ISOLATION OF SOME COMPOUNDS FROM ETHYL ACETATE EXTRACT FROM THE STEMS OF *ROUREA MINOR*

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Abstract: *Rourea minor is a species which belongs to the family of Connaraceae. According to traditional medicine, the species Rourea minor has biological abilities such as antibiotic, antibacterial, hemostatic and wound-healing activities. In this study, from the ethyl acetate extract of Rourea minor stems collected in Ben En national park, Thanh Hoa province, three compounds were isolated including β-sitosterol, vanilic acid and sitoindoside I. The structures of these compounds have been identified by NMR, MS spectroscopic data and comparison with the reported literature.*

Keywords: *Rourea minor, isolation, β-sitosterol, vanilic acid, sitoindoside I.*

1. Introduction

Rourea minor subsp. *microphylla* Vidal. is a climbing shrub or small tree of the *Rourea* genus belonging to the Connaraceae family which includes about 100 species [1] [2]. Studies on some species of the genus Rourea have shown that extracts and compounds isolated from this genus have antibacterial, anti-inflammatory, anti-diabetic, antioxidant, hepatoprotective, and anti-fever activities [3] [4] [5]. The plant is widely distributed in China, Vietnam, Laos, India, Sri Lanka, grows and develops in sunny areas. In Vietnam, the tree grows a lot in the middle and plateau provinces. According to folk experience, this plant is used as a tonic for women after childbirth, also used to treat yellow, red urine, urinary incontinence, and boils. The plant is harvested almost all year round and is used in both fresh and dried forms [6]. Studies on the chemical composition and biological activities of *Rourea minor* plants are quite limited. Studies of Preeti Kulkarni, Anu Chaudhary and their colleagues showed that ethanol extract, methanol extract and aqueous extract of Rourea minor root have anti-diabetic and anti-obesity effects in rats [7] [8]. A recent study by Nguyen Ngoc Hieu [9] Chemical composition of *Rourea minor* tree showed that there are phenolic compounds, alkaloids, fatty acids in this plant and two new substances, lethedocin 3′-O-β-D-glucopyranoside and 3-O-(6′–O-vanilloyl)-β-D-glucopyranosyl 4-hydroxyphenethyl alcohol [9]. To further study on the chemical composition of RM, in this study, we report the isolation of three compounds obtained from the ethyl acetate extract of this plant collected in Ben En National Park, Thanh Hoa province.

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2. Materials and Methods

2.1. Plant materials

The plant samples were collected from Ben En National Park in 2018 and identified by Dr. Do Ngoc Dai, Vinh University of Economics with the scientific name as *Rourea minor* subsp. *microphylla* Vidal., belonging to the Connariaceae family. A voucher specimen (MK-301) was deposited at the Faculty of Natural Sciences, Hong Duc University.

2.2. General experimental procedures

The ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded by a Bruker AM500 FT-NMR spectrometer using TMS as an internal standard. The electrospray ionization mass spectra (ESI-MS) were obtained on an Agilent 1260 series single quadrupole LC/MS system. Column chromatography (CC) was performed on silica gel (Merck, 230-400 mesh) or Sephadex LH-20. Thin layer chromatography used precoated silica gel plates (Merck 60 F_{254}). Compounds were visualized by spraying with Ce-Mo stain. Polar rotation angle was measured on a JASCO P-2000 Polarimeter (Institute of Marine Biochemistry).

2.3. Extraction and isolation

The stem of *Rourea minor* was dried in the shade, and then ground into powder. The dried stems (3.0 kg) were extracted with MeOH (10 L) at room temperature for 24 h, then the extract was removed and the medicinal residues were further extracted with MeOH, this process was repeated 5 times. The extract was combined and concentrated under reduced pressure to obtain a MeOH residue. Dissolved the MeOH residue with 1L of distilled water, and then distributed the extract with ethylacetate solvent. The extract after removing the solvent obtained 9.8 grams of EtOAc residue.

