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Original Research Article

Isolation and Characterization of Anti-Inflammatory Compounds from *Radix bupleuri*

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ABSTRACT

Radix bupleuri is traditionally used in Vietnam for its hepatoprotective activity. The herb was mainly imported from China, until it was recently introduced Vietnam. The plant is found mainly in the Northern region of Vietnam. The aim of this research was to isolate and characterize anti-inflammatory compounds from *Radix bupleuri*. The ethanol (EtOH) extract of *Radix bupleuri* was phytochemical investigation and the structure of five isolated known compounds was identified by 1D and 2D NMR data as well as by comparison with data reported in literature. The isolatd compounds were evaluated for their inhibitory effect on NO production in RAW 264.7 cells and the compounds showed moderate activity. Therefore, *Radix bupleuri* could serve as a potential therapeutic herb for the treatment of diseases related to inflammation.

Keywords: *Radix bupleuri*, Saikosaponin, Phenol, Anti-inflammatory.

Introduction

Radix bupleuri has been used widely in folk medicine for more than 2000 years in Asian countries.[1] A wide range of secondary metabolites, including saponins, polyacetylenes, fatty acids, flavonoids, lignans, and sterols have been isolated from this plant.[2,3] Pure compounds and crude extracts from *Radix bupleuri* possess numerous bioactivities, such as hepatoprotective, anti-inflammatory, antipyretic, analgesic, sedative, and immunomodulatory effects.[2] Previous studies revealed that the major components of *Radix bupleuri* were saikosaponins, which were mainly responsible for various bioactivities.[2] However, long-term use with high doses of *Radix bupleuri* may also cause hepatotoxicity.[2] In Vietnamese traditional medicines, the herb has been therapeutically prescribed to treat a variety of illnesses. As part of our ongoing research for hepatoprotective herbal drugs, *Radix bupleuri* was chemically investigated and five compounds were identified. Their structures and anti-inflammatory effect are described herein.

Materials and Methods

General Experimental Procedure

The 1D and 2D NMR spectra were measured using Bruker 500 and 600 MHz spectrometers (Bruker BioSpin GmbH, Rheinstetten, Germany). Analytical grade solvents were used for extraction and purification. Column chromatography was conducted using silica gel (63–200 mm; Merck, Darmstadt, Germany) and RP-18 (75 mm; Merck). Thin layer chromatography was performed on pre-coated silica gel 60 F254 plates and RP-18 F254 plates (both from Merck) and the plates were visualized under UV (254 and 365 nm) and then dipped in a 10% (v/v) sulfuric acid solution and heated at 100°C for 10 min.

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Plant Collection and Identification

The plant was provided by Son Lam pharmaceutical joint stock company, and was identified as *Radix bupleuri* according to Vietnamese Pharmacopeia V. A voucher specimen was deposited at Vietnam Military Institute of Traditional Medicine.

Extraction and Isolation of Compounds

To prepare the extract, 500 g of air-dried and pulverized *Radix bupleuri* was extracted with 95% EtOH (3 × 5.0 L) by ultrasonication for 2 h. The extract was filtered and evaporated under reduced pressure to afford a crude extract. The extract (53 g) was suspended in H_2O and then successively partitioned with n-hexane, and EtOAc. The EtOAc fraction (10.0 g) was applied to a silica gel column chromatography using a gradient solvent system of CH_2Cl_2-MeOH (100:0 to 0:100, v/v) to afford ten fractions (E01–E10). Fraction E03 was subjected to silica gel column chromatography and eluted with n-hexane:EtOAc (from 10:1 to 0:1, v/v) to afford five subfractions (E03-1 to E03-5). Subfraction E03-3 was further purified by silica gel column chromatography using CH_2Cl_2:EtOAc (10:1, v/v) as the eluent to afford compounds **3** (8.0 mg) and **4** (10 mg). Fraction E10 was furified by ODS column chromatography and eluted with MeOH:H_2O (1:3) to obtained compound **5** (12 mg). Fraction E05 was separated by CC on silica gel and eluting with CH_2Cl_2-MeOH-H_2O (9:1:0.1) to yield compounds **1** (13 mg) and **2** (10 mg).

