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DRYNAETHER A, A NEW FLAVAN FROM THE DRYNARIA BONII RHIZOMES

DRYNAETHER A, MỘT HỢP CHẤT MỚI TỪ CÂY TẮC KÈ ĐÁ

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ABSTRACT

In Vietnam, the medicinal plant Drynaria bonii H. Christ is used for the treatment of osteoporosis, bone fractures, to stimulate the growth of hair and to treat tinnitus. In this paper, experiments were designed to investigate the proliferation activity of ethanol, n-hexane, chloroform, ethyl acetate and methanol extracts from D. bonii rhizomes on MG-63 human osteoblast-like cells. The results showed that methanol and hexane extracts had the ability to proliferate MG-63 cells at the concentration varying from 0.1 to 0.01µg/ml. In particular, at the concentration of 0.01µg/ml, hexane and methanol extracts illustrated the highest proliferation ratio with percentage of 9.31% and 6.16%, respectively. By column chromatography, a new compound named drynaether A (1) and five known compounds including uracil (2), 4'-hydroxy-7-methoxyflavan (3), kaempferol (4), indole-3-carboxylic acid (5) and protocatechuic acid (6) were isolated and identified from the methanol extract.

Keywords: *Drynaria bonii; MG63 cell; flavonoids; drynaether A (1); kaempferol.*

TÓM TẮT

Cây tắc kè đá được nhân dân sử dụng điều trị loãng xương, gãy xương, kích thích mọc tóc... Tuy nhiên chưa có nhiều nghiên cứu về thành phần hóa học và hoạt tính sinh học của loài này. Tiến hành khảo sát khả năng tăng sinh nguyên bào xương MG63 trên các cao chiết ethanol tổng, n-*hexane, chloroform, ethyl acetate và methanol cho thấy cao methanol và cao hexane thể hiện khả năng tăng sinh tế bào MG-63 ở các nồng độ từ 0.1 đến 0.01µg/ml. Ở nồng độ 0.01µg/ml, tỷ lệ tăng sinh tế bào trên cao hexane và methanol lần lượt là 9.31% và 6.16%. Từ phân đoạn methanol, một chất mới drynaether A (1) cùng 5 chất đã biết uracil (2), 4'-hydroxy-7-methoxyflavan (3), kaempferol (4), indole-3-carboxylic acid (5) và protocatechuic acid (6) đã được xác định.*

Từ khóa: *Drynaria bonii; tế bào MG63; flavonoids; drynaether A (1); kaempferol.*

1. INTRODUCTION

The genus *Drynaria* is comprised of 19 species around the world [1] and Vietnam has 7 species [2]. *Drynaria bonii H. Christ* has been used to heal bone fractures, stimulate the growth of hair in folk medicines of Vietnam [2-3]. Major chemical components of this genus are flavonoids, phenyl propanoids, chromone and triterpenoids [4-8]. As a part of our continuing studies on chemical constituents of *Drynaria* species in Vietnam [9-10], this paper informs the bioassay-guided isolation and characterization of a new flavan derivative (1) and five known secondary metabolites (2-6) from the methanol extract of the rhizomes of *Drynaria bonii*.

2. MATERIALS AND METHODS

2.1 Materials

The rhizomes of *Drynaria bonii* were collected in in Nui Chua National Park, Ham Thuan Nam district, Binh Thuan province, Vietnam in February 2013 and authenticated by the Centre of Medicinal Plants and Ginseng in Ho Chi Minh City (National Institute of Medicinal Material, Vietnam). A voucher specimen (No 1912) has been preserved in the Institute of Chemical Technology.

2.2 Apparatus

Specific rotation was measured on digital polarimeter (Kruss, Hamburg, German). NMR spectra were recorded on a Bruker AM500 FTNMR spectrometer (Bruker, Karlsruhe, Germany) using TMS as an internal standard, Institute of Chemistry (Vietnam Academy of Science and Technology, Hanoi, Vietnam). ESI-MS and HR-ESI-MS were performed on a MicroOTOF-Q mass spectrometer (Bruker, Karlsruhe, Germany), University of Science (National University, Ho Chi Minh City, Vietnam). TLC was performed on silica gel 60 F254 (1.05554.0001, Merck) and RP-18 F254S plates (1.15685.0001, Merck). The zones were detected using UV at 254 or 365 nm or a solution of $FeCl_3$/EtOH or H_2SO_4/EtOH. Column chromatography was performed on silica gel (240-430 mesh, Merck) or ODS (70-230 mesh, Merck, Darmstadt, Germany) or Sephadex LH 20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

