

Anti-infective Activities of Secondary Metabolites from *Vitex pinnata*

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ABSTRACT

The phytochemical investigation of *Vitex pinnata* led to the isolation of a mixture of steroids β -sitosterol and stigmasterol (**1a and 1b**) and three known flavonoid identified as 5-hydroxy-3, 7, 4'-trimethoxyflavone (**2**), 5-hydroxy-7,4'-dimethoxy-flavone (**3**) and 5-hydroxy-3,3',4',7-tetramethoxyflavone (**4**). The structures of all isolated compounds were carried out by NMR and mass spectrometry. The isolated compounds were evaluated for their anti-infective activities against *Trypanosoma brucei brucei* and *Mycobacterium marinum*. Compound 1-4 showed moderate antitrypanosomal activity with MIC values of 6.25 μ g/ml, 19.0, 21.0 and 17.0 μ M, respectively while no activity observed on anti-mycobacterial. This study is the first to report the presence of three flavones and their antitrypanosomal activity from *V. pinnata*.

INTRODUCTION

Plants play a pivotal role in drug discovery by producing various types of bioactive compounds including anti-cancer (Pezzuto, 1997), anti-diabetic (Marles and Farnsworth, 1995), anti-inflammatory (Nam, 2006), antiviral (Mukhtar *et al.*, 2008) and also anti-protozoan neglected diseases drugs (Schmidt *et al.*, 2012). *Vitex pinnata* is a woody plant which can be found in primary, secondary forests and savannahs. This species is under the genus *Vitex* with the family *Lamiaceae* (formerly was under family *Verbenaceae*). It can be found in Southeast Asia like in Malaysia, Indonesia, Thailand, Cambodia and Philippines (de Kok, 2008). Traditionally the young leaves of *V. pinnata* are used by Malay communities for antipyretic treatment and bark is used to treat gastric ulcer (Corner, 1951). Other previous study described the leaves of *V. pinnata* were applied on cuts and wounds (Ong and Nordiana, 1999). Human African trypanosomiasis (HAT) or sleeping sickness is one of neglected tropical diseases listed by World Health Organisation (WHO) (WHO, 2013a). It is a fatal vector-borne parasitic disease provoked by *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) or *Trypanosoma brucei gambiense* (*T. b. gambiense*) and transmitted by the tsetse fly (*Glossina* spp.). This infectious disease happens specifically only in rural areas of sub-Saharan Africa (Simarro *et al.*, 2011). Only few drugs have been

used to treat this disease and these include suramin, pentamidine, melarsoprol and eflornithine and the combination of nifurtimox/eflornithine. Most of the drugs are old, having been discovered in the 1940s and 1950s, and also have adverse effects on patients. For this reason, mining and developing new HAT drugs from natural products is still crucial and indispensable (Jacobs *et al.*, 2011). Tuberculosis (TB) is an infectious disease and the pathogen responsible for TB is *Mycobacterium tuberculosis*. TB still remains as a public health disease and infected millions of people each year. According to WHO report, TB is a second leading cause death after the human immunodeficiency virus (HIV) and estimated nearly 8.6 million new TB cases and 1.3 million TB deaths will occur in 2012 (WHO, 2013b). Although TB is curable, however the rise of multidrug-resistance TB (MDR-TB) cases is very concerning which therefore require voluminous new anti MDR-TB drug candidates to be discovered to combat and eradicate this infectious diseases (WHO, 2013b). The present study reports the chemical investigation of extract from *V. pinnata*. Previous phytochemical study revealed the isolation of ecdysteroids, which are pinnatasterone, 20-hydroxyecdysone and turkesterone (Suksamram and Sommechai, 1993). Other study reported a new iridoid glucoside, pinnatoside and three known flavonoids namely viscioside, apigenin, and luteolin were isolated from the bark of *V. pinnata* (Ata *et al.*, 2009). In this paper the isolation of secondary metabolites and its anti-infective activities against *T. b. brucei* and *M. marinum* of isolated compounds were described.

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MATERIALS AND METHODS

General Equipment and Experimental Procedures

The optical rotations of the compounds were measured on a 341 Polarimeter from PerkinElmer, Inc., USA. Analytical Thin Layer Chromatography (TLC) was performed on pre-coated TLC plates with normal silica gel 60 F254 and reverse phase TLC silica gel 60 RP-18 F254S (layer thickness 0.2 mm, Merck, Germany).

