Structural characteristics and biological activities of extracts and secondary metabolites from Millettia phuwuaensis Mattapha & Suddee

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Research Article

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Structural characteristics and biological activities of extracts and secondary metabolites from *Millettia phuwuaensis* Mattapha & Suddee

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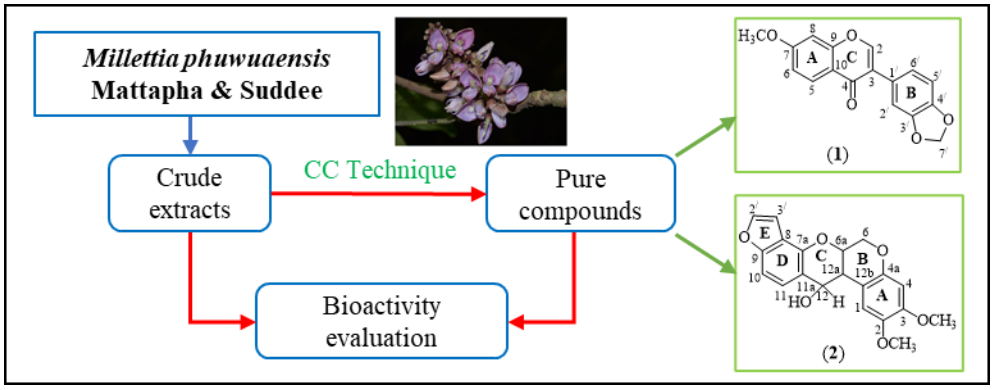
Abstract:

A study of the phytochemicals of stems, *Millettia phuwuaensis*. This led to the discovery of solvent extracts and pure substances from chromatography. Compound (**1**), 7-methoxy-5′,6′-methylenedioxyisoflavone, isoflavonoid, and compound (**2**), 12-deoxo-12α-hydroxyelliptone, rotenoid were structurally proven using NMR-based spectroscopy. Both extracts and pure compounds were evaluated for their biological activity, including antibacterial activity, AIDs, and cancer. 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-AIDs 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** was also exhibited RT, moderately active with IC_{50} 55.19 % inhibition. More than that, compounds **1** and **2** were also exhibited to MC99 at 50% (EC_{50}) values of > 3.01 (TI >1.70) and 1.78 (TI >1.70), respectively.

Graphical abstract:



Keywords: *Millettia phuwuaensis*, Fabaceae, Isoflavonoid, Rotenoid, Biological activity

1. Introduction

The genus *Millettia* belongs to the family of 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 diseases, rheumatic arthritis, cardiovascular diseases, and skin diseases. Previous phytochemical investigations have shown that plants in this genus contain diverse chemical structures [**2**]. This genus *Millettia* is well recognized for its medicinal priority due to the presence of a number of secondary metabolites. An extensive and depth investigation of different *Millettia* species has derived isolation and characterization of various secondary metabolites belonging to alkaloids, triterpenoids, coumarins, flavonoids, isoflavonoids, phenols, and phytosterols [**3**]. *Millettia phuwuaensis* was also known in Thai as "Panerai Phu Wua", It is a vine. The leaves are composed of feathers, arranged in a spiral, with 5-7 leaflets, inflorescences in the axillary or branches, reddish-purple pink flowers, flat pods, round seeds [**4**]. As part of our ongoing program to search for the bioactive compounds, various biological assays on the isolated compounds from the hexane, ethyl acetate, methanol extracts of the stems of *Millettia phuwuaenis* were carries 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. Thus, we decided to investigate the chemical constituents and isolate the bioactive compounds from this plant. The structure elucidation of the two described compounds, their biological activities were also investigated.

2. Results and discussion

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

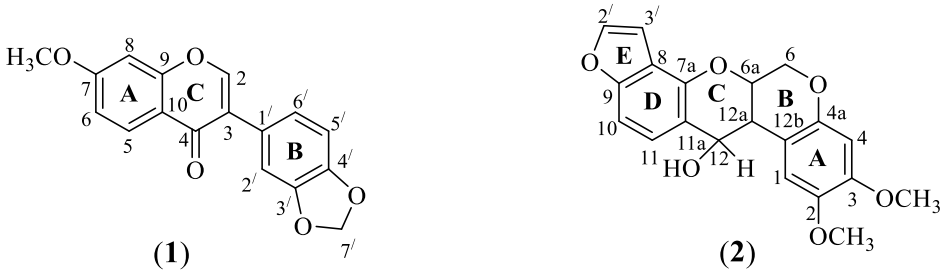
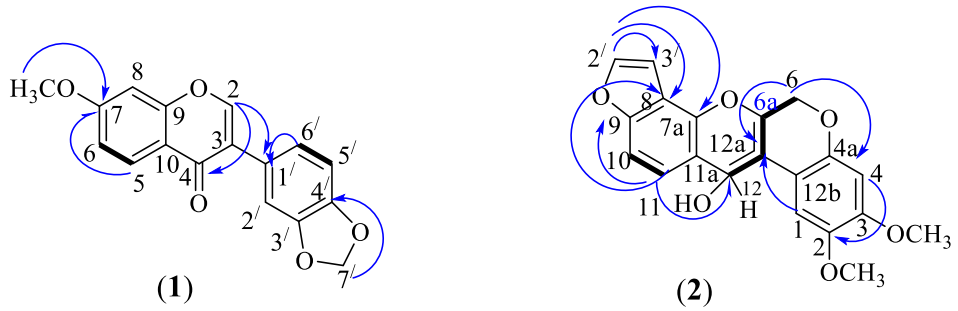