The MG-63 cells (CRL-1427, Korea) were grown in Dulbecco's Modified Eagle's medium (DMEM; Gibco BRL, NY, USA) supplemented with 10% bovine serum (FBS; Gibco BRL, NY, USA), 100 U/ml penicillin G (Gibco BRL, NY, USA), and 100 µg/ml streptomycin (Gibco BRL, NY, USA). Ethanol, n-hexane, chloroform, ethyl acetate and methanol extracts were tested for cell proliferation using EZ cyto kit (ITSBIO, Seoul, Korea) at different concentration after dissolve in DMSO then diluted with culture medium.

2.3 Methods

Dried rhizomes of *D. bonii* (2.0 kg) were extracted with EtOH 96% (3x8 L). The extract was evaporated to dryness (205.0 g) under reduced pressure. The ethanol extract was eluted on a silica gel column sequentially with petroleum ether, chloroform, ethyl acetate and methanol.

The methanol fraction (110.0 g) was fractionated by column chromatography on silica gel using a mixture of petroleum ether:ethyl acetate and ethyl acetate:methanol with increasing polarity to yield six fractions (M1 – M6). Fraction M5 was repeatedly chromatographed over a silica gel column and eluted with $CHCl_3$:MeOH (20:1, v/v) to give 5 subfractions (M51-M55). Subfraction M52 was passed through Sephadex LH-20 (MeOH) and followed by a silica gel column and eluted with $CHCl_3$:MeOH (25:1, v/v) to obtain 2 (6.4 mg). Subfraction 5.4 was subjected to a column chromatography on silica gel using a mixture of $CHCl_3$:MeOH with increasing polarity (from 20:1 to 8:1) to yield 5 (5.0 mg) and 6 (18.8 mg). Fraction M1 was further separated on a silica gel column and eluted with $CHCl_3$:MeOH (1:0, 9:1, 7:3, and 1:1, v/v) to give 6 subfractions (M11-M16). Subfraction M12 was subjected to sephadex LH 20 column using $CHCl_3$:MeOH (1:1, v/v) as eluent, followed by ODS column chromatography with MeOH:H_2O (3:1, v/v) to give 4 (8.3 mg). Subfraction M15 was further separated by ODS column using MeOH–H_2O (1:2, v/v) as eluent and then purified on Sephadex LH-20 using $CHCl_3$:MeOH (1:1, v/v) to obtain 5 (8.6 mg).

Biological evaluation

Cell Culture: MG-63 cells were seeded at 1x104 cells per well in 96- well microtiter plate with 100 µl culture medium then incubated for 24 hours at 37 0C in 5% CO_2 incubator.

Cell Proliferation Assay: 10 µl of tested samples that were prepared at final concentration varying from 0.01 µg/ml to 0.1 µg/ml; 1 µg/ml; 10 µg/ml and 100 µg/ml by diluting with the growth medium were added into each well. In the control group, DMSO was used instead of tested samples and each treatment was performed in five replicates. After 48 hours incubation at 37 0C in 5% CO_2 incubator, 10 µl of EZ cyto kit reagent were added into each well and incubated for 4 hours. The optical density (OD) was measured by microplate reader at 450 nm. Optical density of the samples at different concentrations without cells was also determined at 450 nm by microplate reader at 450 nm to exclude the effect of the sample color. Thus,

$$\mathrm{OD}_{\text{sample}} = \mathrm{OD}_{\text{sample with cells}} - \mathrm{OD}_{\text{sample without cells}} \qquad (1)$$

The cell proliferation rates were calculated using the OD values at 450nm and were represented as percentages of the vehicle control following the formula below. Statistical differences in the assays were determined using one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test ($p<0.05$ and $p<0.01$). Experimental data were performed as mean ± standard deviation (SD) of five replicated experiments. Proliferation ratio (PR, %):

$$\mathrm{PR}(\%) = \frac{\mathrm{OD}_{\text{sample}} - \mathrm{OD}_{\text{control}}}{\mathrm{OD}_{\text{control}}} \times 100 \qquad (2)$$

