH	6.98 (dd, J = 1.4, 6.4)	12a	43.6, CH	3.13 (dd, J = 9.7, 10.7)
6′	130.0, CH	7.47 (dd, J = 2.8, 6.4)	12b	119.8, C	-
7′	101.2, CH_2	5.99 (s)	2′	144.5, CH	7.56 (d, J = 2.0)
7-OMe	54.4, CH_3	3.83 (s)	3′	103.8, CH	6.82 (dd, J = 1.0, 2.2)
			2-OMe	56.4, CH_3	3.86 (s)
			3-OMe	55.8, CH_3	3.83 (s)
			12-OH	-	1.96 (d, J = 10.2)

[a]Data were recorded at 500 MHz for ^{1}H and125 MHz for ^{13}C NMR
[b]Data were recorded at 400 MHz for ^{1}H and100 MHz for ^{13}C NMR
[c]Chemical shift values (in ppm) and J values (in Hz) in parentheses

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Compound 2 was obtained as white needles, melting point 140.8-141.9 °C, and its molecular formula was deduced as $C_{20}H_{16}O_6$ based on the sodiated molecular ion peak at m/z 352 in the EIMS and $[\alpha]_{589}^{26.8}$: -30.01 (*c* 0.20 g/100 mL, $CHCl_3$). The IR spectrum exhibited the characteristic signals for hydroxyl (3470 cm^{-1}), CH_3, CH_2 stretching (2968, 2870 cm^{-1}), carbonyl (1620 cm^{-1}), aromatic ring (1586, 1502, 1450 cm^{-1}), C-O-C stretching, C-O stretching and O-H deformation (1313, 1273, 1165 cm^{-1}). The UV spectrum showed absorptions at 231 (1.13), 265 (1.07), 275 (1.03), 286 (1.92), 316 (0.97) nm. In addition, the EIMS mass spectrum (found *m/z* 354, [M^+]) showed a typical rotenoid structure. The key fragmentation ions in the mass spectrum, base peak at *m/z* 192, 179, 151, and 121 were useful to obtain the structure of **2.** The fragment ion, *m/z* 192 was associated with aromatic ring A and pyran, ring B derived from initial cleavages of ring C, aromatic ring D, and furan. The presence of Ring A and Ring B was confirmed by the fragment ions, at *m/z* 151. Further, the fragment ions at *m/z* 121 indicated the presence of ring A of rotenoid. Analysis of the ^{13}C NMR and DEPT data (Table 1) of 2 indicated 20 carbon resonances, corresponding to two methoxy groups (δ_C 56.4 and 55.8), nine methines (δ_C 144.5, 123.7, 111.1, 105.7, 103.8, 100.2, 71.8, 70.7, and 43.6), one sp^3 methylene (δ_C 67.1), and 8 quaternary carbons (δ_C 155.9, 149.2, 148.0, 147.4, 143.9, 119.8, 116.8, and 123.7). The NMR spectroscopic data of **2** indicated it to be an analogue of elliptinol.[14,15] In compound **2**, ^1H and ^{13}C NMR signals for a furan ring were observed at δ_H 7.56 (1H, d, J = 2.0 Hz, H-2′)/ δ_C 144.5 (C-2′) and δ_H 6.82 (1H, dd, J = 1.0, 2.2 Hz, H-3′)/ δ_C 103.8 C-3′). The furan ring fused in an angular position at C-8 and C-9 of ring A, is supported by the presence of an aromatic proton at δ_H 7.46 (1H, d, J = 8.6 Hz, H-11) showing HMBC correlations to δ_C 116.8 (C-8) and 147.4 (C-9). The ^1H-^1H COSY spectrum showed connectivity between H-6, H-6a, H-12a, and H-12 (Figure 2). The proton NMR spectrum along with the ^1H-^1H COSY displayed two ortho-coupled protons at δ_H 7.2 (dd, J = 1.0, 8.6 Hz) and 7.46 (d, J = 8.6 Hz) which were readily assigned to H-10 and H-11, respectively. The HMBC correlations between the methoxy protons at δ_H 3.86 and C-2 (δ_C 143.9)/ δ_H 3.83 and C-3 (δ_C 149.2) were used to place the two methoxy groups at C-2 and C-3. HMBC cross-peaks were also used to assign the methine carbon position C-12 which attached the hydroxy group by proton H-11. Further, the one methylene proton showed HMBC correlation with C-12a and C-4a that confirmed the location of ring B. Exciting, the single stand-out doublet at δ_H 1.96 belongs to hydroxyl proton at position C-12. The relative configuration at positions 6a, 12a, and 12 of compound (**2**) was determined using the NOESY technique and calculates the interaction angle of protons with J values as follows. In NOESY experiments, there are cross-peaks between H-6 (δ_{Ha} 4.57) with H-6a (δ 4.32). This observed correlation was secured and reliably suggests the co-facial plane between these two protons. Furthermore, the co-facial plane between proton H-6a, H-12a, and H-12 is confirmed by the Karplus equation calculation; $^3Jxy = A\cos^2\Theta + B\cos\Theta + C$.[16] It was found that when replacing the coupling constant (^3J) of proton the interaction between H-6a with H-12a is 10.7 Hz. The calculated result is 24 degrees, indicating that the two protons are angular and oriented in the same direction. Similarly, when the ^3J value of the proton acting between H-12 with H-12a is calculated, a torsion angle of 30 degrees indicated that the two protons point in the same direction. Therefore, from the NOESY experiment and calculating the interconnected angles with the coupling constant of the Karplus equation, we conclude the four protons Ha, H6a, H12a, and H-12 all align in the same direction which means the hydroxyl group at position 12 is pointing downwards. On the basis of the above spectroscopic data, compound 2 (Figure 1) was determined to be eliptinol or 12-deoxo-12α-hydroxyelliptone.[14,15]