Assay for NO Inhibitory Effect Using RAW264.7 Cells

The assay for NO inhibitory effect was carried out as previously described.[4] RAW 264.7 macrophage cells (ATCC®-TIB-71TM) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) added by 10% foetal bovine serum (FBS), 100 U penicillin/mL, 100 µg streptomycin/mL, and 0.25 µg gibco amphotericin B/mL. The cells were seeded in 96-well plates at 2 ×10^5 cells per well and maintained for 24 hours at 37 °C under 5 % carbon dioxide (CO_2). The culture medium was then replaced by DMEM without FBS, and the cells were incubated for 3 hours. The cells were continued to pre-treat with 1-5 and L-NG-methyl arginine acetate (L-NMMA) as a positive control at 128, 32, 8, 2, and 0.5 µg/mL for 2 h, followed by treatment with 10 µg/mL lipopolysaccharides (LPS) for 24 h. The levels of NO production were assessed by the Griess Reagent System (Promega Cooperation, WI, USA), and L-NMMA (sigma) was used as a reference. A microplate reader was used for measuring the absorbance at 540 nm to determine the nitrile concentration in the medium. The

standard curve was generated based on the concentrations of $NaNO_2$. Each experiment was conducted in triplicate. Half-maximal inhibitory concentration (IC_{50}) values for the inhibition of NO production were calculated by Table Curve 2Dv4. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was also performed to determine the viability of RAW 264.7 cells.

Results and Discussion

Compound **1** was isolated as a colorless solid. The ^{1}H-NMR spectrum of **1** (Table 1) showed the presence of six methyl singlets at δ_H 0.86-1.36; a methyl doublet at δ_H 1.42 (3H, d, J = 6.2 Hz, H-6'); two olefinic protons at δ_H 5.98 (1H, d, J = 10.3 Hz, H-11) and 5.64 (1H, d, J = 10.3 Hz, H-12); two anomeric protons at δ_H 5.30 (1H, d, J = 7.8 Hz, H-1'') and 4.97 (1H, d, J = 7.8 Hz, H-1'); signals for sugar protons at δ_H 3.33-4.51; and upfield signals at δ_H 1.03-2.48 ppm. The ^{13}C-NMR spectrum revealed signals of two olefinic carbons at δ_C 131.3 (C-11) and 131.1 (C-12); two anomeric carbons at δ_C 105.8 (C-1') and 106.2 (C-1''); fourteen signals of oxygenated sp^3 carbons at δ_C 62.6-84.9; twenty-four upfield sp^3 carbons at δ_C 13.0-53.1 ppm. Detailed analysis of HSQC and HMBC data and comparison with the published data identified the structure of **1** as saikosaponin A,[5, 6] a major compound isolated from *Radix bupleuri*.

Compound **2** was isolated as a colorless solid. The ^{1}H-NMR spectrum of **2** (Table 1) displayed the existence of six methyl singlets at δ_H 0.85-1.57; a methyl doublet at δ_H 1.40 (3H, d, J = 6.2 Hz, H-6'); two olefinic protons at δ_H 5.98 (1H, d, J = 10.3 Hz, H-11) and 5.65 (1H, d, J = 10.2 Hz, H-12); two anomeric protons at δ_H 5.25 (1H, d, J = 7.8 Hz, H-1'') and 4.95 (1H, d, J = 7.8 Hz, H-1'); signals for sugar protons at δ_H 3.29-4.46; and upfield signals at δ_H 1.02-2.58 ppm. The ^{13}C-NMR spectrum revealed signals of two olefinic carbons at δ_C 132.0 (C-11) and 131.6 (C-12); two anomeric carbons at δ_C 105.5 (C-1') and 105.7 (C-1''); fourteen signals of oxygenated sp^3 carbons at δ_C 62.2-84.9; twenty-four upfield sp^3 carbons at δ_C 12.8-52.8 ppm. Detailed analysis of HSQC and HMBC data identified the planar structure of **2** to be the same as that of **1**. The only significant difference was the chemical shift of C-16 (δ_C 64.0 for **1** and 76.8 for **2**) and comparison with the published data identified **2** as saikosaponin D,[5] another major saponin isolated from *Radix bupleuri*.

