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CYTOTOXIC CONSTITUENTS FROM AERIAL PARTS OF *HELICTERES HIRSUTA* COLLECTED IN BINH PHUOC PROVINCE

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Abstract. *Helicteres hirsuta* is a member of *Helicteres* genus of the plant family Sterculiaceae and widely found in countries of South Asia, such as Vietnam, Lao, and Thailand. In recent years, it is known as a new folk medicine protecting and securing people against human lung carcinoma, hormone-dependent human prostate carcinoma, and human liver. In this work, three compounds were isolated and structurally elucidated from the aerial parts of *Helicteres hirsuta* collected in Binh Phuoc province (October 2016). They are β-stigmasterol (**1**), protosta-17(20),24-dien-3β-ol (**2**), and icosanoic acid (**3**). Compound **2** has a remarkable cytotoxic activity against SK-LU-1, Hep-G2, and Hela cell lines with IC_{50} values from 32.86 to 77.31 μg/mL. Meanwhile, compound **3** shows a moderate cytotoxic activity against SK-Mel-2, AGS, SK-LU-1, Hep-G2, and Hela cell lines with IC_{50} values from 59.02 to 80.87 μg/mL.

Keywords: *Helicteres hirsuta*, cytotoxicity, icosanoic acid, protosta-17(20),24-dien-3β-ol

1 Introduction

Helicteres hirsuta, called "An Xoa" in Vietnamese, has been used as a folk medicine for curing lung carcinoma, human liver, etc. [1, 2]. Previous studies on *Helicteres* species have disclosed that flavonoids, neolignans, quinines, and triterpenoids [3–6] are the major chemical constituents. There have been no reports on the biological activity or phytochemical investigation of *Helicteres hirsuta* in Vietnam. In this paper, we describe our preliminary phytochemical study on the chemical constituents of the aerial parts of *Helicteres hirsuta* collected in Binh Phuoc province.

2 Material and methods

2.1 General experimental procedures

NMR (^{1}H, ^{13}C NMR, DEPT) spectra were recorded on a Bruker Avance 500 MHz. The chemical shift (δ) values are given in ppm with tetramethylsilane (TMS) as an internal standard, coupling

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constant *J* (by Hz) for ^{1}H NMR spectrum. ESI-LC-MS spectra were recorded on an Agilent LC mass spectrometer. Silica gel (Merck Co., Germany) was used for flash chromatography. Thin-layer chromatography (TLC) was carried out on precoated Si gel GF_{254} (Merck Co., Germany), and TLC spots were viewed at 254, 302 and 366 nm and visualized by spraying with a vanillin – 10% H_2SO_4 solution.

2.2 Plant material

The aerial parts of *Helicteres hirsuta* were collected in the Ta Thiet mountain, Loc Thinh commune (Loc Ninh Dist., Binh Phuoc province) in October 2016. The plant material was identified by Do Huu Thu, Institute of Ecology, Natural Resource and Biology, Vietnam Academy of Science and Technology, Vietnam. A voucher specimen was deposited in the Department of Organic Chemistry, Hanoi National University of Education (AX10.2016).

2.3 Extraction and isolation

The air-dried aerial parts of *H. hirsuta* (2.0 kg) were ground into powder and extracted with 80% methanol (MeOH, 10 L × 3) at room temperature to obtain a crude extract (145 g). The crude extract was subjected to fractional extraction and then vacuum evaporation, giving *n*-hexane extract (15 g), ethyl acetate extract (EtOAc, 86 g), butanol extract (28 g), and MeOH extract (41 g). The EtOAc extract was subjected to column chromatography over silica gel and eluted with *n*-hexane:EtOAc gradient from 10:1 to 1:100. Ten fractions were successively obtained. Fraction 2 (1.20 g) was precipitated as white crystals, recrystallized in EtOAc to give compound **1** (25 mg). Fraction 5 (520 mg) was separated by column chromatography (SiO_2), eluting with *n*-hexane:EtOAc gradient from 10:1 to 1:100 to give compound **2** (12 mg). Fraction 8 (72 mg) was separated by column chromatography (SiO_2), eluting with EtOAc:MeOH gradient from 100:0 to 1:100 to give compound **3** (5 mg).