The EtOAc residue (9.8 g) was separated on a normal-phase silica gel chromatographic column, eluting the gradient with the solvent system n-hexane/EtOAc (0-100% ethylacetate). Checked by thin layer chromatography, combined tubes of the same composition and remove the solvent to obtain the corresponding 9 fractions from K1-K9. The K2 fraction (1.0 g) was crystallized the fraction to obtain compound **1** (40 mg).

The K5 fraction (0.32 g) was purified through a silica gel column with the solvent system n-hexane/EtOAc (8/2) to get 5 fractions K5.1 to K5.5. The K5.2 fraction (15.3 mg) was purified through a normal phase silica gel column, eluted with the solvent system n-hexane /EtOAc (6/4) to obtain compound **2** (2.1 mg). The K5.5 fraction (0.11 g) was purified through a normal phase silica gel column, eluted with the n-hexane/acetone solvent system (7/3) to yield compound **3** (3.2 mg).

2.4. Properties and spectral values of the isolated compounds

β-sitosterol **(1)**: White powder, ESI-MS *m/z* 415.1 $[M+ H]^{+}$. ^{1}H-NMR (500MHz, $CDCl_3$), δ (ppm): 5.34 (1H, br d, J = 4.5 Hz), 3.52 (1H, m), 2.20-2.31 (2H, m), 1.95-2.02

(2H, m), 1.83-1.86 (3H, m), 1.47-1.70 (9H, m), 1.20-1.38 (6H, m), 0.90-1.20 (8H, m), 1.01 (3H, s), 0.92 (3H, d, J = 6.5 Hz), 0.84 (3H, t, J = 7.5Hz), 0.83 (3H, d, J = 7.0 Hz), 0.80 (3H, d, J = 7.0 Hz), 0.68 (3H, s). ^{13}C-NMR (125 MHz, $CDCl_3$), δ (ppm): 140.7, 121.7, 71.8, 56.7, 56.0, 50.1,45.8, 42.3, 42.3, 39.8, 37.2, 36.5, 36.1, 33.9, 31.9, 31.9, 31.6, 29.1, 28.2, 26.1, 24.3, 23.1, 21.1, 19.8, 19.4, 19.0, 18.7, 11.9, 11.8.

Vanilic acid (**2**): Light yellow solid. ESI-MS *m/z* 169 $[M+H]^+$. ^{1}H-NMR (500 MHz, CD_3OD) δ (ppm): 7.58 (1H, d, J = 1 Hz, H-2), 7.56 (1H, dd, J = 2.0, 8.0 Hz, H-6), 6.85 (1H, d, J = 8.5 Hz, H-5), 3.91 (3H, s, Ome). ^{13}C-NMR (125 MHz, CD_3OD) δ (ppm): 168.0 (COOH), 151.5 (C-3), 147.6 (C-4), 124.1 (C-1), 123.0 (C-6), 116.7 (C-2), 114.7 (C-5), 55.4 (OCH_3).

Sitoindoside I (**3**): White solid. ESI-MS *m/z* 815 $[M+H]^+$. ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 5.37 (1H, m, H-6), 4.48 (1H, dd, J = 12.5, 4.5 Hz, H-6′a), 4.38 (1H, d, J = 7.5 Hz, H-1′), 4.28 (1H, br d, J = 12.5Hz, H-6′b), 3.4-3.6 (5H, m, H-3, 2′, 3′, 4′, 5′), 1.01 (3H, s, H-19), 0.92 (3H, d, J = 6,5Hz, H-21), 0.88 (3H, t, J = 7.0Hz, Me-16″), 0.85 (3H, t, J = 7,5Hz, Me-29), 0.84 (3H, d, J = 6.5 Hz, Me-27), 0.82 (3H, d, J = 6.5 Hz, Me-26), 0.68 (3H, s, Me-18). ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm): 174.7 (C-1″), 140.3 (C-5), 122.2 (C-6), 101.2 (C-1′), 79.6 (C-3), 76.0 (C-3′), 74.0 (C-5′), 73.6 (C-2′), 70.1 (C-4′), 63.2 (C-6′), 56.8 (C-14), 56.1 (C-17), 50.2 (C-9), 45.9 (C-24), 42.3 (C-13), 39.8 (C-12), 38.9 (C-4), 37.3 (C-1), 36.7 (C-10), 36.2 (C-20), 34.2 (C-2″), 34.0 (C-22), 31.9 (C-7, 8, 14″), 29.2–29.7 (C-2, 4″-13″), 28.3 (C-16), 26.1 (C-23), 25.0 (C-3″), 24.3 (C-15), 23.1 (C-28), 22.7 (C-15″), 21.1 (C-11), 19.8 (C-27), 19.4 (C-19), 19.0 (C-26), 18.8 (C-21), 14.1 (C-16″), 12.0 (C-29), 11.9 (C-18).