Plant material

Leaf parts of *V. pinnata* were collected from Marang Kuala Terengganu, situated approximately 500 kilometres northeast of Kuala Lumpur on the east coast of Peninsular Malaysia in September 2009. The plant was authenticated by Dr. Nashriyah Mat from Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin and voucher specimen was deposited (collection number VP 01).

Extraction and Isolation

The ground leaves of *V. pinnata* (1 kg) were extracted in a Soxhlet apparatus at 40°C by utilising three different solvents based on their polarity. The extraction was started with *n*-hexane followed by ethyl acetate then methanol with solvent volumes of 7.5 L each for 72 hours.

Later the extracts were concentrated *in vacuo* by using rotary evaporator (BUCHI Labortechnik AG, Switzerland) at 40°C and the yield was 6.33g (*n*-hexane extract), 6.5g (ethyl acetate extract) and 5.2g (methanol extract). All dried extracts were stored at -20°C freezer. Fractionation of the *n*-hexane extract (6.33g) was accomplished by using Medium Pressure Liquid Chromatography (MPLC).

Linear gradient elution was employed with hexane (A) and ethyl acetate (B) as solvents at a flow rate of 100ml/min. Pre-packed VersaPak silica cartridge (particle size 20–45 µm, diameter and length 40 x 150 mm) from Supelco was used in this run. 100% A was run for 5 min (isocratic) followed by 100% A to 100% B for 20 min (gradient) then with 100% B for the last 5 min (isocratic).

The run time was 30 min in total. For the first and last 5 min of the run, the fractions were collected for every 100 ml and followed by 50 ml volume fractions for 20 min resulting to 50 fractions. Fractions with similar TLC profiles were pooled together yielding 28 fractions. Fraction 12 contained a mixture of two compounds assigned as **1** (15mg).

Fraction 13 was further subjected to MPLC over a pre-packed VersaPak silica cartridge (particle size 20–45 µm, diameter and length 23 x 53 mm) utilising an isocratic gradient system with 80% hexane and 20% ethyl acetate for 30 min at a flow rate of 20 ml/min. Fraction 13 afforded six sub-fractions and gave one pure compound **2** (20 mg). Fraction 16 afforded one major compound **3** (4 mg). Fraction 19 afforded the major compound **4** (130mg) by crystallisation after washing with mixtures of hexane and methanol.

NMR Instrumentation

One and two dimensional Nuclear Magnetic Resonance (NMR) experiments were measured in Strathclyde Institute of Pharmacy and Biomedical Sciences by using 400 MHz on an AS-400 JEOL NMR instrument (Tokyo, Japan). All isolated samples were dissolved in 650 µl of deuterated chloroform (CDCl₃).

HR-LCMS analysis

In High Resolution-Liquid Chromatography Mass Spectrometry (HR-LCMS) analysis, all samples were dissolved in MeOH HPLC grade to give final concentration of 1mg/ml. HR-LCMS analysis was carried out using a Accela 600 High Performance Liquid Chromatography (HPLC) pump with Accela autosampler and UV/Vis detector (Thermo Scientific, Bremen, Germany) and Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific Inc, Hemel Hempstead UK). A reversed phase silica C18 HPLC column, 75.0 x 3.0 mm² (Hichrom Limited, UK) with particle size of 5 µm, pore size 100 Å was used. The approximate pressure was at 37 bar while the temperature was maintained at 22 °C. The mobile phase consisted of purified water (A) and acetonitrile (B) with 0.1 % formic acid in each solvent. The samples were eluted on a linear gradient of 90% A and 10% B to 100% B for 30 min and changed to isocratic mode for 5 min before decreasing back to 10% of B for 1 min. Then the column was re-equilibrated with 10% of B for 9 min before the next sample injection. The flow rate used in this method was 300µl/min and the injection volume was 10 µl.

GCMS Analysis

Sample (1 µl) was injected into the the Gas Chromatography Mass Spectrometry (GCMS) (Focus GC-DSQ2) system from Thermo Fisher Scientific (Bremen, Germany) using with 30 m long, 0.25 mm i.d., and 0.25 µm film thickness InertCap 1 MS capillary column from GL Sciences (Japan). The oven temperature was set at 80°C for 1 minute and the temperature was increased at a rate of 15°/min until it reached to 200°C and was maintained for 15 min. Then the temperature was again increased at a rate of 5°C/min until the final temperature of 320°C (held for 10 min). The base temperature of the split/splitless (SSL) was 250°C. The mode was splitless. The split flow was on at 15 mL/min. The splitless time was 1 minute. The carrier method was set to constant flow. The initial value was 1.50 mL/min and the initial time was 1 minute. The MS transfer line was maintained at a temperature of 320°C. The source temperature of the DSQ II mass spectrometer was set to 250°C. The mass range used was 50.0-800.0.

