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STUDY ON THE CHEMISTRY AND ANTIMICROBIAL ACTIVITY OF *PSYCHOTRIA REEVESII* WALL. (RUBIACEAE)

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SUMMARY

The first chemical investigation on Vietnamese medicinal plant Psychotria reevesii Wall. (Rubiaceae) led to the isolation and structural determination of β-sitosterol and stigmasterol as a mixture, 1-octacosene, and asperglaucide from n-hexane- and $CHCl_3$-soluble fractions of MeOH extract from the aerial parts of P. reevesii. Phytochemical screening based on color reactions, HPLC analysis, and NMR spectroscopy revealed the concentration of condensed tannins in EtOAc- and n-BuOH soluble fractions. The high accumulation of tannins may be responsible for the antibacterial activities of the polar fractions against Staphylococcus aureus, Pseudomonas aeruginosa, Shigella sonnei, and Shigella flexneri. However, they did not exhibit any inhibitory effect against Escherichia coli, Candida albicans, and Candida stellatoides.

Keywords: *Psychotria reevesii*; Rubiaceae; asperglaucide; antibacterial activity; antifungal activity.

I - INTRODUCTION

Psychotria reevesii Wall. (syn. *Psychotria rubra* (Lour.) Poir.) of the family Rubiaceae is a medicinal plant known as Lau or Bo chat in Vietnam [1, 2]. *P. reevesii* is a plant of 1 - 9 m high, widely distributed in Vinh Phu, Thai Nguyen, Lang Son,... The roots and leaves of *P. reevesii* (*Radix et Folium Psychotriae Rubrae*) are used in the treatment of throat inflammation, dysentery, and rheumatic fever; leaves are also used externally to cure wounds. This paper deals with the chemical study and the investigation of antimicrobial activity of the aerial parts of *P. reevesii*.

II - EXPERIMENTAL

General Melting points were recorded on a Boetius micromelting point apparatus without correction. Optical rotations were measured on a Union Giken PM-101 digital polarimeter. Infrared (IR) spectra were recorded on an Impact 410-Nicolet FT-IR spectrometer. Electron impact mass spectra (EIMS) were recorded at 70 eV on a Hewlett Packar 5989B spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV 500 spectrometer. High performance liquid chromatography (HPLC) was run on Dionex HPLC equipment with a Photodiode array detector and an automated sample injector (injection volume 10 µl). Analytical HPLC was performed on an YMC HPLC analytical column (J'sphere ODS-H80, 150×4.6 mm I.D., S-4 µm, 8 nm) using gradient elution from 20% to 100% MeOH in H_2O in 25 min., and MeOH in 5 min. at a flow rate of 1 ml/min. Column chromatography (open CC and flash FC) was performed on silica gel Merck (63 - 100 µm). Thin-layer chromatography (TLC) was performed on Aluminium precoated sheets

(silica gel Merck, 60 F_{254}). Spray reagent vanilin/H_2SO_4 1% and UV light at λ 366 nm were used for visualization.

Plant Material The aerial parts of *P. reevesii* were collected in June 2000 in province Thai Nguyen, Northern Vietnam. The plant was identified by Dr Nguyen Hoanh Coi, Military Institute of Pharmaceutical Control and Research, Hanoi, Vietnam.

