CHEMICAL CONSTITUENTS FROM THE ARIAL OF *Crinum asiaticum* L.

PHAM VAN CONG [(1)], NGO VAN HIEU [(1)], NGO VIET DUC [(1)],
VU THI TRANG [(1)], DO THI THU HIEN [(1)], DO THANH TUAN [(2)],
NGUYEN THI THU HIEN [(3)], PHAM NGOC THUAT [(4)],
NGUYEN TUAN ANH [(5)], CU LAN HUONG [(5)], HOANG LE TUAN ANH [(1)]

1. INTRODUCTION

The genus *Crinum* (Amaryllidaceae family) with about 130 species, is characterized by that have large showy flowers on leafless stems, and developing from bulbs [1]. *Crinum* leaves are basal, typically long and strap-shaped, with colors ranging from light green to green [2]. They are distributed in seasonally moist and warm temperate areas of the world in Asia, Africa, America and Australia [3]. Eight species of genus *Crinum* have been found and identified in Vietnam: *C. asiaticum, C. amabile, C. giganteum, C. moorei, C. ensifolium, C. latifolium* and *C. zeylanicum*.

Crinum asiaticum L., as known as "Nang hoa trang", has been a well-known traditional medicine in various countries to treat gastrointestinal complaints, tonsillitis, urinary difficulties, vomiting, boils, contusions, edema, pain, rheumatism, wounds, swelling, aching joints and sores [4]. Previous studies indicated that its chemical constituents include alkaloids, fatty acids, sterols, triterpenes, anthraquinones and phenolic compounds. Furthermore, modern pharmacological studies reported these constituents to possess a lot of interesting activities, such as antibacterial and antifungal, cytotoxic, antioxidant, and anti-inflammatory activities… [4].

Inflammation is the body's response to an injurious stimulus, such as physical damage, ultraviolet irradiation, bacteria, virus and immune reactions [5]. Nitric oxide (NO), an inflammatory mediator, is known as a critical cellular signaling molecule involved in many physiological and pathological processes of both acute and chronic inflammatory disorders. Overproduction of NO results in the development of inflammatory diseases such as rheumatoid arthritis and autoimmune disorders. Thus, the regulation of NO production can be a treatment for neutralizing excessive inflammatory responses [6].

The enzyme acetylcholinesterase (AchE), one of the well-known enzymes that catalyze the cleavage of acetylcholine in the synaptic cleft after depolarization, plays an important role in the central nervous system. AChE inhibitors (AChEIs) are currently the most highly recommended approved therapy for the treatment of Alzheimer's disease, as well as for the production of insecticides [7].

In this research, we describe the isolation and structural elucidation of six compounds (**1**–**6**) from the aerial of *C. asiaticum* and their inhibitory activities on NO production and enzyme acetylcholinesterase.

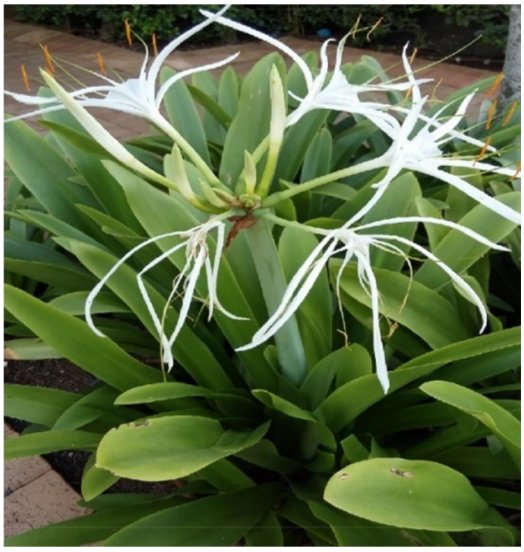

Figure 1. *Crinum asiaticum* L.

2. MATERIALS AND METHODS

2.1. General experimental procedures

^{1}H-(500, 600 MHz), ^{13}C-(125, 150 MHz) NMR, and 2D-NMR spectra were recorded on a Bruker Avance Digital 500 MHz NMR spectrometer (Karlsruhe, Germany) in ppm relative to tetramethylsilane (TMS) as an internal standard, *J* in Hz at 294 K. Thin-layer chromatography was performed using glass plates pre-coated with silica gel (60F254 and RP-18 F254s; Merck, Germany). Chromatography column (CC) was carried out on a Merck silica gel (60-200 μm) and Merck Lichroprep RP-18 gel (40-63 μm).