Fig. 1. Structures of compounds **1-2**

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 point (245.1-245.9) ℃. The IR spectrum showed characteristic absorptions for conjugated ketone (1637 cm^{-1}), two aromatics (1620, 1597, 1570, 1514), C-O-C stretching 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, which was produced by the molecular ion losing CO, also supporting the structure of the one methylenedioxy unit in the structure. The fragment ion at *m/z* 146 in the mass spectrum due to retro Diel-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 ^1H 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 ^1H NMR spectrum showed a signal from a methylenedioxy group (δ_H 5.99, s). Three aromatic protons 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′ (Fig. 2). The signal proton at position 2 (*(*δ_H 8.16) was correlated with both C-4 and C-1′ (δ_C 176.7, 124.1).



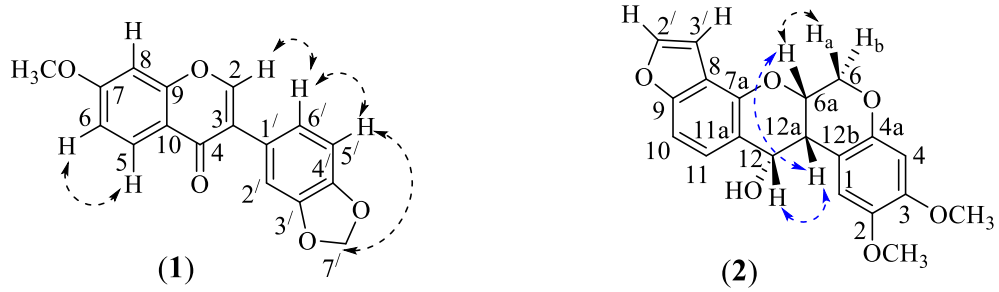


Fig. 2. The COSY, HMBC, and NOESY data of compounds **1-2**

The correlation between H-7′ (δ_H 5.99) and C-5′ (δ_C 113.5) and between H-7-OMe (δ_H 3.83) and C-7 (δ_C 159.7) revealed the orientation of methylenedioxy and methoxy, respectively. In addition, H-5 (δ_H 8.06) with C-7 (δ_C 159.7) suggested that the methoxy group was located at C-7 on the Ring A of the isoflavone skeleton. 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 (Fig.2). A NOESY experiment revealed a strong interaction between this proton and aromatic proton (H-2′) resonance at 7.01, thus requiring the placement of the aromatic ring B. The presence of methylenedioxy being placed at positions 3′, 4′ by NOESY correlation between the methylene proton (δ_H 5.99) with the δ_H 6.98 (H-5′). These data are in agreement with structure **1** for this first new isoflavone compound of *Millettia phuwuaensis*, for which the trivial name 7-methoxy-3′,4′-methylenedioxyisoflavone is suggested [**5**].

Compound 2 was obtained as a white needle solid, melting point 140.8-141.9 °C, and its molecular formula was assigned as $C_{20}H_{16}O_6$ base on the sodiated moleular 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 & 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 sp3 methylene (δ_C 67.1), 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** (**Table 1**) indicated it to be related structurally to elliptinol [**6, 7**]. In this compound, ^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, as supported by the presence of aromatic proton at δ_H 7.46 (1H, d, J=8.6 Hz, H-11)/ showed HMBC correlations with δ_C 116.8 (C-8) and 147.4 (C-9). The ^1H-^1H COSY spectrum showed connectivities among H-6, H-6a, H-12a, and H-12(Fig. 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, 8.6) which were readily assigned to H-10 and H-11, respectively. The HMBC correlation 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) are used to place two methoxy groups on C-2 and C-3. HMBC cross-peaks were also used to assign the methine carbon position 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 12. The relative configuration at positions 6a, 12a, and 12 of structure compound (**2**) uses 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 suggested the co-facial plane between those two

protons. Furthermore, the co-facial plane among proton H-6a, H-12a, and H-12 is confirmed by the Karplus equation calculation; $^{3}Jxy = A\cos^{2}\Theta + B\cos\Theta + C$ [**8**]. It was found that when replacing the coupling constant (^{3}J) 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 ^{3}J 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 value 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 inevitably point downward. On the basis of the above spectroscopic data, compound 2 was determined as eliptinol or 12-deoxo-12α-hydroxyelliptone [**6, 7**]. The chemical composition of the tree and full spectroscopic data for this compound are reported here for the first time.

Biological Activities

Anti-bacterial Activity

Our research exhibited the antibacterial properties of *Milletia phuwuaensis*. These extracts were generally active against Gram-negative and Gram-positive bacteria. The results of the anti-bacterial activity of *Milletia phuwuaensis* are shown in Table 1. From Table 1, the extracts of stems hexane extract 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 extracts of stems ethyl acetate extract 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.