Compound **3** was isolated as a yellow amorphous powder. The ^{1}H-NMR (Table 2) demonstrated a pair of AABB aromatic system protons at δ_H 8.05 (2H, d, J = 8.8 Hz, H-2', H-6') and 6.94 (2H, d, J = 8.4 Hz, H-3', H-5'); two meta-coupling protons at δ_H 6.20 (1H, d, J = 1.9 Hz, H-6) and 6.44 (1H, d, J = 1.9 Hz, H-8). The ^{13}C-NMR spectrum indicated the presence of fifteen carbons, including a keto carbonyl at δ_C 176.3 (C-4); two protonated signals, each representing two carbons at δ_C 129.9 (C-2' and C-6') and 115.8 (C-3' and C-5'); and ten remaining signals at δ_C 93.9-164.3. The ^{1}H and ^{13}C NMR data of **3** were identical to the published data[7] and **3** was identified as kaempferol.

Compound **4** was isolated as a yellow amorphous powder. The ^{1}H-NMR spectrum of **4** (Table 2) showed three protons of an ABX spin system including two doublets at δ_H 7.68 (1H, d, J = 2.2 Hz, H-2'), 6.89 (1H, d, J = 8.5 Hz, H-6'), 6.90 (1H, d, J = 8.5 Hz, H-5'), and a double doublet at δ_H 7.55 (1H, dd, J = 8.5, 2.0 Hz, H-6′); and two meta-coupled aromatic protons at δ_H 6.18 (1H, d, J = 1.8 Hz, H-6) and 6.40 (1H, d, J = 1.7 Hz, H-8). The ^{13}C-NMR spectrum of **4** indicated the presence of fifteen carbons, typical of a flavonoidal nucleus, which comprised of a keto carbonyl at δ_C 176.3 (C-4); seven oxygenated sp^2 carbons at δ_C 136.2-164.3; and eight remaining aromatic carbons at at δ_C 93.8-122.4. The ^{1}H and ^{13}C NMR data of **4** were similar to those of **3**, except for the presence of an additional hydroxy group and by comparison with existing data in literature,[7] the structure of **4** was determined as quercetin.

Compound **5** was isolated as a white amorphous powder. The ^{1}H-NMR spectrum (Table 3) exhibited two olefinic doublets at δ_H 7.56 (1H, d, J = 15.9 Hz, H-7′) and 6.27 (1H, d, J = 15.9 Hz, H-8′); three protons of an ABX spin system including two doublets at δ_H 7.05 (1H, d, J = 2.0 Hz, H-2′) and 6.79 (1H, d, J = 8.2 Hz, H-5′), and a double doublet at δ_H 6.96 (1H, dd, J = 8.2, 2.0 Hz, H-6′); three methines at δ_H 5.34 (1H, m, H-5), 4.18 (1H, m, H-3) and 3.73 (1H, dd, J = 3.0, 8.5 Hz, H-4); two methylene signals at δ_H 2.07-2.21 (4H, m, H-2a,b and H-6a,b). The ^{13}C-NMR spectrum confirmed sixteen carbon signals, which were classified into two carbonyls at δ_C 177.1 (C-7) and 168.7 (C-9′); eight olefinic carbons at δ_C 115.3-149.6; four sp^3 oxygenated carbons at δ_C 71.3-76.2; two methylene signals at δ_C 38.2 (C-2) and 38.6 (C-6). When compared to the published data,[8] **5** was identified as chlorogenic acid.

The anti-inflammatory activity of **1**-**5** was investigated by NO assay, and their effect on the viability of RAW 264.7 cell was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide) assay. The cells were pre-treated with **1**-**5** at the concentrations of 128, 32, 8, 2, and 0.5 µg/mL. The results revealed that at concentrations of 128 and 64 µg/mL, the viability of RAW 264.7 cells was found to be less than 20 % for **1** and **2**, thus the inhibition effect of **1**-**2** at these concentrations was not measured. Cytotoxicity reduced with lower concentrations. According to the results, compounds **1**-**4** demonstrated moderate NO inhibitory activity against lipopolysaccharide (LPS)-induced nitric oxide production with the IC_{50} values ranging from 2.5 to 15.7 µM (Table 4). Notably, these compounds showed no significant effect on the viability of RAW 264.7 cells at their IC_{50} values for NO inhibitory activity. However, compound **5** showed no significant effect at the concentration of 128 µg/mL.