Extraction and Isolation The aerial parts of *P. reevesii* were dried at 40 - 50°C, then powdered, and extracted with 70% EtOH in H_2O at room temperature for five times (each time for three days). Successive fractionation of the concentrated EtOH extract between H_2O and solvents of increasing polarities gave the following corresponding soluble fractions: *n*-hexane- (**PH**, 0.2 g, 0.04% on the basis of the dried material), $CHCl_3$- (**PC**, 1.7 g, 0.3%), EtOAc- (**PE**, 116 g, 20.4%), and *n*-BuOH-soluble fractions (**PB**, 17 g, 3%). The *n*-hexane-soluble fraction (0.15 g) was subjected to silica gel CC eluting with 0 - 15% EtOAc in *n*-hexane to give a mixture of *β*-sitosterol and stigmasterol (20 mg), which were determined by comparison of their ^{1}H- and ^{13}C-NMR data with those of an authentic sample. The $CHCl_3$-soluble fraction (1.5 g) was fractionated on a silica gel column eluting with *n*-hexane-EtOAc-$(CH_3)_2CO$ (19:1:0 - 1:2:1) to give 14 fractions. Recrystallization of the precipitated solid from fraction 1 with $CHCl_3$ gave 1-octacosene (**1**) (15 mg). Fraction 8 was purified by a silica gel CC (*n*-hexane-EtOAc-$(CH_3)_2CO$, 5:5:1), followed by recrystallization in $CHCl_3$ to give asperglaucide (**2**) (10 mg).

1-Octacosene (1): white needles, m.p. 30 - 32°C. R_f = 0.82 (silica gel TLC, *n*-hexane-EtOAc-$(CH_3)_2CO$, 5:2:1).

IR (KBr): ν_{max} (cm^{-1}) 3070, 2920, 2860, 1637, 1464, 1375, 995, 909, 723.

EIMS: m/z (%) 392 ($M^{+\cdot}$, $C_{28}H_{56}$, < 0.1), 223 (9.3), 209 (4.2), 195 (5.9), 181 (7.6), 167 (10.2), 153 (14.4), 139 (21.2), 125 (39.8), 111 (62.7), 97 (100), 83 (86.4), 69 (73.7), 57 (96.6), 55 (77.1).

^{1}H-NMR (supported by ^{1}H-^{1}H COSY) ($CDCl_3$, ppm): δ 5.81 (1H, ddt, *J* = 10 Hz, 17 Hz, 7 Hz, H-2), 4.99 (1H, dd, *J* = 17 Hz, 2 Hz, H-1a), 4.92 (1H, dd, *J* = 10 Hz, 2 Hz, H-1b), 2.04 (2H, dt, *J* = 7 Hz, 7.5 Hz, 2H-3), 1.38 (2H, m, 2H-4), 1.25 (46H, m, 2H-5→2H-27), 0.88 (3H, t, *J* = 7 Hz, 28-CH_3).

^{13}C-NMR (supported by DEPT 135, DEPT 90, and HMQC) ($CDCl_3$, ppm): δ 139.3 (d, C-2), 114.1 (t, C-1), 33.8 (t, C-3), 29.7, 29.6, 29.5, 29.4, 29.2, 28.9 (all t, C-4→C-26), 22.7 (t, C-27), 14.1 (q, C-28).

Asperglaucide (2): white needles, m.p. 184 - 188°C (*Lit*. m.p. 185 - 187°C [3]), $[\alpha]^{30}_D$ -45 (c = 1.0, $CHCl_3$). R_f = 0.59 (silica gel TLC, *n*-hexane-EtOAc-$(CH_3)_2CO$, 5:2:1).

IR (KBr): ν_{max} (cm^{-1}) 3315, 3070, 3030, 2950, 2921, 2850, 1726, 1661, 1630, 1600, 1532, 1450, 1380, 1370, 1261, 1055, 745, 703, 604.

EIMS: m/z (%) 444 ($M^{+\cdot}$, $C_{27}H_{28}O_4N_2$, 1.5), 384 (1.1), 368 (0.5), 353 (3.9), 323 (3.4), 311 (6.2), 293 (2.0), 269 (12.8), 253 (11.5), 252 (66.4), 232 (7.8), 225 (15.8), 224 (51.1), 194 (1.7), 190 (2.0), 176 (4.2), 172 (11.7), 131 (8.5), 105 (100), 91 (9.7), 77 (18.4), 60 (2.0).