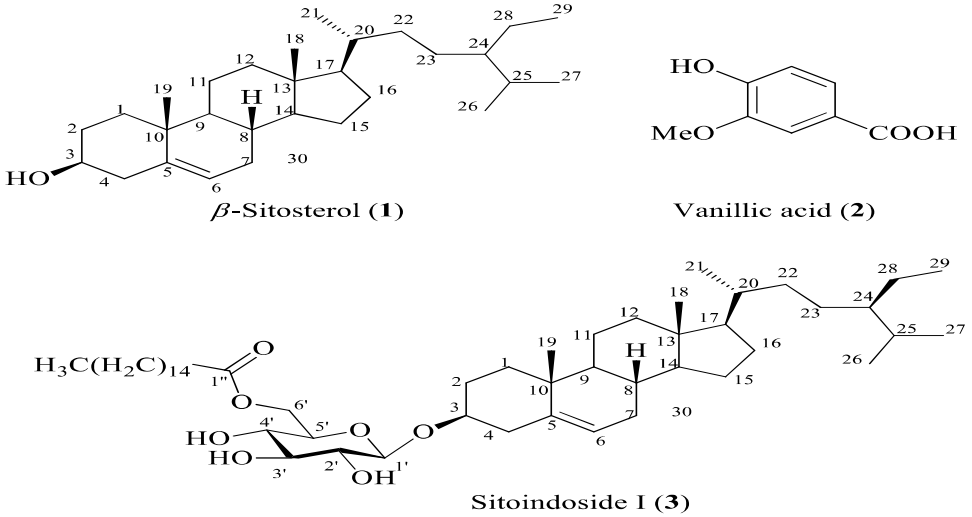

Figure 1**. Chemical structures of the isolated compounds **1-3

3. Results and Discussion

3.1. Determination of the structure of β-sitosterol (1)

Compound **1** was obtained as a white powder, mass spectrometry (ESI-MS) has a pseudo molecular ion peak of *m/z* $[M+H]^+$ of 415.1, indicating that the molar mass of compound **1** is 414.1 corresponding to the molecular formula $C_{29}H_{50}O$. On the ^{1}H-NMR

spectrum of **1**, there are typical signals for a sterol compound with a double bond with a signal proton at position δ_H 5.34 (br d, J = 5 Hz). There is a hydroxyl group with signal CH-OH at position δ_H 3.52 (1H, m) and 6 methyl groups including 2 singlets at δ_H 0.68 (3H, s) and 1.01 (3H, s), 3 doublet signals at δ 0.92 (3H, d, J= 6.5 Hz), 0.83 (3H, d, J= 7.3 Hz), 0.80 (3H, d, J= 6.8 Hz), and one triplet signal at δ 0.84 (3H, t, J= 7.5 Hz). In addition, other signals of the sterol ring, ^{13}C-NMR and DEPT spectra for **1** have 29 carbons, including 6 CH_3 groups, 11 CH_2 groups, 9 CH groups and 3 quaternary carbons. From the physical properties, the ^{1}H-NMR, ^{13}C-NMR, ESI-MS spectral data of **1** and the comparison of the spectral data with reference material is suggested that this compound is β-sitosterol [10].