Drynaether A (1)

Ivory-white powder. $[\alpha]^{25}_{D}$-16.7 (c = 0.59, CHCl3). ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) data are given in Table 2. ESI-MS (positive mode) *m/z* 413.4 $[M+Na]^+$ and 279.2 $[M+Na-C_8H_6O_2]^+$. ESI-MS (negative mode) *m/z* 255.3 $[M-H-C_8H_6O_2]^-$ and 151.1 $[M-H-C_{16}H_{14}O_2]^-$. HR-ESI-MS *m/z* 413.2596 $[M+Na]^+$.

3. RESULTS AND DISCUSSIONS

On premilary screening, the 96% ethanol extract of *D. bonii* stimulated the proliferation of MG-63 cells by 5.34%, 5.06% and 5.01 % at concentrations of 1.0, 0.1 and 0.01 µg/ml, respectively (Table 1). The ethanol extract was fractionated by flash column chromatography eluting with hexane, chloroform, ethyl acetate and methanol to give four fractions. The methanol fraction promoted proliferation of osteoblastic MG63 cells by 6.16% at 0.01 µg/l (Table 1) and was chromatographed on silica gel, sephadex LH-20 and octadecyl silane column to give a new compound named drynaether A (**1**), together with 5 known compounds: uracil (**2**) [11], 4'-hydroxy-7-methoxyflavan (**3**) [12], kaempferol (**4**) [13], indole-3-carboxylic acid (**5**) [14] and protocatechuic acid (**6**) [15].

Drynaether A (**1)** was obtained as ivory-white powder. The HR ESI-MS gave pseudo-molecular ion peak $[M+Na]^+$ at m/z 413.2596, which corresponded to molecular formula of $C_{24}H_{22}O_5$.

The ^{1}H NMR spectrum revealed the resonance signals of a 1,2,4-trisubstituted benzene ring at [δ_H 6.97 (1H, d, 8.0 Hz; H-5); 6.48 (1H, dd, 2.0 and 8.5 Hz; H-6) and 6.46 (1H, d, 2.0 Hz; H-8)], two 1,4-disubstituted benzene ring at [δ_H 7.29 (2H, d, 8.5 Hz; H-2' and H-6'); 6.85 (2H, d, 8.5 Hz; H-3' and H-5'); 7.95 (2H, d, 8.5 Hz; H-3" and H-5") and 6.85 (2H, d, 8.5 Hz; H-2" and H-6")], one oxymethine proton at δ_H 4.95 (1H, dd, 2.0 and 10.0 Hz; H-2), two methylene groups at [δ_H 2.05 (1H, m; H-3a) and 2.15 (1H, m; H-3b)] and [δ_H 2.72 (1H, m; H-4a) and 2.91 (1H, m; H-4b)] , two methoxy groups at δ_H 3.75 (3H, s, H-11), and 3.89 (3H, s, H-8").

The ^{13}C NMR together with DEPT spectrum exhibited the resonance of 24 carbons including two methoxy [δ_C 55.3 (C-11) and 52.0 (C-8")], eleven olefinic methine [δ_C 129.9 (C-5); 107.4 (C-6); 101.6 (C-8); 127.6 (C-2' and C-6'); 115.3 (C-3' and C-5'); 115.2 (C-2" and C-6"); 131.9 (C-3" and C-5")]; 4 oxygenated sp^2 tertiary [δ_C 158.9 (C-7); 155.8 (C-9) and 155.4 (C-4'); 160.1 (C-1")], three olefinic quaternary [δ_C 114.0 (C-10) and 133.8 (C-1'); 122.3 (C-4")], one sp^3oxymethine δ_C 77.7, two sp^3 methylene [δ_C 29.8 (C-3) and 24.5 (C-4)] and one carboxyl carbon δ_C 167.3 (C -7").