Anti-infective assays

The *in vitro* antitrypanosomal and antimycobacterial activities were examined according to the standard protocols previously published (Viegelmann *et al.*, 2014).

RESULT AND DISCUSSION

The substance **1** was observed as a white powder. From the GCMS result, it showed two peaks at 19:23 and 19:53 minutes

with the molecular ion peaks at m/z 412.51 $[M]^+$ and 414.51 $[M]^+$, respectively. Online GCMS NIST library database suggested this fraction is a mixture of two compounds. GCMS spectrum at m/z 414.51 suggested the compound is β -sitosterol **1a**, with the molecular formula $C_{29}H_{50}O$ while at m/z 412.51 is stigmasterol **1b** with the molecular formula $C_{29}H_{48}O$. The HMQC spectrum was done to confirm the identity of the individual compounds in each of the mixture which were elucidated as β -sitosterol and stigmasterol by observing the specific signals at positions C-22 and C-23.

In the HMQC spectrum of stigmasterol the chemical shifts at positions C-22 and C-23 are 138.0 and 129.2 ppm, respectively which suggested an alkene group while in β -sitosterol, chemical shift signals at C-22 and C-23 were at 34.0 and 26.1 ppm, respectively for an alkyl group. Based on comparison to ^{13}C NMR data of **1a** and **1b** to the previous literatures (Alam *et al.*, 1996; Mahato and Kundu, 1994) (Table 1) supported that compound **1a** and **1b** is a mixture of β -sitosterol and stigmasterol.

Table 1: ^{13}C NMR data of β -sitosterol (1a) and stigmasterol (1b) in Chloroform-*d*

Carbon Position	1a	β-sitosterol (Alam <i>et al.</i>, 1996) in Chloroform-<i>d</i>	1b	Stigmasterol (Mahato and Kundu, 1994) in Chloroform-<i>d</i>
	δ_c	δ_c	δ_c	δ_c
1	37.4	37.3	37.1	37.3
2	31.9	31.9	31.6	31.7
3	71.8	71.9	71.8	71.9
4	42.3	42.3	41.5	42.3
5	140.3	140.8	140.3	140.8
6	121.7	121.8	121.7	121.8
7	31.7	31.7	31.7	32.1
8	32.0	32.0	32.0	32.0
9	50.1	50.2	50.1	50.2
10	36.5	36.6	36.7	36.6
11	21.1	21.2	20.8	21.2
12	39.6	39.8	39.6	39.8
13	42.3	42.4	42.3	42.4
14	56.7	56.8	56.7	56.8
15	24.3	24.4	24.3	24.4
16	28.6	28.3	28.6	28.3
17	55.9	56.1	55.9	56.1
18	11.6	11.9	11.6	11.9
19	19.4	19.5	19.2	19.1
20	36.2	36.2	40.1	40.6
21	18.8	18.9	20.5	20.0
22	34.0	34.0	138.0	138.4
23	26.1	26.2	129.2	129.2
24	45.8	46.0	51.1	51.3
25	29.2	29.2	32.0	34.0
26	19.8	19.9	19.0	18.9
27	19.0	19.1	21.2	21.3
28	23.1	23.1	25.4	25.5
29	12.0	12.1	12.0	12.1

Compound **2** was obtained as white needles, m.p. 427°C. ESI-MS peak in the positive mode was found at m/z 329.1026 $[M+H]^+$ (base peak) which revealed a molecular weight of 328.32 g/mol and a molecular formula of $C_{18}H_{16}O_6$. Consequently, compound **2** had 11 degree of unsaturation. The 1H NMR spectrum