Compound 1 (β-Stigmasterol): White needle crystals; mp. 174-176 °C. ^{1}H NMR (500 MHz, $CDCl_3$), δ_H (ppm): 3.53 (1H, m, H-3), 5.25 (1H, m, H-5), 0.90 (3H, d, *J* = 6.5 Hz, H-19), 5.07 (1H, dd, *J* = 15.0, 8.0 Hz, H-20), 5.21 (1H, dd, *J* = 15.0, 8.0 Hz, H-21), 0.84 (3H, t, *J* = 7.0 Hz, H-24), 0.82 (3H, d, *J* = 7.0 Hz, H-26), 0.80 (3H, d, *J* = 7.0 Hz, H-27), 0.67 (3H, s, H-28), 0.96 (3H, s, H-29); ^{13}C NMR (125 MHz, $CDCl_3$) (Table 1).

Compound 2 (Protosta-17(20),24-dien-3β-ol): White amorphous powders; ^{1}H NMR (500 MHz, $CDCl_3$), δ_H (ppm): 3.22 (1H, dd, *J* = 11.0, 4.5 Hz, H-3), 5.26 (1H, t, *J* = 3.5 Hz, H-24), 0.77 (3H, s, H-

18), 1.08 (3H, s, H-19), 0.87 (3H, s, H-26), 0.85 (3H, s, H-27), 0.98 (3H, s, H-28), 0.79 (3H, s, H-29), 0.93 (3H, s, H-30); ^{13}C NMR (125 MHz, $CDCl_3$) (Table 1).

Compound 3 (Icosanoic acid): white amorphous powders. ESI-LC-MS (m/z): 313.1 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) (Table 2).

2.4 Cytotoxicity

Pure compounds **1-3** were tested against Hep-G2 (human hepatocellular carcinoma), SK-Mel-2 (human malignant melanoma), AGS (human gastric adenocarcinoma), SK-LU-1 (human lung carcinoma), and Hela (human cervix carcinoma) cell lines from American Type Culture Collection according to the method described by Scudiero et al. [7]. The cell lines were cultured in the RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) under standard conditions, sterilised with 5% CO_2, 37 °C, 98% humidity and harvested at log phase for assays. In this assay, 200 µL of cells at the concentration of 3×10^4 cells/mL were inoculated into a 96-well plate in the RPMI 1640 medium. Pure compounds **1–3** were applied at final concentrations 128, 32, 8, 2 and 0.5 µg/mL, and the cultures were incubated for 3 days at 37 °C with 5% CO_2. Then, 50 µL of MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide prepared at 1 mg·mL^{-1} in FBS was added to the microculture well. After 4 h of incubation, 250 µL of the supernatant was removed from each well and 100 µL of DMSO was added and thoroughly mixed. The absorbance was measured at 540 nm in the Genios TECAN spectrophotometer. The IC_{50} value was calculated on the basis of the percentage of growth inhibition $(OD_{control} – OD_{sample})/OD_{control}$. Ellipticine was used as a referential compound.