Biological activities

Anti-bacterial activity

Our research exhibited the antibacterial properties of *M. phuwuaensis*. These extracts were generally active against Gram-negative and Gram-positive bacteria. The results of the anti-bacterial activity of *M. phuwuaensis* are given in Table 1. The hexane extract of the stems showed antibacterial activity against two species, *S. typhimuriam* and *P. mirabilis* at MIC/MBC (mg/mL) to 50/50 and 50/100 mg/mL, respectively. The ethyl acetate extract of the stems showed antibacterial activity against nine species, *S. aureus*, *E. aerogenes*, *E. coli* O157:H7, *E. coli* (ETEC), *E. coli* (EPEC), *S. typhimuriam*, *S. flexneri*, *P. mirabilis* and *V. cholerae* at MIC/MBC (mg/mL) to 50/50, 50/200, 12.5/200, 25/25, 25/25, 25/25, 25/50, 25/25 and 25/25 mg/mL, respectively. The stems methanol extract showed antibacterial activity against eight species, *S. aureus*, *E. aerogenes*, *E. coli* O157:H7, *E. coli* (ETEC), *E. coli* (EPEC), *S. typhimuriam*, *P. mirabilis*, and *V. Cholerae* at MIC/MBC (mg/mL) to 50/100, 50/200, 25/200, 50/50, 50/100, 50/50, 25/50 and 25/25 mg/mL, respectively.

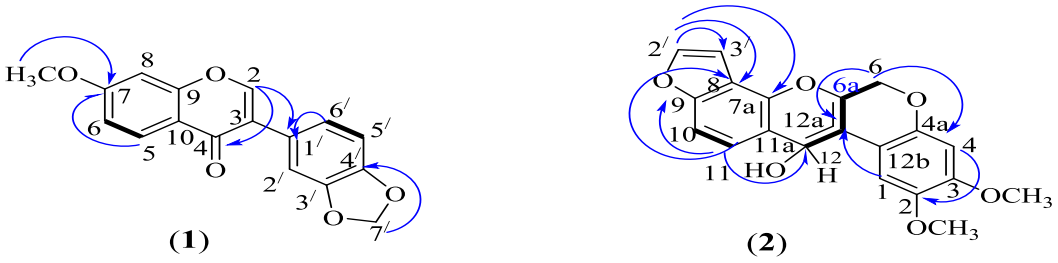

Figure 2: The COSY, HMBC, and NOESY data of compounds **1-2**

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Table 2: Determination of MIC and MBC for crude extracts and isolated compounds from *M. phuwuaensis*

Type	Extracts	Concentration of MIC/MBC (mg/mL)								
		S. aureus	***E. aerogenes***	***E. coli* O157:H7**	***E. coli* (ETEC)**	***E. coli* (EPEC)**	***S. typhimuriam***	***S. flexneri***	***P. mirabilis***	***V. cholerae***
Stems	Hexane	200/>200	200/>200	200/>200	200/>200	200/>200	50/50	200/>200	50/100	200/>200
	EtOAc	50/50	50/200	12.5/200	25/25	25/25	25/25	25/50	25/25	25/25
	MeOH	50/100	50/200	25/200	50/50	50/100	50/50	200/>200	25/50	25/25
Compound **1**	-	1.5/6	0.188/6	6/>6	6/>6	6/6	1.5/3	6/>6	0.75/1.5	1.5/3
Compound **2**	-	6/>6	0.188/>6	6/>6	6/>6	6/>6	6/>6	6/>6	6/>6	6/>6
Chloramphenicol (control)	-	<0.03/0.5	0.0625/0.25	<0.03/0.25	<0.03/0.25	<0.03/1	<0.03/0.0625	<0.03/0.25	<0.03/0.03	<0.03/1