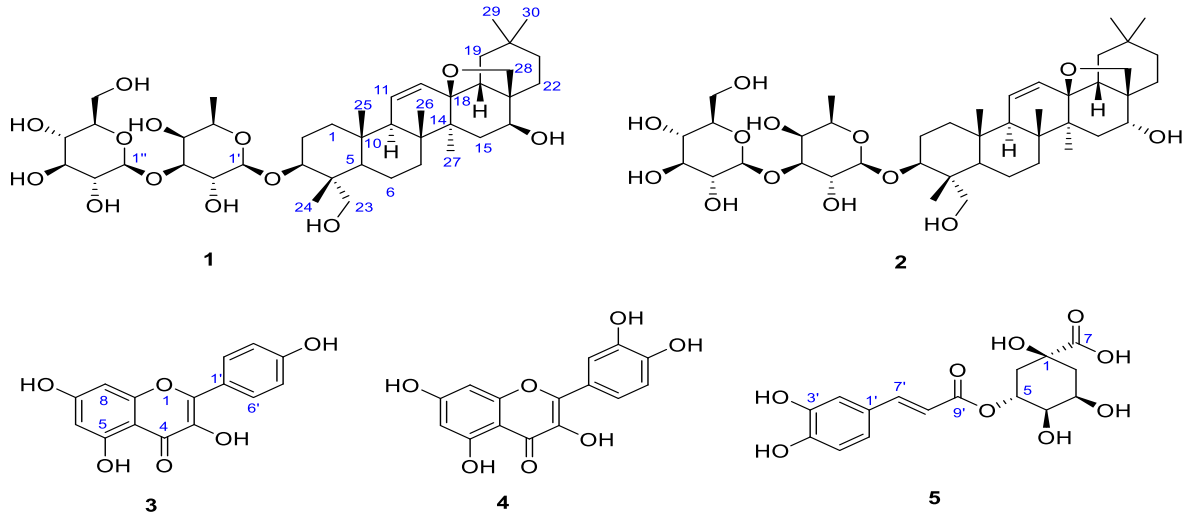

Figure 1: Structures of Compounds **1**-**5** isolated from *Radix bupleuri*.

Table 1: ^{1}H- and ^{13}C-NMR data of compounds **1** and **2**

No	1[a]		2[a]	
	δ_H, mult, *J* in Hz	δ_C	δ_H, mult, *J* in Hz	δ_C
la	1.03, m	38.6	1.02, t, 12.4	38.4
1b	1.83, m		1.81, brd, 12.8	
2a	2.30, brd, 12.8	26.1	2.27, m	25.8
2b	2.03, m		2.01, m	
3a	4.24, m	81.7	4.18, m	81.7
4	—	43.7		43.4
5a	1.64, d, 11.6	47.3	1.60, m	47.1
6a	1.75, d, 12.2	17.6	1.72, d, 12.0	17.3
6b	1.53, m		1.47, d, 13.8	
7a	1.57, m	31.6	1.57, m	31.3
7b	1.23, d, 11.3		1.17, m	
8	-	42.2	-	41.7
9a	2.02, brs	53.1	2.11, brs	52.8
10	-	36.3		36.1
11	5.98, d, 10.3	132.3	5.98, d, 10.4	131.6
12	5.64, d, 10.3	131.1	5.65, d, 10.2	132
13		84.1		84.6
14	-	45.7	-	43.4
15a	1.70, dd, 12.3, 5.5	36.0	1.47, d, 13.8	35.0
15b	2.00, m		2.08,m	
16b	4.49, m	64.0	4.17, m	76.8
17	-	47.0	-	45.1
18b	1.95, d, 13.4	52.2	1.93, bd, 14.3	51.1
19a	1.81, m	37.8	2.59, t, 13.2	38.2
19b	1.29, m		1.32, bd, 11.8	
20		31.5		31.7
21a	1.58, m	34.7	2.43, dt, 13.0, 4.2	36.6
21b	1.17, bd, 13.1		1.17, m	
22a	2.48, d, 13.2	25.8	1.88, m	31.0
22b	1.33, m		1.52, m	
23	3.66, m	63.9	3.62, d, 10.8	63.8
23	4.31, m		4.25, m	
24	0.88, s	13.0	0.85, s	12.8
25	0.96, s	18.7	0.95, s	18.6
26	1.36, s	20.0	1.28, s	19.3
27	1.07, s	20.9	1.56, s	18.0
28	3.33, d, 6.9	73.0	3.29, d, 6.9	77.6
28	4.39, d, 6.9		3.52, d, 6.9	
29	0.90, s	33.6	0.95, s	33.5
30	0.86, s	23.8	0.89, s	24.2
		Fucose		
1’	4.97, d, 7.8	105.8	4.95, d, 7.7	105.5
2’	3.70, m	70.9	4.08, m	71.2
3’	4.07, dd, 9.7, 2.8	85.0	4.11, m	84.9
4’	4.18, m	72.0	4.20, m	71.4
5’	4.15, m	71.5	3.70, m	70.6
6’	1.42, d, 6.2	17.2	1.40, d, 6.2	17.0
		Glucose		
1’’	5.30, d, 7.8	106.2	5.25, d, 7.8	105.7
2’’	4.03, t, 8.5	75.6	4.00, t, 8.5	75.2
3’’	4.24, m	78.1	4.21, m	77.8
4’’	4.50, m	71.6	4.46, m	71.7
5’’	3.97, m	78.5	3.95, m	78.2
6’’	4.50, m 4.20, m	62.6	4.46, m 4.22, m	62.2