3 Results and discussion

3.1 Structural identification of isolated compounds

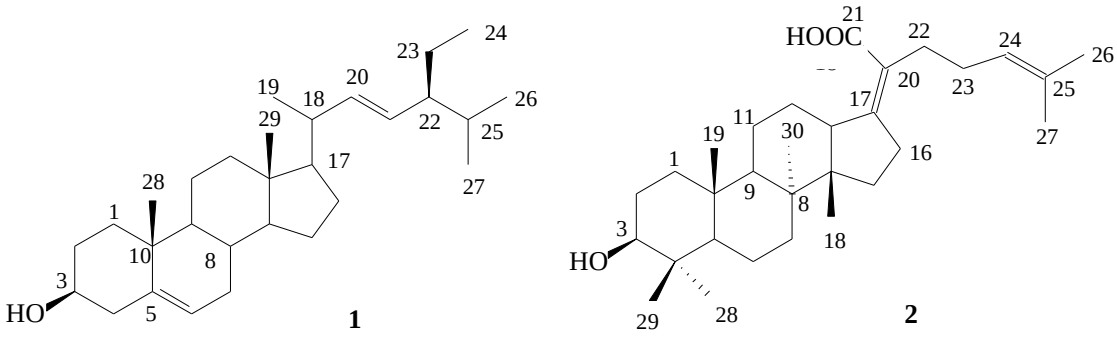
Compound 1

Compound **1** was isolated as a white crystal, well soluble in chloroform and DMSO. The ^{1}H NMR spectrum of compound **1** shows the presence of two methyl singlets at δ 0.67 and 0.96 ppm, three methyl doublets at δ 0.80, 0.82 and 0.90 ppm, and a methyl multiplet at δ 0.84 ppm. The ^{1}H NMR spectrum together with ^{13}C NMR and HSQC spectra also shows the presence of three olefinic protons at δ 5.07, 5.21, and 5.25 ppm, suggesting that they belonged to two double bonds: >C=CH– and –CH=CH–. The proton that appears as a multiplet at δ 3.53 ppm was assigned to a methine proton, bonded to the carbinol carbon (–C**H**OH–). From the analysis above, we suggested that compound **1** should belong to the group of sterols.

The ^{13}C NMR spectrum of compound **1** shows the presence of six methyl (δ 11.4, 11.5, 18.7, 19.2, 20.3, and 22.5), nine methylene, eleven methine, and three quaternary carbons. Four carbon signals at δ 141.1 and 119.8, and 137.0 and 128.7 were assigned to two double bonds: a cyclic >C=CH– and an acyclic –CH=CH–, respectively. The carbon signal at δ 69.8 was assigned to a cyclic carbinol carbon of a sterol (C-3). The spectral data above support the presence of a sterol skeleton having a hydroxyl group at the C-3 position with two double bonds at C-5/C-6 and C-20/C-21 with six methyl groups (Table 1). Thus, compound **1** was assigned as the known **β-stigmasterol** (Fig.1). The physical and spectral data are consistent with the reported literature values [8]. This sterol is very popular in plants.

Table 1. ^{13}C NMR (125 MHz, $CDCl_3$) spectral data (δ_c, ppm) of compound **1** and **2**

No.	1	2	No.	1	2	No.	1	2
1	36.7	32.9	**11**	20.6	22.4	**21**	128.7	171.8
2	33.3	29.7	**12**	39.0	27.2	**22**	45.1	37.0
3	69.8	79.1	**13**	41.6	42.0	**23**	25.8	28.1
4	41.9	39.5	**14**	56.0	52.7	**24**	11.4	125.9
5	141.1	47.6	**15**	24.3	30.6	**25**	30.9	137.9
6	119.8	18.3	**16**	28.7	31.5	**26**	20.3	15.5
7	31.7	36.0	**17**	55.9	157.4	**27**	19.2	17.1
8	31.3	38.8	**18**	41.5	16.9	**28**	18.7	28.0
9	50.2	47.9	**19**	22.5	23.6	**29**	11.5	13.9
10	35.8	36.7	**20**	137.0	125.4	**30**	–	21.2



1 **2**

Fig. 1. Compounds **1** and **2**

Compound 2

Compound **2** was isolated as a white amorphous powder, well soluble in chloroform and DMSO. The ^{1}H NMR spectrum of compound **2** shows the presence of six methyl singlets at δ 0.77, 0.79, 0.85, 0.87, 0.93, and 0.98 ppm. The ^{1}H NMR spectrum also shows the presence of one olefinic proton at δ 5.26 ppm, suggesting that it belongs to double bond >C=CH–. The proton that appears as a double of doublet at δ 3.22 ppm was assigned to the methine proton bonded to carbinol carbon C-3 (–C**H**OH–). The α-orientation of this H-3 (or the β-orientation of the 3-OH) was deduced from the coupling constants, J = 11.0 and 4.5 Hz, between *axial* H-3 and *equatorial* H-28, *axial* H-5, and from reference [9]. From the analysis above, we suggested that compound **2** should belong to the group of sterols.

The ^{13}C NMR and DEPT spectra of compound **2** show the presence of seven methyl (δ 13.9, 15.5, 16.9, 17.1, 21.2, and 23.6 ppm), ten methylene, four methine, and seven quaternary carbons. Among them, four carbon signals at δ 125.4 (C), 125.9 (CH), 137.9 (C), and 157.4 (C) ppm were assigned to two double bonds: >C=C< and >C=CH–; one carboxylic carbon conjugated with double bond >C=C< at δ 171.8 ppm. The carbon signal at δ 79.1 ppm was assigned to the cyclic carbinol carbon of a sterol (C-3). The spectral data above supported the presence of a sterol skeleton having a hydroxyl group at the C-3 position with two double bonds at C-17/C-20 and C-24/C-25 (Table 1).

