https://doi.org/10.48048/tis.2023.6366

Chemical Constituents and Their Anti-inflammatory Activities from the Stems of *Cryptolepis buchanani*

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Received: 16 November 2022, Revised: 10 December 2022, Accepted: 11 December 2022, Published: 12 December 2022

Abstract

The stems of *Cryptolepis buchanani* are traditionally used for the treatment of inflammation, including arthritis, muscle and joint pain. In the present study, 3 crude extracts (hexane, EtOAc and methanol) from the stems of *C. buchanani* were evaluated for anti-inflammatory effects using an *in vitro* model of RAW 264.7 macrophages. At a concentration of 50 μg/mL, the hexane and EtOAc crude extract have inhibited nitric oxide (NO) production by 84.33±2.01 and 82.49±0.92 %, respectively. 3,4-Dihydroxyl benzoic acid (**1**), vanillic acid (**2**), syringaldehyde (**3**), isoscopoletin (**4**) and stigmast-4-ene-3,6-dione (**5**) were isolated from hexane and EtOAc crude extracts. Their structures were identified on the basis of ^{1}H and ^{13}C NMR, as well as a comparison of the data from previous reports. Compounds **1-5** were evaluated for anti-inflammatory activity. Compound **3** showed activity of NO inhibition with IC_{50} values of 49.07 μM (Indomethacin IC_{50} values of 39.21 μM). In addition, compounds **1-3** and **5** were isolated for the first time from this plant.

Keywords: Anti-inflammatory, *Cryptolepis buchanani*, Nitric oxide (NO), Thao En On, Chromatography

Introduction

Cryptolepis buchanani belongs to the family Asclepiadaceae, a climbing tree found in evergreen forest in Thailand, China, India, Nepal, and Indo-China [1]. In Thailand, *C. buchanani* which is known as "Thao En On", is used as folk medicine. The stems were crushed and used as a poultice on the inflamed area [2]. The aqueous and alcoholic extracts of this plant were evaluated for anti-inflammatory activity [3,4]. Moreover, the alcoholic extracts of *C. buchanani* can restore the strength of the bone and reduced bone repairing period [5].

The secondary metabolites from *C. buchanani* were reported in leaves, stems and roots. The cardenolide, cryptosin, armentogenin, sarmentocymarin and cryptanosides A-D were reported from the leaves [6-8]. Germanicol docosanoate is a major constituent of the root extract [8]. Other chemicals including isoscopoletin, (+)-3-hydroxy-*β*-ionone, (3*R*, 6*R*, 7*E*)-3-hydroxy-4, 7-megastigmadien-9-one, ficusic acid, (+)-pinoresinol, (+)-8-hydroxypinoresinol, (+)-syringaresinol, diaaurantiamide acetate, loliolide, (-)-balanophonin, chrysoeriol, 9-hydroxy-10*E*, 12*Z*-octadecadienoic acid methyl ester and ficusesquilignan A were isolated from the stems and leaves of *C. buchanani*. [9]. The buchananine and 1, 3, 6-*O*-trinicotinoyl-*α*-D-glucopyranose were isolated from the stems [10-11].

Herein, we report the chemical constituents from stems of *C. buchanani*. The 4 compounds (**1-3** and **5**) are isolated from this plant for the first time and also found isoscopoletin (**4**). The anti-inflammatory activity of the crude extracts (hexane and EtOAc) and all pure compounds were analyzed by *in vitro* tests.

Materials and methods

General experimental procedures

The NMR spectra were recorded on a Varian Mercury plus spectrometer (California, USA) and Bruker AVANCE NEO (Rheinstetten, Germany) operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). Thin layer chromatography (TLC) was carried out on MERCK silica gel 60 F254 TLC aluminium sheets. Column chromatography was done with silica gel 0.063 - 0.200mm or less than 0.063 mm (Darmstadt, Germany). Preparative thin layer chromatography (PTLC) was carried out on glass supported silica gel

plates using silica gel 60 PF254 for preparative layer chromatography (Darmstadt, Germany). All solvents were routinely distilled prior to use.

Plant material

The stems of *C. buchanani* were collected from Sop Prap District, Lampang Province, Thailand, in October 2020. A voucher specimen (SNRU012019) was deposited at the Department of Chemistry, Faculty of Science and Technology, Rajabhat Sakon Nakhon University, Thailand.