Table 3: Anti-HIV-1 RT, Anti-syncytium (MC99+1A2, and cytotoxicity study of crude extracts and isolated compounds of stems *M. phuwuaensis*

Crude extracts / compounds	Anti-HIV-1 RT[a] (% inhibition)		Anti-syncytium (MC99+1A2)[b]				AZT			Cytotoxicity ED_{50}(μg/mL)[d]							
			IC_{50}	EC_{50}	TI[c]	Activity	EC_{50}	IC_{50}	TI[c]	KKU-M213	FaDu	HT-29	MDA-MB-231	A 549	SH-SY5Y	MNN-K1	Hep G2
Hexane extract	16.55	I	81.52	21.5	3.79	Active	$>10^{-8}$	$4.14x10^{-9}$	>2.41	-	-	-	-	-	-	-	-
Ethyl acetate extract	75.93	VA	22.77	8.9	2.56	Active	$>10^{-8}$	$4.14x10^{-9}$	>2.41	-	-	-	-	-	17.58	-	-
Methanol extract	0	I	168.6	125.2	1.35	Active	$>10^{-8}$	$4.14x10^{-9}$	>2.41	-	-	-	-	-	-	-	-
Ellipticine	-	-	-	-	-	-	-	-	-	0.62	0.50	0.58	0.60	0.57	0.48	0.55	0.52
Compound **1**	55.19	M	>125	41.59	>3.01	Active	$>10^{-8}$	$5.89x10^{-9}$	>1.70	-	-	-	-	-	-	-	-
Compound **2**	0.00	I	110.99	62.36	1.78	Active	$>10^{-8}$	$5.89x10^{-9}$	>1.70	-	-	-	-	-	-	-	-

[a]Anti-HIV-1 RT activity express as % inhibition at 200 μg/mL: very active (VA) = >70% inhibition, moderately active (MA) = 50% to 69% inhibition, weakly active (WA)= 30% to 50% inhibition and inactive (I)= <30 % inhibition; For determination of IC_{50} in the HIV-1 RT assay, the coefficients of determination, R^2 , were 0.98−0.99 in all assays for 50% end point. Positive control nevirapine IC_{50} 1.960 μg/mL

[b] Anti-syncytium (MC99+1A2) EC_{50} = dose of compound that reduced 50% syncytium formation by ΔTat/RevMC99 virus in 1A2 cells. AZT, averaged from three experiments, EC_{50} 3.95×10^{-3}μM; [c]TI, Therapeutic Index: IC_{50}/EC_{50}

[d]Cytotoxic assay: ED_{50} less than 20 μg/mL were considered active for extracts and ED_{50} less than 4 μg/mL were considered active for pure compounds. Cancer cell lines: KKU-M213 (Human cholangiocarcinoma) FaDu (Human squamous cell carcinoma) HT-29 (Human colon adenocarcinoma) MDA-MB-231 (Human mammary gland/breast adenocarcinoma) A 549 (Human lung adenocarcinoma) SH-SY5Y (Human neuroblastoma) MNN-K1(highly differentiated immortalized human cholangiocyte cell line) Hep G2 (Human hepatocellular carcinoma)

Anti-HIV1-RT Activity and Anti-syncytium (MC99+1A2) Assay
The results of the anti-HIVs of crude extracts were evaluated for their anti-HIV-1 activity employing reverse transcriptase (RT) and syncytium reduction assays using the $^{\Delta Tat/Rev}$MC99 virus in 1A2 cell lines systems as shown in Table 3. In the reverse transcriptase assay, the ethyl acetate extract exhibited very high activity with IC_{50} of 75.93 % inhibition. All extracts displayed potent activity in syncytium inhibition assay with an effective concentration (EC_{50}) of 50 % value of 1.35 µM (TI>2.41). Further, the ethyl acetate extract showed marked cytotoxicity (ED_{50} = 17.58 µg/ml against the SH-SY5Y cancer cell line. Furthermore, compound **1** also exhibited moderate activity with IC_{50} 55.19 % inhibition. More than that, compounds **1** and **2** also exhibited to MC99 at 50% (EC_{50}) values of >3.01 (TI >1.70) and 1.78 (TI >1.70), respectively.

Cytotoxicity
The results of the cytotoxicity of the crude extracts of the three-part of *M. phuwuaensis* are shown in Table 3**.** From Table 3, The stem ethyl acetate extracts exhibited cytotoxicity against SH-SY5Y with an ED_{50} value of 17.58**.**

Conclusion

The phytochemical screening of *M. phuwuaensis* has led to the isolation of two pure substances. The extracts and pure compounds showed activity against bacteria, HIVs, and some cancer cell line lines. This research led to data proving the structures of compound **1** and **2**., such as optical rotation, 2D NMR, NOESY, which helped to clarify the relative configuration of the rotenoid chiral carbon. In addition, both compounds were found to be present in this tree for the first time.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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