of **2** (Table 2) displayed a singlet signal at δ 12.65 due to the strongly hydrogen bonded phenolic hydroxyl moiety. The presence of two doublet signals at δ_H 6.44 (d, $J=2$) and δ 6.35 (d, $J=2$) were characteristic of two *meta*-related H-6 and H-8 as in a 5,7 disubstituted A-ring flavonoid. In the B ring system, two sets of symmetric proton doublet signals at δ 8.07 (2H, d, $J=9$) and 7.02 (2H, d, $J=9$) were detected for an AA' BB' system which indicated that C-4' was substituted. In addition, the 1H NMR spectrum of this compound exhibited three singlets at δ_H 3.89, 3.87 and 3.85 showing the existence of three methoxy groups. By comparing the data with previous literature (Rossi *et al.*, 1997), the compound **2** was identified as 5-hydroxy-3, 7, 4'-trimethoxyflavone previously isolated from *Aniba* species. Compound **3** was obtained as white needles, m.p. 381°C. The ESI-MS spectrum showed a molecular ion at negative mode with m/z 297.2434 $[M-H]^-$ suggested a molecular formula of $C_{19}H_{18}O_7$. The 1H NMR spectrum (Table 2) showed of two methoxy signals at δ_H 3.88 and 3.89. One downfield singlet at δ_H 12.80 assigned to a phenolic hydroxyl group. The 1H NMR spectrum of compound **3** was found to be similar to spectrum compound **2** except that compound **3** showed additional singlet peak at δ_H 6.58 suggested its position at C-3. In the A ring, AB system was assigned to two doublet protons (H-6 and H-8) at δ_H 6.37 and 6.48 with typical *meta* coupling constant of $J=2.2$ Hz. In the B ring system, two sets of symmetric proton signals δ 7.84 (2H, d, $J=9$) and 7.01 (2H, d, $J=9$) were detected for an AA' BB' system which indicated that C-4' was substituted. The structure of **3** was identified as 5-hydroxy-7, 4'-dimethoxyflavone based on comparison to the previous published data (Kolak *et al.*, 2009). Compound **4** was obtained as white crystalline needles, m.p. 473°C. A molecular formula of $C_{19}H_{18}O_7$ was deduced by ESI-MS with molecular ion peak at m/z 359.1134 $[M+H]^+$ (base peak) with 11 degrees of unsaturation.

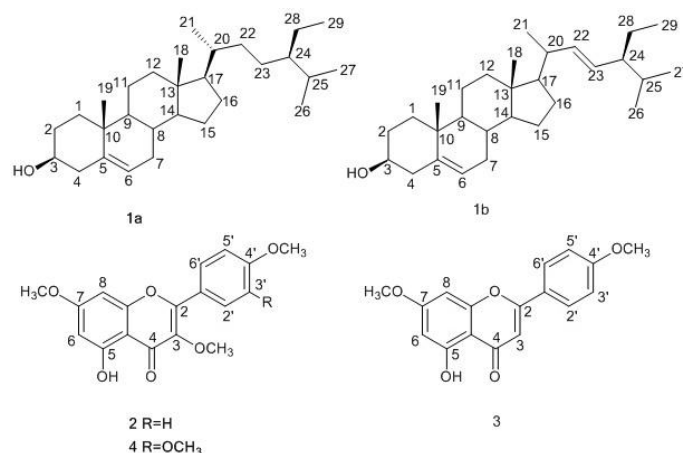


Fig. 1: Structures of isolated secondary metabolites from *V. pinnata*

The 1H NMR spectrum (Table 3) showed four methoxy signals at δ_H 3.86, 3.88, 3.96 and 3.97. One downfield singlet at δ_H 12.64 was assigned to a phenolic hydroxyl group. In the A ring, AB system was assigned to two doublet protons (H-6 and H-8) at δ_H 6.36 and 6.45 with a typical *meta* coupling constant of $J=2.2$ Hz.

Table 2: ¹³C NMR data of 5-hydroxy-3, 7, 4'-trimethoxyflavone (2) and 5-hydroxy-7, 4'-dimethoxyflavone (3) in Chloroform-*d*.