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, ethyl acetate extracts exhibited very actively with IC_{50} values of 75.93 % inhibition. All extracts displayed

potent activity in syncytium inhibition assay with an effective concentration at 50% (EC_{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** was also exhibited moderately active with IC_{50} 55.19 % inhibition. More than that, compounds 1 and 2 were 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 *Milletia phuwuaensis* are shown in Table 3. From Table 3, The stem's ethyl acetate extracts exhibited cytotoxicity against SH-SY5Y at the ED_{50} values of 17.58.

3. Conclusions

The phytochemical processes of *Milletia phuwuaensis* lead to the acquisition of extracts and pure substances. Both the extract and the pure compound showed activity against bacteria, HIVs, and some cancer cell line lines. This research led to data on proving the structure of substance 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.

4. Experimental

4.1. General experimental procedures

^{1}H (500, 400 MHz), ^{13}C (125, 100 MHz), and 2D NMR spectra were noted on a BrÜker AV-500 spectrometer in deuterated chloroform ($CDCl_3$) solution. Melting points were explained by a Büchi 322 micro melting point apparatus and have to be uncorrected. Optical rotations were acquired using a Rudolph Research Analytical Autopol, Automatic Polarimeter. UV-visible absorption spectra were carried out using a UV-2550 (SHIMADZU) UV-Vis spectrometer (Shimadzu). Infrared spectra (IR) were written down as KBr pellets using a Shimadzu 8900 FT-IR spectrophotometer and major bands were taken down in wavenumber (cm^{-1}). The mass spectra were recorded on a Thermo Finnigan Polaris Q mass spectrometer at 70 eV (probe) and EIMS were estimated by a BrÜker Esquire apparatus Column chromatographies (CC) were demanded using silica gel 60 H from E. Merck. 70-230 mesh ASTM, cat. No. 7734 and No.7736. The thin-

layer chromatography (TLC) technique was preceded on silica gel 60 PF254 at aluminum sheets ultraviolet light.

4.2. Plant Materials

The stems of *Milletia 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 recognized by Dr. Narong Nantasean, a botanist at the Forest Herbarium, Ministry of Natural Resources and Environment. Bangkok.

4.3. Extraction and isolation

The air-dried powdered stems from *Milletia 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 EtOAc extract (46.38 g) and crude MeOH 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 disparate by column chromatography on silica gel eluted with a gradient system between hexane, EtOAc, and MeOH to give four fractions (A_1-A_4). Fraction A_3 (3.44 g) was isolated by flash CC with hexane: EtOAc (100:0-0:100) then with EtOAc: MeOH (100:0-0:100) to yield two subfractions (B_1-B_2). Subfractions B_2 were further segregated by flash CC with hexane: EtOAc (100:0-0:100) to afford D_1-D_3. The subfraction D_2 was also crystallized by ethanol to a white needle 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 between hexane, EtOAc, and MeOH to give eight fractions (E_1-E_8). Fraction E_7 (2.6 g) was separated by flash CC with hexane: EtOAc (100:0-0:100) then with EtOAc: MeOH (100:0-0:100) to yield three subfractions (F_1-F_3). Subfraction F_2 was further separated by flash CC with hexane: EtOAc (100:0-0:100) to afford G_1-G_3. The subfraction G_3 was as well crystallized by ethanol to a white needle of 12-deoxo-12α-hydroxyelliptone 0.54 mg (**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 (long-range coupling)	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, 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
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 125 MHz for ^{13}C NMR

[b]Data were recorded at 400 MHz for ^{1}H 100 MHz for ^{13}C NMR

[c]Chemical shift values (in ppm) and J values (in Hz) are presented in parentheses

4.4 Anti-bacterial Activity

4.4.1. 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).

4.4.2. Minimum Inhibitory Concentration (MIC) [**9**]

The samples were diluted 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 attenuat 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.

4.4.3. Minimum Bactericidal Concentration (MBC) [**9**]

The MBC assay was operated for samples that did not show any visible growth and was 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.

4.5. HIVs assay

4.5.1 Anti-HIV1-RT (Reverse Transcriptase) Assay [**10**]

Anti-HIV1-RT and cytotoxicity assay of the extracts of *Milletia 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 [**11, 12**]. 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 of 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.

4.5.2 Cell-based assay for anti-HIV-1

The syncytium assay was performed in triplicate using $^{\Delta Tat/rev}$MC99 virus and 1A2 cell system [**13, 14**], 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: $TI=IC_{50}/EC_{50}$.

4.6. Cytotoxicity Assay

Cytotoxicity activity of the extracts of *Milletia phuwuaensis* was also investigated using the standard Sulforhodamine B (SRB) assay. Ellipticine was operated as a positive control [**15, 16**]. 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 were spending for culturing the cell lines. The cell lines were kept at temperature 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.

Table 2. Determination of MIC and MBC for crude extracts and isolated compounds from *Millettia 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 *Millettia 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)

5. Declaration of Competing Interest

The authors declare no competing financial interest.

6. Acknowledgments

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