3.2. Determination of the structure of vanillic acid (2)

Compound **2** was isolated as a pale yellow solid. ESI-MS mass spectroscopy for pseudo-molecular ion peak *m/z* 169 $[M+H]^+$ allows prediction of compound 3 with molecular formula $C_8H_8O_4$ (M = 168). On the ^{1}H-NMR spectrum of compound **2**, the resonance signal of 3 aromatic ring protons of the ABX system appears at δ_H 7.58 (1H, d, J = 1 Hz, H-2), 7.56 (1H, dd, J = 2.0) , 8.0 Hz, H-6), 6.85 (1H, d, J = 8.5 Hz, H-5) and 1 methoxy group. ^{13}C-NMR spectrum shows carboxylic acid group signal at 168.0 (COOH) and 6 aromatic carbon signal at 151.5 (C-3), 147.6 (C-4), 124.1 (C-1), 123.0 (C-6), 116.7 (C-2), 114.7 (C-5), methoxy group at 55.4 (OCH_3). From the above analyzed mass spectrometry and nuclear magnetic resonance spectroscopy data and compared with reference literature [9], compound 2 was identified as a vanillic acid or 4-hydroxy-3-methoxybenzoic acid compound.

3.3. Determination of the structure of sitoindosid I (3)

Compound **3** is a white solid. ESI-MS spectrum gives pseudo molecular ion peak at *m/z* $[M+H]^+$ 815, corresponding to the molecular formula $C_{51}H_{90}O_7$ (M=814). On the ^{1}H-NMR spectrum, there are signals specific to sterol glucoside ester compounds. The sterol framework signals were observed with a double bond at δ_H 5.37 (1H, m, H-6); an oxymethin group at δ_H 3.5 (1H, m, H-3) and 6 methyl groups consisting of 2 singlets at δ_H 0.68 (3H, s) and 1.01 (3H, s), 3 doublets at δ_H 0, 92 (3H, d, J = 6.5 Hz), 0.84 (3H, d, J = 6.5Hz), 0.82 (3H, d, J = 6.5 Hz), and a triplet at δ_H 0.85 (3H, t, J = 7.5Hz). The signal of glucose molecule was detected by 4 oxymethin groups at position δ_H 3.4-3.6 (4H, m), oxymethylene group signal at δ_H 4.48 (1H, dd, J = 12.5, 4.5 Hz, H-6′a) and 4.28 (1H, br d, J = 12.5 Hz, H-6′b); and the sugar anomer proton signal at δ_H 4.38 (1H, d, J = 7.5 Hz). The signal of a fatty acid was detected based on the CH_2 group signal at δ_H 2.35 (2H, t, J=7.5 Hz), the long-terminal CH_3 methyl group at δ_H 0.88 position (3H, t). , J = 7.0 Hz) and long-chain CH_2 groups signal at δ_H 1.25 (m). On the ^{13}C-NMR and DEPT spectra, there are also signals characteristic of sterol glucoside ester compound. Signals of the sterol nucleus include 6 methyl groups at δ_C 19.8 (C-27), 19.4 (C-19), 19.0 (C-26), 18.8 (C-21), 12, 0 (C-29), 11.9 (C-18); oxymethin group at δC 79.6 (C-3) and signal of 2 olefinic carbon at δ_C 140.3 (C-5) and 122.2 (C-6). The signal of the sugar group consists of a carbon anomer

signal at δ_C 101.2 (C-1′) along with 5 other signals of 1 oxymethylene group and 4 oxymethin groups at δ_C 76.0 (C-3′), 74.0 (C-5′), 73.6 (C-2′), 70.1 (C-4′) and 63.2 (C-6′). The ester functional group was detected by the C=O group signal at δC 174.7 (C-1″). Other long-chain fatty acid signals include a signal at δ_C 34.2 (C-2″), a saturated circuit signal at CH_2 29.2-29.7 (C-4″-13″) and a methyl group, at δ_C 14.1 (C-16″). From the data of mass spectrometry and NMR spectrum analyzed above, combined with comparison with published material [12], compound **3** was identified as sitoindoside I.

4. Conclusion

A phytochemical investigation of the ethyl acetate extract of the stems of *Rourea minor* led to the isolation of three compounds including *β*-sitosterol, vanilic acid and sitoindoside I. This is the first study on the chemical composition of *Rourea minor* species collected in Ben En National Park, Thanh Hoa province. Investigation on the chemical composition as well as biological activities of this species will be continued in the near future.

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