The NMR data proved that **1** was a flavan derivative which bearing one 1,4-disubstituted benzene ring (D ring). In HMBC spectrum, methoxy proton at δ_H 3.89 (3H, s, H-8") gave correlation with carboxyl carbon δ_C 167.3 (C-7") demonstrated that this methoxy attached to carboxyl. In addition, two proton signals at δ_H 7.95 (2H, d, 8.5 Hz) correlated with carboxyl carbon and one oxygenated tertiary carbon δ_C 160.1. Therefore, the carbon at δ_C 160.1 was represented as C-1" and two proton signals were indicated as H-3" and H-5". The HMBC correlation between proton signal at δ_H 6.85 (2H, d, 8.5 Hz) with C-1" indicates that two protons were H-2" and H-6". The carbon resonating at δ_C 122.3 ppm is characteristic for C-4".

The remaining disubstituted benzene residue should be B-ring of flavan. The HMBC spectrum showed correlations between proton at δ_H 7.29 and 6.85 with oxygenated tertiary carbon δ_C 155.4 indicating this carbon was C-4'. Additionally, there was no HMBC correlation between methoxy proton with C-4' demonstrated that C-4' bearing a hydroxyl group. Based on the effect of oxygen atom at C-4', chemical shifts of H-2'/H-6' and H-3'/H-5' were 7.29 and 6.85 ppm, respectively.

The oxymethine proton at δ_H 4.95 (1H, dd, 2.0 and 10.0 Hz) was assigned to H-2 of flavan skeleton. The J-coupling of H-2 (dd, 2.0 and 10.0 Hz) proving that C-2 was S -configuration. In HMBC spectrum, H-2 gave J^3 and J^2 HMBC correlation with carbon at δ_C 24.5 and 29.8 indicated that two carbons were C-4 and C-3, respectively. Moreover, the proton H-4 showed HMBC correlations with one quaternary olefinic carbon δ_C 114.0; one tertiary olefinic carbon δ_C 129.9 and one oxygenated tertiary carbon δ_C 155.8; hence three carbons mentioned above were C-10; C-5 and C-9. In HSQC spectrum, H-5 was a doublet peak at 6.97 ppm with spin-spin coupling 8.0 Hz. This confirmed that two protons at δ_H 6.48 (1H, dd, 2.0 and 8.5 Hz) and 6.46 (1H, d, 2.0 Hz) were H-6 and H-8, respectively. The HMBC correlations of H-5 and methoxy proton δ_H 3.75 (3H, s) with oxygenated olefinic carbon δ_C 158.6 established that the carbon was C-7 and the methoxy group attached to C-7 of flavan.

Furthermore, HMBC correlations between H-3'/H-5' with C-1" and H-2"/H-6"with C-4' indicated that ring D attached to flavan skeleton at C-4' via ether bonding. The ether bonding of ring B and D was confirmed by ESI-MS spectrum. The fragmentation peaks in the ESI-MS (positive) at m/z 413.4 $[M+Na]^+$ and 279.2 $[M+Na-C_8H_6O_2]^+$ were due to the cleavage of ether bond. The negative ESI-MS spectrum showed its fragmentation at m/z 255.3 $[M-H-C_8H_6O_2]^-$ and 151.1 $[M-H-C_{16}H_{14}O_2]^-$ further confirming the ether bonding between ring D and B. Based on these data, structure of 1 was determined as 4'-O-(4-carboxylmethyl-1-phenyl)-7-methoxyflavan. To our best knowledge, this is a new compound and named drynaether A (Fig. 1).

4. CONCLUSSION

The crude ethanol 96% extract of the rhizomes of *Drynaria bonii* and its sub-fractions had the ability to proliferate MG-63 cells at the concentration varying from 0.1 to 0.01µg/ml. At the concentration of 0.01µg/ml, the hexane and methanol sub-fractions illustrated the highest proliferation ratio with percentage of 9.31% and 6.16%, respectively. From the methanol sub-fraction, a new flavan named drynaether A (**1**) and five known compounds including uracil (**2**), 4'-hydroxy-7-methoxyflavan (**3**), kaempferol (**4**), indole-3-carboxylic acid (**5**) and protocatechuic acid (**6**) were isolated and identified.