2.2. Plant material

The dried aerial part of *Crinum asiaticum* L. was collected from Thai Binh province, Viet Nam in January 2021, and identified by Dr. Do Thanh Tuan, Thai Binh University of Medicine and Pharmacy. The voucher specimen (CA-2021) was deposited at the Centre for Research and Technology Transfer, Vietnam Academy of Science and Technology.

2.3. Extraction and isolation

The dried aerial parts (3.0 kg) were extracted with MeOH (10L) at room temperature. The solvent was removed under reduced pressure to obtain the crude MeOH extract (250.0 g), which was extracted subsequently with *n*-hexane, dichloromethane (CH_2Cl_2), ethylacetate (EtOAc), and water layer. The EtOAc extract (45.0 g) was applied to silica gel CC and eluted with *n*-hexane/ EtOAc (9/1, v/v) to obtain six fractions (1A-1F). Fractions 1B (12.0 g) was further subjected to a silica gel CC eluting with CH_2Cl_2/ EtOAc (30/1, v/v) to get seven fractions (2A-2G).

Subfraction 2A (4.0 g) was isolated by RP-18 with MeOH/H_2O (3/1, v/v) to get compound **1** (12.0 mg) and compound **2** (8.0 mg). Fraction 2D (2.0 g) was further subjected to a Sephadex™ LH-20 column using solvent MeOH to afford compound **3** (4.0 mg). Fraction 2E (3.5 g) was subjected to a silica gel CC and eluted with CH_2Cl_2/ EtOAc (40/1, v/v) to yield compound **4** (5.0 mg). Sub-fraction 2F (1.5 g) was chromatographed on an RP-18 gel CC and eluted with MeOH/ H_2O (3/1,v/v) to afford compound **5** (3.5 mg) and compound **6** (6.0 mg).

Chrysophamol (**1**): orange needles; ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.08 (1H, s, H-2), 7.63 (1H, s, H-4), 7.80 (1H, d, J = 6.6 Hz, H-5), 7.65 (1H, t, J = 8.4, 6.6 Hz, H-5), 7.27 (1H, d, J = 8.4 Hz, H-7), 12.09 (1H, s, 1-OH), 11.98 (1H, s, 8-OH); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 162.4 (C-1), 124.5 (C-2), 149.3 (C-3), 121.3 (C-4), 133.3 (C-4a), 119.9 (C-5), 136.9 (C-6), 124.3 (C-7), 162.7 (C-8), 115.9 (C-8a), 192.5 (C-9), 113.7 (C-9a), 181.9 (C-10), 133.7 (C-10a), 22.3 (C-11) [10].

Emodin (**2**): orange needles; ^{1}H-NMR (600 MHz, acetone-d_6): δ_H 6.65 (1H, d, J = 2.0 Hz, H-2), 7.23 (1H, d, J = 2.0 Hz, H-4), 7.55 (1H, brs, H-5), 7.12 (1H, brs, H-7), 2.46 (3H,s, H-11), 12.06 (1H, s, 1-OH), 12.17 (1H, s, 8-OH); ^{13}C NMR (150 MHz, acetone-d_6): δ_C 163.2 (C-1), 124.9 (C-2), 149.5 (C-3), 121.4 (C-4), 134.2 (C-4a), 109.7 (C-5), 166.6 (C-6), 108.8 (C-7), 166.2 (C-8), 110.2 (C-8a), 191.6 (C-9), 114.4 (C-9a), 182.2 (C-10), 136.5 (C-10a), 21.9 (C-11) [11].

Physcion (**3**): orange needles; ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.07 (1H, s, H-2), 7.61 (1H, s, H-4), 7.35 (1H, d, J = 2.0 Hz, H-5), 6.67 (1H, d, J = 2.0 Hz, H-7), 2.44 (3H, s, H-11), 3.93 (3H, s, OCH_3), 12.28 (1H, s, 1-OH), 12.09 (1H, s, 8-OH); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 162.5 (C-1), 124.5 (C-2), 149.4 (C-3), 121.3 (C-4), 133.2 (C-4a), 108.2 (C-5), 166.6 (C-6), 106.8 (C-7), 165.2 (C-8), 110.3 (C-8a), 190.8 (C-9), 113.7 (C-9a), 182.0 (C-10), 135.3 (C-10a), 22.1 (C-11), 56.1 (OCH_3) [12].