Position	2	5-hydroxy-3, 7, 4'-trimethoxyflavone (Rossi <i>et al.</i> , 1997) in Chloroform- <i>d</i>	3	5-hydroxy-7, 4'-dimethoxyflavone (Kolak <i>et al.</i> , 2009) in Chloroform- <i>d</i>
	δ_H (J Hz)		δ_H (J Hz)	
3			6.58, s	6.58, s
6	6.35, d (<i>J</i> =2)	6.33, d (<i>J</i> =2)	6.36, d (<i>J</i> =2.2)	6.37, d (<i>J</i> =2.4)
8	6.44, d (<i>J</i> =2)	6.43, d (<i>J</i> =2)	6.48, d (<i>J</i> =2.2)	6.48, d (<i>J</i> =2.4)
2' and 6'	8.07, d (<i>J</i> =9)	8.07, d (<i>J</i> =9)	7.84, d (<i>J</i> =9.0)	7.85, d (<i>J</i> =9.0)
3' and 5'	7.02, d (<i>J</i> =9)	7.0, d (<i>J</i> =9)	7.01, d (<i>J</i> =9.0)	7.02, d (<i>J</i> =9.0)
3-OCH ₃	3.87, s	3.84, s	3.89, s	3.84, s
7-OCH ₃	3.89, s	3.84, s	3.88, s	3.84, s
4'-OCH ₃	3.85, s	3.84, s	12.80, s	12.80, s
5-OH	12.65, s	12.6, s		

Table 3: ¹³C NMR data of 5-hydroxy-3,3',4',7-tetramethoxyflavone (4) in Chloroform-*d*.

Position	4	5-hydroxy-3,3',4',7-tetramethoxyflavone (Li <i>et al.</i> , 2006) in DMSO- <i>d</i> ₆
	δ_H (J Hz)	
6	6.36, d (<i>J</i> =2.2)	6.39, d (<i>J</i> =2.2)
8	6.45, d (<i>J</i> =2.2)	6.80, d (<i>J</i> =2.2)
2'	7.69, d (<i>J</i> =2.0)	7.67, d (<i>J</i> =2.0)
5'	6.99, d (<i>J</i> =8.9)	7.17, d (<i>J</i> =9.0)
6'	7.73, dd (<i>J</i> =8.9, 2.0)	7.74, dd (<i>J</i> =9.0, 2.0)
3-OCH ₃	3.86, s	3.83, s
7-OCH ₃	3.88, s	3.87, s
3'-OCH ₃	3.96, s	3.87, s
4'-OCH ₃	3.97, s	3.88, s
9-OH	12.64, s	12.62, s

Compound **1a** and **1b**, **2**, **3** and **4** showed moderate antitrypanosomal activity with MIC values of 6.25 µg/ml, 19.0, 21.0 and 17.0 µM. However no activity was observed in all isolated compounds against *M. marinum* (Table 4). Previous study reported that compound **4** was inactive against *T. b. rhodesiense* and *T. cruzi* (IC₅₀ >80 µM) however showed adequate antileishmanial activity against *Leishmania donovani* with IC₅₀ value of 21.77 µM (Tasdemir *et al.*, 2006).

Table 4: Summary of anti-infective activities of isolated compounds from *V. pinnata*.

Compounds	Antitrypanosomal activity against <i>T. b. brucei</i> , MIC (µM)	Anti-mycobacterial activity against <i>M.</i> <i>marinum</i> MIC (µM)
1	6.25 µg/ml	>100
2	19.0 ± 3.5	>100
3	21.0 ± 4.1	>100
4	17.0 ± 2.3	>100
Suramin	0.11 ± 0.0	
Gentamycin		13.48 ± 0.0

Tumor necrosis factor- α (TNF- α) is a group of cytokines that involved systemic inflammatory reaction. Previous report describes the level of TNF- α was increased in the sera of bacterial, viral, and parasitic-infected patients including malaria and leishmaniasis (Barral-Netto *et al.*, 1991; Scuderi *et al.*, 1986; Shaffer *et al.*, 1991).

The level of TNF- α in serum of *T. b. gambiense*-infected patient also corresponding with the disease severity (Okomo-Assoumou *et al.*, 1995). Other finding also reported the level of TNF- α was high in patient with late-stage of *T. b. gambiense* infection and was declined dramatically after treatment with melarsoprol (Rhind *et al.*, 1997). Traditionally, Malay community used leaves of *V. pinnata* to treat inflammation including fever and

wounds (Burkill, 1966; Ong and Nordiana, 1999) and based from these evidences suggested the anti-inflammatory response in treating fever and wounds is possibly from compound **2**, **3** and **4** which exhibited moderate antitrypanosomal activity against *T. b. brucei*.

CONCLUSIONS

In conclusion, this finding reported that compound **2**, **3** and **4** were isolated efficiently for the first time from *V. pinnata* by using MPLC. In this study as well reported a moderate antitrypanosomal activity of these compounds against *T. b. brucei* for the first time.

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