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Table 1. *Proliferation ratio of extracts at different concentrations on MG-63 cell.*

Samples	Concentration (µg/ml)	A control at 450 nm (mean±SD)		A sample at 450 nm (mean±SD)		Proliferation ratio (%)
		A control (mean)	SD	A sample (mean)	SD	
ethanol extract	100.00	1.696	0.139	1.613	0.080	-4.901
	10.00	2.206	0.078	2.096	0.084	-5.001
	1.00	2.201	0.161	2.319	0.127	5.344
	0.10	2.023	0.209	2.125	0.219	5.061
	0.01	1.972	0.250	2.071	0.128	5.016
n-hexane extract	100.00	1.696	0.139	1.671	0.134	-1.459
	10.00	2.206	0.078	2.112	0.052	-4.285
	1.00	2.201	0.161	2.006	0.193	-8.871
	0.10	2.023	0.209	1.937	0.229	-4.219
	0.01	1.972	0.250	2.156	0.245	9.310
chloroform extract	100.00	1.696	0.139	0.868	0.097	-48.798
	10.00	2.206	0.078	2.065	0.114	-6.384
	1.00	2.201	0.161	1.946	0.236	-11.562
	0.10	2.023	0.209	1.856	0.321	-8.253
	0.01	1.972	0.250	1.787	0.377	-9.412
ethyl acetate extract	100.00	1.696	0.139	0.958	0.109	-43.513
	10.00	2.206	0.078	1.955	0.125	-11.379
	1.00	2.201	0.161	1.706	0.362	-22.479
	0.10	2.023	0.209	1.902	0.269	-5.972
	0.01	1.972	0.250	1.849	0.341	-6.269
methanol extract	100.00	1.696	0.139	1.684	0.231	-0.731
	10.00	2.206	0.078	1.769	0.302	-19.798
	1.00	2.201	0.161	1.829	0.436	-16.887
	0.10	2.023	0.209	1.759	0.199	-13.015
	0.01	1.972	0.250	2.094	0.169	6.164

Table 2. *NMR data (500 MHz for 1H and 125 for 13C) in CDCl3 for compound 1.*

Position	δ_H	δ_C
2	4.95, dd, *J* 10.0 and 2.0, 1H	77.7
3	2.05, m, 1H	29.8
	2.15, m, 1H	
4	2.72, m, 1H	24.5
	2.91, m, 1H	
5	6.97, d, *J* 8.0, 1H	129.9
6	6.48, dd, *J* 8.5 and 2.0, 1H	107.4
7		158.9
8	6.46, d, *J* 2.0, 1H	101.6
9		155.8
10		114.0
11	3.75, s, 1H	55.3
1’		133.8
2’	7.29, d, *J* 8.5, 1H	127.6
3’	6.85, d, *J* 8.5, 1H	115.3
4’		155.4
5’	6.85, d, *J* 8.5, 1H	115.3
6’	7.29, d, *J* 8.5, 1H	127.6
1”		160.1
2”	6.85, d, *J* 8.5, 1H	115.2
3”	7.95, d, *J* 8.5, 1H	131.9
4”		122.3
5”	7.95, d, *J* 8.5, 1H	131.9
6”	6.85, d, *J* 8.5, 1H	115.2
7”		167.3
8”	3.89, s, 1H	52.0

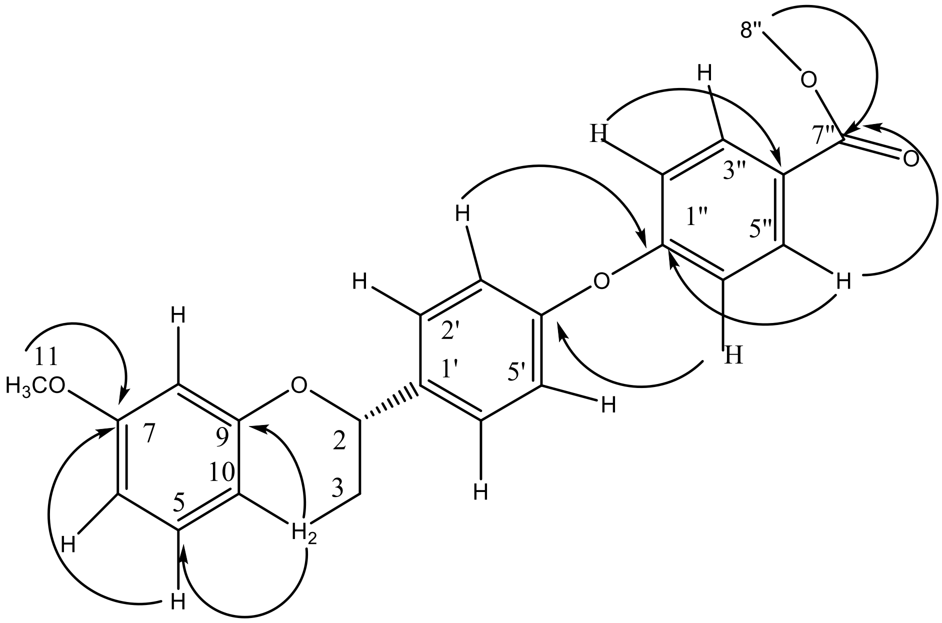
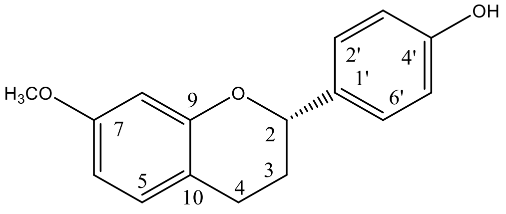