^{1}H-NMR (supported by ^{1}H-^{1}H COSY and HMBC) ($CDCl_3$, ppm): δ 7.71 (2H, d, *J* = 8.5 Hz, H-3″, H-7″), 7.52 (1H, t, *J* = 8.5 Hz, H-5″), 7.43 (2H, t, *J* = 8.5 Hz, H-4″, H-6″), 7.26 (2H, m, H-5′, H-9′), 7.26 (3H, m, H-6′, H-7′, H-8′), 7.15 (3H, m, H-6, H-7, H-8), 7.07(2H, d, *J* = 8.3 Hz, H-5, H-9), 6.76 (1H, d, *J* = 7.5 Hz, NH-1′a), 6.0 (1H, d, *J* = 8.5 Hz, NH-1″a), 4.76 (1H, m, H-2′), 4.34 (1H, m, H-2), 3.92 (1H, dd, *J* = 11 Hz, 5 Hz, H-1b), 3.81 (1H, dd, *J* = 11 Hz, 4.5 Hz, H-1a), 3.21(1H, dd, *J* = 13.5 Hz, 6 Hz, H-3′b), 3.06 (1H, dd, *J* = 13.5 Hz, 8.5 Hz, H-3′a), 2.75 (2H, m, 2H-3), 2.02 (3H, s, C<u>H</u>$_3$COO-).

^{13}C-NMR (supported by DEPT 135, DEPT 90, HMQC, and HMBC) ($CDCl_3$, ppm): δ 170.8 (s, CH_3<u>C</u>OO-), 170.3 (s, C-1′), 167.1 (s, C-1″), 136.7 (s, C-4), 136.6 (s, C-4′), 133.7 (s, C-2″), 131.9 (d, C-5″), 129.3 (2d, C-5, C-9), 129.1 (2d, C-5′, C-9′), 128.8 (2d, C-4″, C-6″), 128.7 (2d, C-6, C-8), 128.6 (2d, C-6′, C-8′), 127.2 (d, C-7′),

127.1 (2d, C-3″, C-7″), 126.8 (d, C-7), 64.6 (t, C-1), 54.99 (d, C-2′), 49.5 (d, C-2), 38.4 (t, C-3′), 37.4 (t, C-3), 20.8 (q, $\underline{C}H_3COO$-).

Preparation of test fractions ***PE$_1$*** *and* ***PE$_2$*** *from fraction* ***PE***

The EtOAc-soluble fraction **PE** (5 g) was washed several times with EtOAc. After removing the insoluble material, toluene was added to the soluble part up to a volume corresponding to a 1/1 (EtOAc-toluene, v/v). The soluble fraction, which was separated from insoluble **PE$_2$** fraction, was concentrated under reduced pressure at 50°C to give the **PE$_1$** fraction.

Antibacterial and Antifungal Assay *Staphylococcus aureus* ATTC 29213, *Staphylococcus aureus* BN, *Pseudomonas aeruginosa* ATTC 27853, *Shigella sonnei* BN, *Shigella flexneri* BN, *Escherichia coli* ATCC 25922, *Candida albicans* BN, and *Candida stellatoides* BN were used for the assay. The disk diffusion method (8 mm filter papers) was used for the preliminary screening [4]. Gentamycin and mycostatin were used as reference antibiotics.

III - RESULTS AND DISCUSSION

The dried aerial parts of *P. reevesii* Wall. (Rubiaceae) were extracted with 70% EtOH in H_2O at room temperature to give an EtOH extract. Then, the extract was subjected to the fractionation between H_2O and solvents of increasing polarities to afford the corresponding *n*-hexane- (**PH**), $CHCl_3$- (**PC**), EtOAc- (**PE**), and *n*-BuOH-soluble (**PB**) fractions.

Phytochemical screening of soluble fractions of the MeOH extract from P. reevesii

The phytochemical analysis was carried out to detect the main classes of phytochemical constituents in *n*-hexane-, $CHCl_3$-, EtOAc-, and *n*-BuOH-soluble fractions using the characteristic color reactions [5]. The results were summarized in the table 1.