Extraction and isolation

The stems of *C. buchanani* (3.0 kg) were ground and extracted with methanol (MeOH, 3×10 L, 72 h each) at room temperature to yield a crude extract (250.0 g) after under reduced pressure. The dried crude MeOH was dissolved in distilled water (200 mL) and partitioned successively using different solvents of increasing polarity (hexane and EtOAc) in a separatory funnel. After partitioning, a hexane-soluble extract (20.0 g) and an EtOAc-soluble extract (7.6 g) were obtained.

The hexane-soluble extract (20.0 g) was subjected to column chromatography (CC) using 60 - 120 mesh silica gel and eluted successively with varying concentrations of EtOAc and n-hexane. The eluates were collected in 100 mL flasks and they were combined on the basis of their similarity of their TLC spots to obtain total 4 fractions (H1 - H4). Fraction H2 (2.4 g) was subjected to silica gel CC eluted with isocratic of 50 % CH_2Cl_2–hexane to yield 3 fractions (H2.1 - H2.3). Subfraction H2.3 was subjected to RP-C18 column chromatography with an isocratic system of 10 % CH_2Cl_2–MeOH affording 2 subfractions (H2.3.1 and H2.3.2). Compound **5** was crystallized from subfraction H2.3.1 with EtOAc.

The EtOAc-soluble extract (7.6 g) was subjected on 60 - 120 mesh silica gel CC (0 - 100 % EtOAc–hexane and 0 - 50 % MeOH–EtOAc) to provide 7 fractions (E1 - E7). Fraction E2 (285.9 mg) was subjected on Sephadex LH-20 CC (MeOH) to obtain 2 subfractions (E2.1 and E2.2). Subfraction E2.1 (86.3 mg) was purified by preparative TLC using 30 % EtOAc:Hexane as developing solvent to afford compound **3** (7.1 mg). Re-chromatography of fraction E4 (1.5 g) was performed using RP-C18 CC and eluted with MeOH to obtain 2 subfractions (E4.1 and E4.2). Subfraction E4.1 (1.0 g) was subjected successively to Sephadex LH-20 CC (MeOH) to obtain 4 subfractions (E4.1.1 - E4.1.4). Subfraction E4.1.2 (0.5 g) was chromatographed over a silica gel CC with isocratic system of hexane:CH_2Cl_2:EtOAc (30:50:20) to afford 6 subfractions (E4.1.2.1 - E4.1.2.6). Compound **4** (20.1 mg) was crystallized from subfraction E4.1.2.2 with MeOH. Subfraction E4.1.2.5 was purified by preparative TLC using 5 % MeOH:CH_2Cl_2 as developing solvent to afford compound **2** (11.8 mg). Compound **1** (47.0 mg) was purified from subfraction E4.1.2.6 (0.3 g) by RP-C18 CC (MeOH) and Sephadex LH-20 CC (MeOH).

Nitric oxide measurement

RAW 264.7 cells were seeded at a density of 2×10^5 cells/well in a 96-well plate. After overnight incubation, cells were pretreated for 1 h by replacing medium with complete Dulbecco's modified Eagle's medium (DMEM) containing, *Salmonella enterica* serovar Minnesota lipopolysaccharide (LPS), was then added. Treated cells were incubated for 24 h. Subsequently, supernatants were collected and transferred into a round bottom 96-well plate. Sulfanilamide solution (1 % sulfanilamide in 5 % phosphoric acid) was added to the wells and incubated in the dark for 15 min, followed by an equal volume of NED solution (1 % N-1-napthylethylenediamine dihydrochloride). The resulting solutions were incubated again in the dark for 15 min, and absorbance was determined at 540 nm using a microplate reader [12].

Results and discussion

The stems of *C. buchanani* were powdered and extracted with MeOH at room temperature to give a crude extract, which was suspended in H_2O and successively partitioned with hexane and EtOAc. The hexane-soluble and EtOAc-soluble fraction was submitted to various chromatographic methods, to result in the isolation of 3,4-Dihydroxyl benzoic acid (**1**), vanillic acid (**2**), syringaldehyde (**3**), isoscopoletin (**4**) and stigmast-4-ene-3,6-dione (**5**) as shown in **Figure 1**.