Figure 1. *Structure and key HMBC correlations of 1.*



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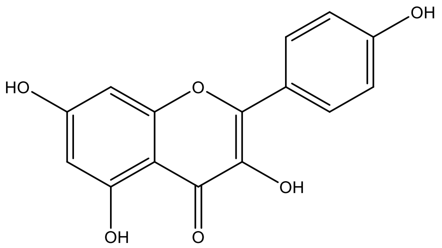

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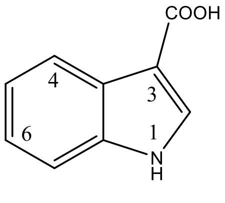

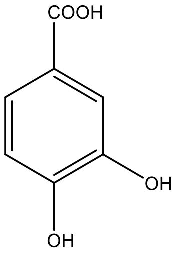

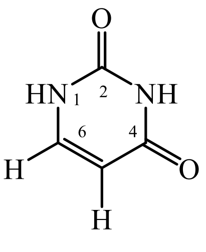

5 **6** **2**

Figure 2. *Structure of compounds 2-6.*

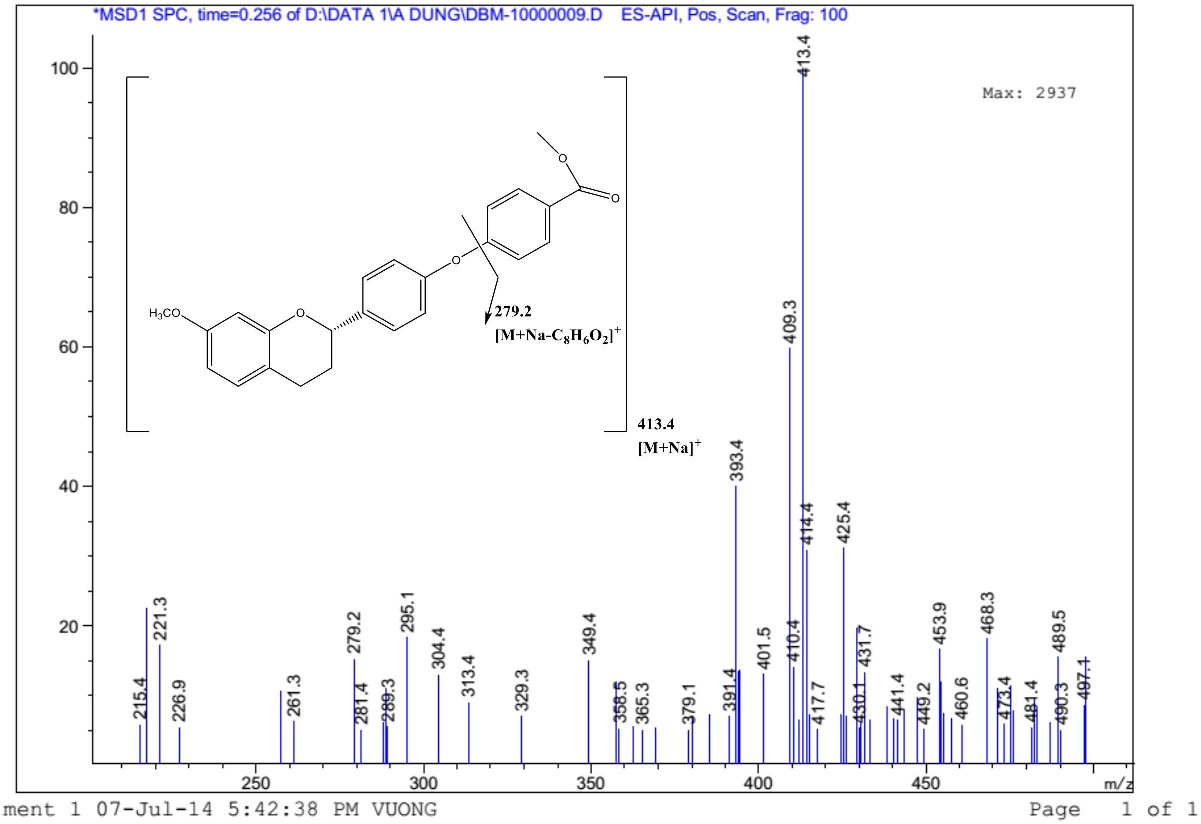

Figure 3. The positive ESI-MS fragment of 1.

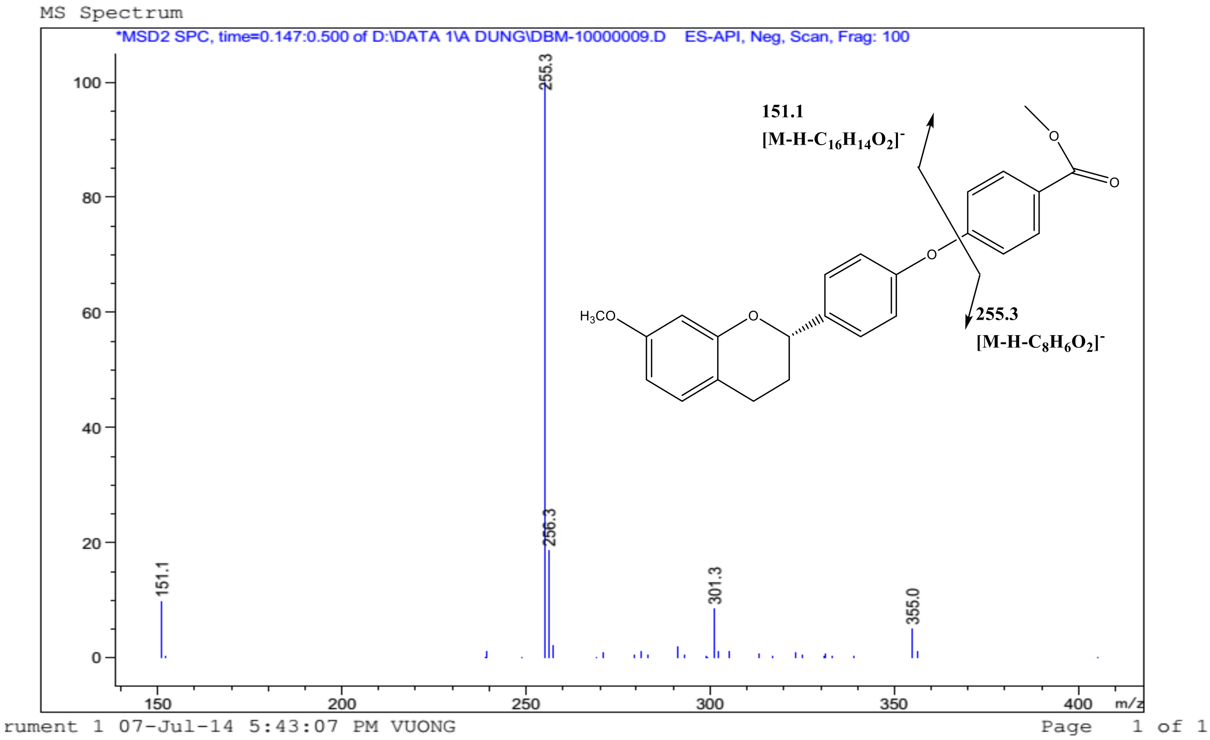

Figure 4. The negative ESI-MS fragment of 1.

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