[a] ^{1}H and ^{13}C NMR spectra were recorded in pyridine-d_5

Table 2: ^{1}H- and ^{13}C-NMR data of compounds **3** and **4**

No	3[a]		4[a]	
	δ_H, mult, *J* in Hz	δ_C	δ_H, mult, *J* in Hz	δ_C
2		147.2		147.2
3		136.0		136.2
4		176.3		176.3
5		156.6		156.6
6	6.20, d, 1.9	98.6	6.18, d, 1.8	98.6
7		164.3		164.3
8	6.44, d, 1.9	93.9	6.40, d, 1.7	93.8
9		161.1		161.2
10		103.4		103.4
1’		122.1		122.4
2’	8.05, d, 8.8	129.9	7.68, d, 2.0	115.5
3’	6.94, d, 8.8	115.8		145.5
4’		159.6		148.1
5’	6.94, d, 8.8	115.8	6.89, d, 8.5	116.0
6’	8.05, d, 8.8	129.9	7.55, dd, 8.5, 2.0	120.4

[a] ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6

Table 3: ^{1}H- and ^{13}C-NMR data of compound **5**

No	δ_H[a], mult, *J* in Hz	δ_C[a]	No	δ_H[a], mult, *J* in Hz	δ_C[a]
1		76.2	2’	7.05, d, 2.0	115.3
2	2.07, m	38.2	3’		146.8
3	4.18, m	71.3	4’		149.6
4	3.73, dd, 8.5, 3.1	73.6	5’	6.79, d, 8.2	116.5
5	5.34, m	72.0	6’	6.96, dd, 8.2, 2.0	123.0
6	2.21, m	38.8	7’	7.56, d, 15.9	147.1
7		177.1	8’	6.27, d, 15.9	115.3
1’		127.9	9’		168.7

[a] ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD

Table 4: Half-maximal inhibitory concentration (IC_{50}, μM) of **1**–**5** against NO assay

IC_{50}	**1**	**2**	**3**	**4**	**5**	L-NMMA
IC_{50}, μM	8.3 ± 0.69	2.5 ± 0.27	15.7 ± 0.81	6.6 ± 0.50	>128	17.6 ± 2.01
IC_{50}, μg/mL	6.5 ± 0.54	1.95 ± 0.21	4.5 ± 0.52	2.0 ± 0.15	>128	4.38 ± 0.5

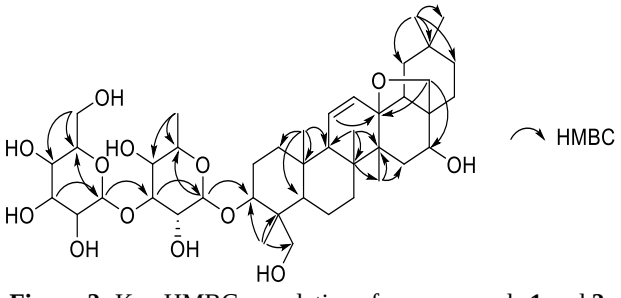

Figure 2: Key HMBC correlations for compounds **1** and **2**.

Conclusion

Phytochemical investigation of the EtOH extract of *Radix bupleuri* resulted in the isolation of five compounds, saikosaponin A (**1**), saikosaponin D (**2**), kaempferol (3), quercetin (**4**), and chlorogenic acid (**5**). The isolated compounds (**1**-**4**) exhibited moderate anti-inflammatory activity. However, further research is still needed to better understand the safety and benefits of this plant.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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