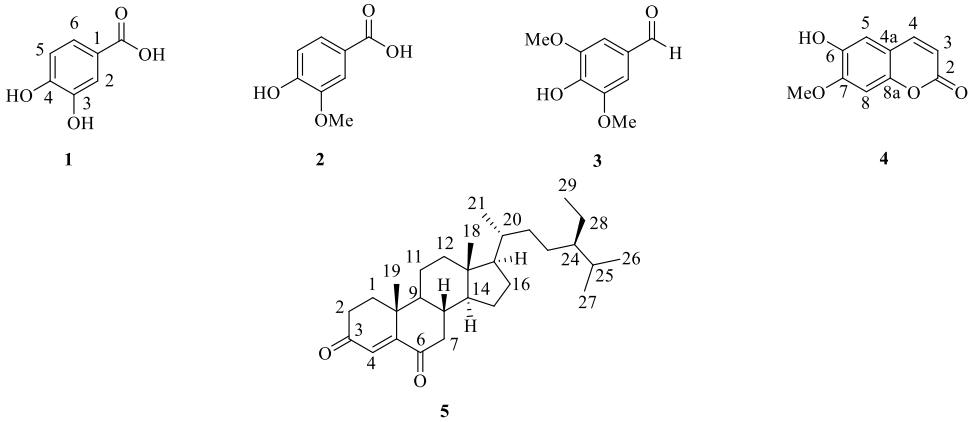

Figure 1 The chemical constituents from the stems of *C. buchanani*.

The ^{1}H NMR spectrum of compound **1** showed the presence of 3 proton signal peaks of an ABX system of a benzene ring at δ_H 7.46 (1H, m, H-2), 6.81 (1H, d, J = 8.8 Hz, H-5) and 7.46 (1 H, m, H-6) (**Table 1**). The ^{13}C NMR showed 2 oxygenated aromatic carbons at δ_C 144.1 (C-3), 149.6 (C-4) and a quaternary aromatic carbon δ_C 121.6 (C-1). The 3 methine aromatic carbons at δ_C 114.4 (C-2), 116.4 (C-5) and 122.9 (C-6), including for a carbonyl carbon of carboxylic group at δ_C 169.0 (C-1). The ^{1}H NMR and ^{13}C NMR data of **1** were compared with the literature [13,14], and determined to be 3,4-dihydroxyl benzoic acid.

The ^{1}H and ^{13}C NMR data of **2** (**Table 1**) were similar to those of **1**, with the only difference being the show of a methoxy group at δ_H 3.86 (1H, s, 3-OMe) and δ_C 55.9 (3-OMe). The difference of oxygenated aromatic carbon at C-3 of compounds **1** and **2,** the compound **2** showed downfield carbon at δ_C 146.7. So, the C-3 carbon was confirmed the difference of functional group. The compound **2** was identified as vanillic acid by comparison of its ^{1}H and ^{13}C NMR spectral data to the literature [15,16].

The ^{1}H NMR spectrum of **3** contained 2 aromatic protons at δ_H 7.15 (2 H, m, H-2,6), 6 protons of 2 methoxy group at δ_H 3.97 (6H, s, 3,6-OMe) and show a characteristic aldehyde proton at δ_H 9.82 (1H, s). ^{13}C NMR signals displayed 6 aromatic carbons at δ_C 106.8 (C-1), 128.5 (C-2,6), 147.4 (C-3,5) and 140.9 (C-4), 2 methoxy carbons at δ_C 56.6 (C-3,5) (Table 1). The above ^{1}H and ^{13}C NMR spectral data was consistent with the data of syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) reported in the literature [17].

The ^{1}H NMR spectrum of **4** showed 2 characteristic resonances for H-3 and H-4 of a coumarin at δ_H 6.17 (1H, d, J = 9.4 Hz, H-3) and 7.59 (1H, d, J = 9.4 Hz, H-4), 2 methine aromatic protons at δ_H 6.79 (1H, s, H-5) and 6.80 (1H, s, H-8). In addition, the signal at δ_H 3.85 (3H, s, 6-OMe) indicated the presence of a methoxy groups. The ^{13}C NMR spectrum of **4** showed 10 carbon signals, of which 9 were assigned to the coumarin skeleton part (δ_C 162.5, 150.8, 150.1, 145.2, 144.1, 112.3, 111.2, 108.0, 103.2, 56.2) as shown in **Table 1** [18]. A methoxy signal at δ_C 56.2 (6-OMe). Furthermore, all the data was consistent with the data of isoscopoletin reported in the literature [19]. Thus, Compound **4** was elucidated to be isoscopoletin.