Table 1: Phytochemical screening of soluble fractions from *P. reevesii*

Soluble fraction	Reagent										Main class of photochemical
	1	2	3	4	5	6	7	8	9	10	
PH	–	–	–	–	–	–	–	+	–	Violet	Phytosterol
PC	–	–	–	+	–	–	+	–	–	Violet	Polyphenol
PE	–	–	+	+	+	+	+	–	–	Red	Flavonoid, tannin
PB	–	–	+	+	+	+	+	–	–	Red	Flavonoid, tannin

–: negative reaction; +: positive reaction Reagents: 1: Mayer; 2: Dragendorff; 3: Shinoda; 4: Diazo; 5: H_2SO_4; 6: NaOH; 7: $FeCl_3$; 8: Liebermann-Burchardt; 9: formation of foams with NaOH or HCl; 10: Vanilin/H_2SO_4.

It is clear, that the phytosterols were detected in *n*-hexane-soluble fraction, and polyphenols were found in $CHCl_3$-soluble fraction. The high concentration of tannins, which were detected in EtOAc- and *n*-BuOH-soluble fractions, may lead to "non-specific" biological activities of *P. reevesii* in many bioassay systems.

We got further evidences for the presence of tannins in the EtOAc- and *n*-BuOH-soluble fractions since it correlated chromatographically with a broad "hump" eluting over the polar/moderately polar region of the HPLC chromatograms (figures 1 and 2).

NMR methods also proved the concentration of condensed tannins in the EtOAc- and *n*-BuOH-soluble fractions. After fractionation of these soluble fractions on silica gel, the obtained fractions were collected on the basis of major spots on TLC, which showed characteristic ^{1}H- and ^{13}C-NMR signals (data not shown) for catechin moieties.