Thymine (**4**): white solid; ^{1}H-NMR (500 MHz, DMSO): δ_H 7.24 (1H, d, J = 1.0 Hz, H-3), 1.72 (1H, d, J = 1.0 Hz, H-5); ^{13}C NMR (125 MHz, DMSO): δ_C 164.9 (C-1), 151.5 (C-4), 137.7 (C-3), 107.7 (C-2), 11.8 (C-5) [13].

Thymidine (**5**): white solid; ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.83 (1H, d, J = 1.0 Hz, H-4), 6.30 (1H, t, J = 7.0 Hz, H-1'), 2.26 (1H, m, H-2'), 4.42 (1H, m, H-3'), 3.93 (1H, m, H-4'), 3.76 (1H, dd, J = 3.5, 12.0 Hz, H-5'), 3.82 (1H, dd, J = 3.0, 12.0 Hz, H-5'), 1.90 (3H, d, J = 1.0 Hz, 5-CH_3); ^{13}C NMR (125 MHz, CD_3OD): δ_C 152.5 (C-2), 138.2 (C-4), 111.5 (C-5), 166.6 (C-6), 86.3 (C-1'), 41.2 (C-2'), 72.2 (C-3'), 88.8 (C-4'), 62.8 (C-5'), 12.4 (5-CH_3) [14].

5-Hydroxymethyl-2-furancarboxaldehyde (**6**): ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H 9.52 (1H, s, CHO), 7.24 (1H, d, J = 3.0 Hz, H-3), 6.52 (1H, d, J = 3.0 Hz, H-4), 4.69 (1H, s, H-6); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 177.9 (C-1), 161.3 (C-5), 152.1 (C-2), 123.6 (C-3), 110.0 (C-4), 57.2 (C-6) [15].

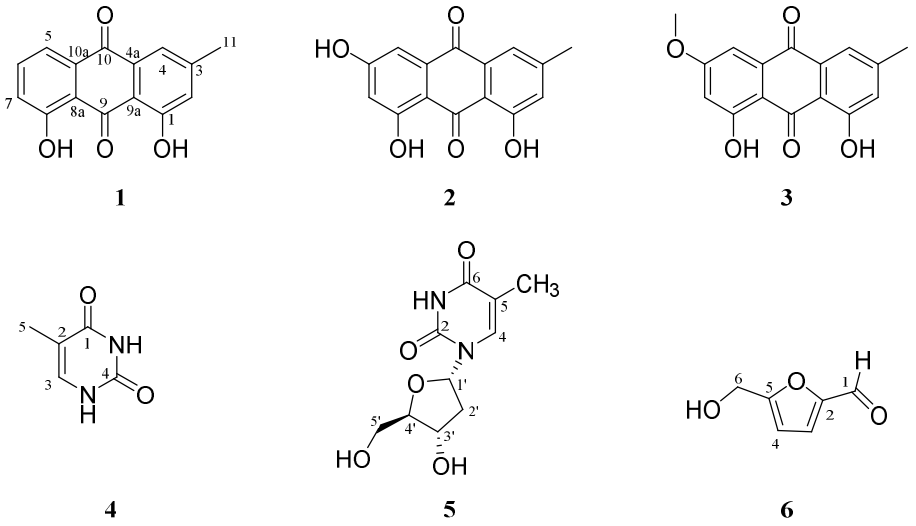

Figure 2. Chemical structures of compounds **1**-**6** isolated from *C. asiaticum* L.

2.4. Anti-inflammatory assays

The level of NO production was determined by measuring the amount of nitrite present in cell culture supernatants, as described previously. Briefly, the RAW264.7 cells (1×10^6 cells/well) were stimulated with or without 1 μg/mL LPS (Sigma Chemical Co., St. Louis, MO) for 24 h in the presence or absence of the test compounds (10 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediaminedihydrochloride in distilled H_2O). The absorbance at 540nm was determined with a microplate reader (MolecularDevices, Emax, Sunnyvale, CA, USA), and the absorption coefficient was calibrated by using a sodium nitrite ($NaNO_2$) solution standard. For this experiment, cell viability was measured with an MTT-based colorimetric assay. N^G-Methyl-L-arginine acetate (L-NMMA) was used as a positive control [8].