Table 1 ^{1}H and ^{13}C NMR spectral data (400 MHz) of compounds **1**-**4**.

Position	**1**[b]		**2**[b]		**3**[a]		**4**[a]	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	121.6		121.9		106.8			
2	114.4	7.46 (1H, m)	112.4	7.49 (1H, d, J = 1.9)	128.5	7.15 (1H, s)	162.5	
3	144.1		146.7		147.4		111.2	6.17 (1H, d, J = 9.4)
4	149.6		150.6		140.9		144.1	7.59 (1H, d, J = 9.4)
4a							112.3	
5	116.4	6.81 (1H, d, J = 8.8)	114.4	6.83 (1H, d, J = 8.3)	147.4		108.0	6.79 (1H, s)
6	122.9	7.46 (1H, m)	124.4	7.55 (1H, dd, J = 8.3, 1.9)	128.5	7.15 (1H, s)	150.1	
7							145.2	
8							103.2	6.80 (1H, s)
8a							150.8	
1-COOH	169.0		168.9					
1-CHO					190.8	9.82 (3H, s)		
3-OMe			55.9	3.86 (3H, s)	56.6	3.97 (3H, s)		
6-OMe					56.6	3.97 (3H, s)	56.2	3.85 (3H, s)

[a]$CDCl_3$, [b]$CDCl_3$+CD_3OD

The ^{1}H and ^{13}C NMR spectra (**Table 2**) of **5** show sterol's characteristics. The ^{1}H NMR spectrum showed methylene proton at δ_H 6.16 (1H, s, H-5) and 6 methyl protons at δ_H 0.93 (3H, d, J = 6.2, H-29), 0.82 (d, 3H, J = 7.9, H-27), 0.85 (3H, d, J = 7.0, H-26), 0.72 (3H, s, H-18), 1.16 (3H, s, H-19), 0.93 (3H, d, J = 6.2, H-21β). The ^{13}C NMR spectrum displayed 29 carbons, including for 2 carbonyl group at δ_C 199.6 (C-3) and 202.5 (C-6). The olefinic carbon at δ_C 125.5 (C-4) and 161.2 (C-5). By means of comparison of its ^{1}H and ^{13}C NMR spectral data to the literature [20], compound **5** was deduced to be stigmast-4-ene-3,6-dione.

Table 2 ^{1}H and ^{13}C NMR spectral data ($CDCl_3$, 400 MHz) of compound **5**.

Position	stigmast-4-ene-3,6-dione [20]		5	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	35.6	1.91 (dd, J = 9.9, 5.7, H-1α) 2.14 (dd, J = 5.0, 2.8, H-1β)	35.6	1.92 (dd, J = 14.0, 5.3, H-1α) 2.12 (d, J = 16.2, H-1β)
2	34.0	2.46 (m, H-2α) 2.53 (m, H-2β)	34.1	2.49 (m, H-2α) 2.53 (m, H-2β)
3	199.5		199.6	
4	125.5	6.16 (s)	125.5	6.17 (s)
5	161.1		161.2	
6	202.3		202.5	
7	46.9	2.01 (dd, J = 12.6, H-7α) 2.67 (dd, J = 4.2, H-7 β)	46.9	2.02 (d, J = 16.0, H-7α) 2.68 (dd, J = 15.9, 3.7, Hβ)
8	34.3	1.90 (–[a], H-8β)	34.3	1.91 (–[a], H-8β)
9	51.1	1.36 (–[a], H-9α)	51.1	1.37 (–[a], H-9α)
10	39.9		39.9	
11	20.96	1.62 (m, H-11α) 1.49 (dd, J = 13.3, 4.2 H-11β)	21.0	1.62 (m, H-11α) 1.50 (dd, J = 12.9, 3.4 H-11β)
12	39.2	2.09 (ddd, J = 13.0, 3.4, H-12α) 1.25 (–[a], H-12β)	39.2	2.08 (d, J = 7.6, H-12α) 1.25 (–[a], H-12β)
13	42.6		42.6	
14	56.6	1.19 (–[a], H-14α)	56.7	1.20 (–[a], H-14α)
15	24.05	1.61 (m, H-15α) 1.12 (m, H-15β)	24.1	1.62 (m, H-15α) 1.13 (m, H-15β)
16	28.1	1.88 (–[a], H-16α) 1.30 (–[a], H-16β)	28.1	1.89 (–[a], H-16α) 1.31 (–[a], H-16β)
17	55.96	1.17 (–[a], H-17α)	56.9	1.20 (–[a], H-17α)
18	11.96	0.72 (s)	12.1	0.72 (s, H-18)
19	17.58	1.16 (s)	17.6	1.16 (s, H-19)
20	36.1	1.38 (m, H-20 β)	36.1	1.40 (m, H-20β)
21	18.78	0.93 (d, J = 6.5, H-21β)	18.8	0.93 (d, J = 6.2, H-21β)
22	33.9	1.04 (m, H_a-22) 1.32 (–[a], H_b-22)	33.9	1.04 (m, H_a-22) 1.31 (–[a], H_b-22)
23	26.2	1.18 (–[a], 2H-23)	26.1	1.22 (–[a], 2H-23)
24	45.9	0.93 (m, H-24)	45.9	0.94 (m, H-24)
25	29.3	1.67 (ddd, J = 6.9, 6.9, 1.8, H-25)	29.2	1.66 (dd, J = 12.7, 7.2, H-25)
26	19.9	0.83 (d, J = 6.9, 3H-26)	19.9	0.85 (d, J = 7.0, 3H-26)
27	19.1	0.81 (d, J = 6.9, 3H-27)	19.1	0.82 (d, J = 7.9, 3H-27)
28	23.2	1.22 (m, Ha-22), 1.27 (–[a], Hb-22)	23.2	1.23 (m, Ha-22), 1.29 (–[a], Hb-22)
29	12.04	0.84 (d, J = 7.6, 3H-29)	12.1	0.93 (d, J = 6.2, 3H-29)