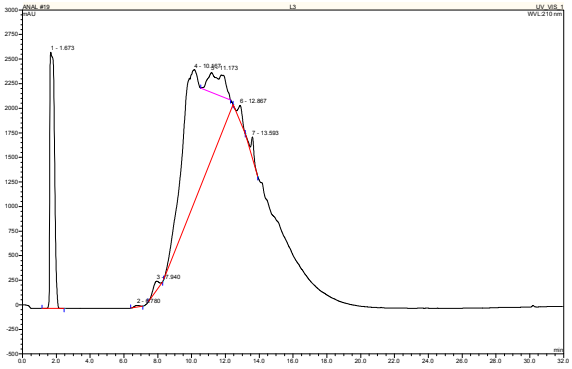


Figure 1: HPLC Profile of the EtOAc-soluble Fraction from *P. reevesii*

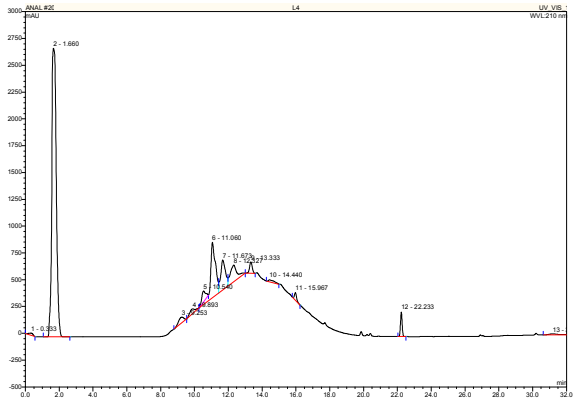


Figure 2: HPLC Profile of the *n*-BuOH-soluble Fraction from *P. reevesii*

Susceptibility of bacteria and fungi to soluble fractions from P. reevesii

The antibacterial and antifungal activities of the tannin-containing fractions **PE** and **PB**, together with the test fractions $\mathbf{PE_1}$ and $\mathbf{PE_2}$ were evaluated in this study. $\mathbf{PE_1}$ and $\mathbf{PE_2}$ were prepared from **PE** on the basis of the solubility of compounds in **PE** in different solvent systems. The data in table 2 showed the noticeable activities of **PE**, **PB**, $\mathbf{PE_1}$, and $\mathbf{PE_2}$ against *S. aureus* strains. In the case of *P. aeruginosa*, the activity was improved from **PE** (0 mm) to the test fractions $\mathbf{PE_1}$ (11.3 mm) and $\mathbf{PE_2}$ (11.8 mm). However, the activities against *S. flexneri* and *S. sonnei* were decreased in case of $\mathbf{PE_2}$, showing the specific concentration of active compounds against *Shigella* strains in $\mathbf{PE_1}$. It is noticeable that all the test fractions did not exhibit any inhibitory effect against *Escherichia coli*, *Candida albicans*, and *Candida stellatoides*.

*Isolation and structure determination of compounds **1** and **2***

The $CHCl_3$-soluble fraction was subjected to repeated column chromatography on silica gel and recrystallization to give compounds **1** and **2**.

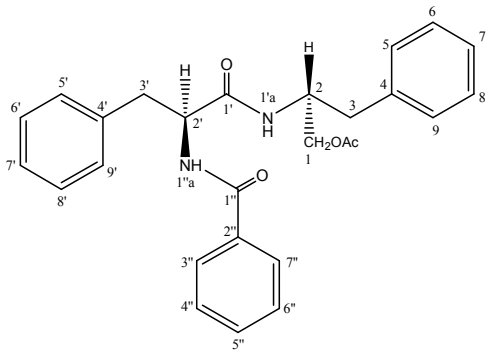
Compound **1** was isolated as a white needle. The IR spectrum showed the presence of a vinyl

Table 2: Susceptibility of bacteria and fungi to soluble fractions from *P. reeversii*

No.	Organism	Diameter of inhibition zone (mm)			
		PE[a)]	**PB**[a)]	$\mathbf{PE_1}$[a)]	$\mathbf{PE_2}$[a)]
1	*Staphylococcus aureus* ATTC 29213	11.8	13.7	15.7	11.4
2	*Staphylococcus aureus* BN[c)]	12.3	13.4	15.5	11.3
3	*Pseudomonas aeruginosa* ATTC 27853	0[b)]	12.4	11.3	11.8
4	*Shigella sonnei* BN[c)]	8.5	12.8	10.7	0[b)]
5	*Shigella flexneri* BN[c)]	9.7	10.9	11.6	0[b)]
6	*Escherichia coli* ATCC 25922	0[b)]	0[b)]	0[b)]	0[b)]
8	*Candida albicans* BN[c)]	0[b)]	0[b)]	0[b)]	0[b)]
9	*Candida stellatoides* BN[c)]	0[b)]	0[b)]	0[b)]	0[b)]

[a)] 3 mg/disk; [b)] 0 means no visible zone of inhibition; [c)] from Bach Mai Hospital patients.

group (ν_{max} 1637, 995, 909 cm^{-1}). The ^1H- and ^{13}C-NMR spectroscopic data showed a vinyl group [δ_H 5.81 (ddt, J = 10 Hz, 17 Hz, 7 Hz), 4.99 (dd, J = 17 Hz, 2 Hz), and 4.92 (dd, J = 10 Hz, 2 Hz); δ_C 139.3 (d), 114.1(t)], a long aliphatic hydrocarbon chain [δ_H 2.04 (dt, J = 7 Hz, 7.5 Hz), 1.38 (m), 1.25 (m)], and a terminal methyl group [δ_H 0.88 (t, J = 7 Hz), δ_C 14.1 (q)]. Thus structure of **1** was deduced to be a natural 1-ankene. The molecular formula of **1** was revealed to be 392 ($M^{+\cdot}$, $C_{28}H_{56}$) by EIMS spectrum, leading to the structure of **1** as 1-octacosene.