Thus, compound **2** was assigned as the carboxylic acid derivative of protosta-17(20),24-dien-3β-ol [9] (Fig.1), synthesized from (*3S*)-oxidosqualene by fungus *Aspergillus fumigatus* and named as ***3*β-hydroxyprotosta-17(20),24-dien-21-oic acid**.

Compound 3

Compound **3** was isolated as a white amorphous powder, well soluble in chloroform and DMSO. Its molecular formula was deduced to be $C_{20}H_{40}O_2$ on the basis of the *pseudo*-molecular ion $[M+H]^+$ peak at *m*/*z* 313.1 in the ESI-LC-MS spectrum and on the basis of its ^{1}H NMR and ^{13}C NMR spectral data.

The ^{1}H NMR spectrum of compound **3** shows the presence of only saturated protons in the high field: one methyl triplet at δ 0.88 ppm, one methylene triplet at δ 2.35 ppm, and one multiplet with 34 protons at δ 1.25–1.30 ppm. This information enables us to suggest that compound **3** should be an aliphatic acid. The ^{13}C NMR spectrum of compound **3** shows the presence of twenty carbons: one methyl carbon (δ 14.1 ppm), eighteen methylene carbons with δ from 22.7 to 33.7 ppm

Table 2. 1H and ^{13}C NMR spectral data (δ_H ppm, *J* Hz, and δ_C ppm) of compound **3**

No.	^{1}H NMR (500 MHz, $CDCl_3$, *J* Hz)	^{13}C NMR (125 MHz, $CDCl_3$)
1	–	177.9
2	2.35 t 7.5 (2H)	33.7
3–19	1.25 – 1.30 (34H)	22.7–31.9
20	0.88 t 7.0 (3H)	14.1

(some of them were overlapped at δ 29.1–29.7 ppm), and one carboxylic carbon at δ 177.9 ppm. This means that compound **3** should be an aliphatic acid, as mentioned above from the ^{1}H NMR spectral analysis (Table 2).

From the MS, ^{1}H NMR and ^{13}C NMR spectral data above, we conclude that compound **3** should be **icosanoic acid**, $CH_3(CH_2)_{18}COOH$. This acid was isolated from *Helicteres hirsuta* for the first time.

3.2 Cytotoxic activity

Isolated compounds **1–3** were tested against SK-Mel-2, AGS, SK-LU-1, Hep-G2, and Hela cell lines from American Type Culture Collection according to the method described in section 2.4.

Compound **2** has a remarkable cytotoxic activity against SK-LU-1, Hep-G2, and Hela cell lines with IC_{50} values ranging from 32.86 to 77.31 μg/mL (Table 3). Meanwhile, compound **3** shows a moderate cytotoxic activity against SK-Mel-2, AGS, SK-LU-1, Hep-G2, and Hela cell lines with IC_{50} values ranging from 59.02 to 80.87 μg/mL.

Table 3. Cytotoxic activity of compounds **1–3** against 5 cell lines

No.	Compounds	IC_{50} (μg/mL)				
		SK-Mel-2	**AGS**	**SK-LU-1**	**Hep-G2**	**Hela**
1	Compound **1**	>100	>100	>100	>100	>100
2	Compound **2**	No test	No test	77.31 ± 3.18	35.74 ± 2.32	32.86 ± 4.27
3	Compound **3**	80.87 ± 6.86	59.02 ± 4.52	53.66 ± 2.38	73.08 ± 3.77	74.81 ± 6.42
4	Ellipticine	0.56 ± 0.08	0.50 ± 0.04	0.47 ± 0.06	0.44 ± 0.07	0.39 ± 0.03

4 Conclusion

This is the first time, three compounds: β-stigmasterol (**1**), protosta-17(20),24-dien-3β-ol (**2**), and icosanoic acid (**3**) were isolated from *Helicteres hirsuta* collected in Binh Phuoc province. Compounds **2** and **3** have a very low cytotoxic activity against SK-LU-1, Hep-G2, and Hela cell lines. Further investigation of the chemical constituents and biological activities of *Helicteres hirsuta* is continued.

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