[a] Overlapped signals

Nitric oxide (NO), a small signaling molecule, is one of the most important proinflammatory mediators. However, there are evidences indicating that the high polar solvent extract of *C. buchanani* was reported. The results give scientific support to the traditional use of this plant for combating inflammation [1,3,4,21,22]. In this research, the anti-inflammatory activity of the crude extracts from low to high polar solvent was compared. As a result, at a concentration of 50 μg/mL, the hexane and EtOAc crude extracts inhibited the NO production by 84.33±2.01 and 82.49±0.92 %, respectively (**Table 3**).

Table 3 Nitric oxide (NO) inhibitory effect of crude extracts from *C. buchanani.*

Crude	Concentration (μg/mL)	% NO inhibition	% Cell Viability Max. conc. at 100 μM (MTT assay)
Hexane	100	Toxic	18.6
	50	84.33 ± 2.01	> 80
EtOAc	100	Toxic	52.3
	50	82.49 ± 0.92	> 80
MeOH	100	Not active	> 80
	50	Not active	> 80

The five pure compounds were evaluated anti-inflammatory by using an *in vitro* model of RAW 264.7 macrophages. Indomethacin was used as a positive control (IC_{50} = 39.2 μM). The compound **3** providing IC_{50} value of 49.07 ± 1.69 μM, without any cytotoxicity observed (**Table 4**). It is believed that the aldehyde group necessitated the activity. Moreover, syringaldehyde (**3**) showed anti-inflammatory properties to exert cardio protective action [23].

Table 4 Nitric oxide (NO) inhibitory effect of isolated compounds from *C. buchanani.*

Compound	IC_{50} (μM) (NO inhibition)	% Cell Viability Max. conc. at 100 μM (MTT assay)
1	> 50	> 80
2	> 50	> 80
3	49.07 ± 1.69	> 80
4	> 50	> 80
5	> 50	> 80
Indomethacin	39.21 ± 1.51	> 80

Conclusions

Five known compounds, phenolic acid: 3,4-dihydroxyl benzoic acid (**1**), vanillic acid (**2**), syringaldehyde (**3**), a coumarins: isoscopoletin (**4**) and a steroid: stigmast-4-ene-3,6-dione (**5**) were isolated from the hexane and EtOAc extracts of *C. buchanani* stems. Compound **3** displayed NO inhibition with IC_{50} value of 49.07 ± 1.69 μM, without any cytotoxicity observed. This is the first report of the presence of compounds **1**-**3** and **5** in this plant.

Acknowledgements

The authors gratefully acknowledge for instrument and laboratory equipment from the Department of Chemistry, Faculty of Science, Khon Kaen University and Sakon Nakhon Rajabhat University, Thailand. Research and Development Institute, Sakon Nakhon Rajabhat University, Thailand, for supporting the research funding (Number: 2/2566).

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