2

Figure 3: Chemical Structure of Asperglaucide (**2**)

Compound **2** was isolated as white needles. The molecular formula of **2** was determined to be 444 ($M^{+\cdot}$, $C_{27}H_{28}O_4N_2$) by EIMS spectrum. The IR spectrum showed the presence of amide (ν_{max} 3315, 1661, and 1630 cm^{-1}) and ester (ν_{max} 1726 and 1261 cm^{-1}) functional groups, and aromatic rings (ν_{max} 1600, 1532, and 1450 cm^{-1}). The ^1H- and ^{13}C-NMR spectroscopic data exhibited the presence of two secondary amide groups [δ_H 6.76 (d, J = 7.5 Hz), 6.0 (d, J = 8.5 Hz); δ_C 171.8 (s), 167.7 (s)], three monosubstituted benzene rings, an acetyloxymethyl group [δ_H 3.92 (dd, J = 5 Hz, 11 Hz), 3.81 (dd, J = 4.5 Hz, 11 Hz), 2.02 (s); δ_C 64.6 (t), 170.8 (s), 20.8 (q)], and two -CH_2-CH(NH-)- groups. Two main structural fragments were constructed on the basis of the ^1H-^1H COSY spectrum and they were connected to the amide centers and benzene rings using HMBC correlations (Fig. 4). Finally the sign and value of the optical rotation were conclusive for the stereochemistries at C-2 and C-2′ [6]. Thus the structure of **2** was deduced to be asperglaucide, an antiallergic compound previously isolated from a *Euphorbia* species [6], *Pteris multifida* Poir. (Pteridaceae) [7], and from the fungus *Aspergillus glaucus* [6]. EIMS fragmentation of **2** (Fig. 5) is in full agreement with this structure.

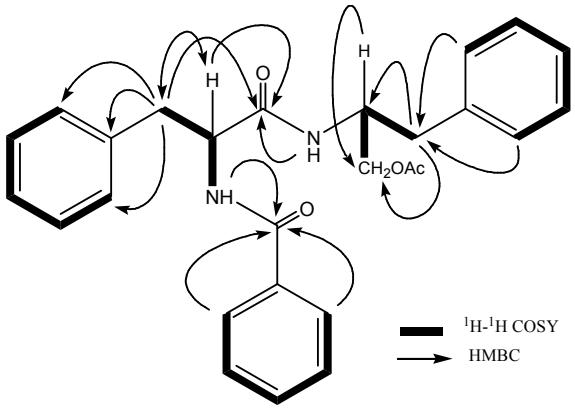


Figure 4: ^1H-^1H COSY and Selected HMBC Correlations of **2**

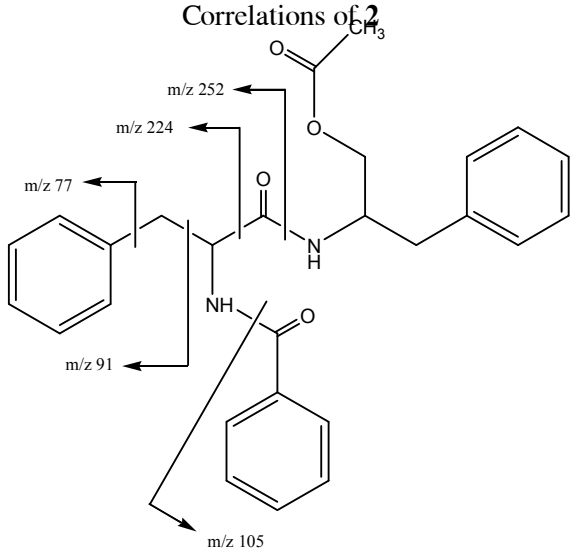


Figure 5: EIMS Fragmentations of **2**

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