2.5. The inhibitory acetylcholinesterase enzyme assay

The acetylcholinesterase activity was determined following the spectrophotometric method using Ellman's reagents. Briefly, 40 μL of sodium phosphate buffer (pH 8.0), 20 μL of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), 20 μL of the test solution, and 20 μL of AChE solution were added into a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 μL of acetylthiocholine iodide. Hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 405 nm utilizing a 96-well microplate reader. The measurements and calculations were evaluated by using TableCurve 2Dv4. Galantamine was used as the positive control. The blank sample was the nonenzymatic hydrolysis of acetylcholine. The experiments were done in triplicate. The percentage of AChE inhibition (%I) was determined by using

the formula **%I = ((Ac-At)/(Ac))*100**, where **Ac** represents the enzyme control absorbance subtracted from the blank, and **At** is the sample absorbance subtracted by extract blank (test samples + substrate + buffer + DTNB) [9].

3. RESULTS AND DISCUSSION

3.1. Isolation of compounds from the aerials of *C. asiaticum*

Purification of the methanol extract of the aerials of *C. asiaticum* by chromatographic techniques gave six compounds, chrysophanol (**1**) [10], emodin (**2**) [11], physcion (**3**) [12], thymine (**4**) [13], thymidine (**5**) [14], 5-(hydroxymethyl)-2-furfural (**6**) [15] (Figure 2).

Compound **1** was isolated as orange needles. The ^{1}H-NMR spectrum of compound 1 showed signals of an ABC system at δ_H 7.80 (1H, d, J = 6.6 Hz, H-5), 7.65 (1H, t, J = 8.4, 6.6 Hz, H-6), 7.27 (1H, d, J = 8.4 Hz, H-7), a pair of singlet protons at δ_H 7.08 (1H, s, H-2), 7.63 (1H, s, H-4), and a methyl group at δ_H 2.46 (3H, s, H-11). The ^{13}C-NMR spectrum of compound **1** exhibited 15 carbons signal, including two carbonyl carbons at δ_C 192.5 (C-9) and 181.9 (C-10), two oxygenated quarternary carbons at δ_C 162.4 (C-1) and 162.7 (C-8), and a methyl carbon at δ_C 22.3 (C-11). These typical signals suggested compound **1** is an anthraquinone derivative. The ^{1}H and ^{13}C NMR data of **1** were consistent with those reported in the previous study [10], thus the structure of compound **1** was identified as chrysophanol (Figure 2).

Compound **2** was obtained as orange needles. The ^{1}H- and ^{13}C-NMR data of compound **2** also indicated that **2** was an anthraquinone derivative. Its ^{1}H-NMR spectrum displayed the presence of two pairs of meta coupling protons at δ_H 7.55 (1H, brs, H-5), 7.12 (1H, brs, H-7), 6.65 (1H, d, J = 2.0 Hz, H-2), 7.23 (1H, d, J = 2.0 Hz, H-4), and a singlet methyl proton at δ_H 2.46 (3H, s, H-11). In the ^{13}C-NMR spectrum of **2**, 15 carbons signals were observed. The 1D-NMR data of compound **2** were very similar to those of compound **1**, except for the addition of a hydroxy group at C-6. By careful analysis of NMR data and compared to the literature data, compound **2** was determined as emodin.

Compound **3** was obtained as orange needles. The ^{1}H- and ^{13}C-NMR spectra of **3** exhibited an anthraquinone skeleton, and were close to those of **2**, except for the addition of a methoxy group (δ_H 3.93 and δ_C 56.1, 6-OCH_3) at C-6. Based on the good agreement of its NMR data with those reported in the literature, compound **3** was elucidated as physcion.

Compound **4** was obtained as a colorless needle. The ^{1}H-NMR spectrum of **4** revealed the presence of a downfield vinylic proton at δ_H 7.24 (1H, d, J = 1.0 Hz, H-3) and a methyl group at 1.72 (1H, d, J = 1.0 Hz, H-5). The ^{13}C-NMR spectrum displayed the signals for two carbonyl carbons at δ_C 164.9 (C-1), 151.5 (C-4), two vinylic carbons at δ_C 137.7 (C-3), 107.7 (C-2), and a methyl carbon at δ_C 11.8 (C-5). The ^{1}H- and ^{13}C-NMR data of **4** was in good agreement with those of thymine, thus, compound **4** was identified as thymine.

Compound **5** was isolated as a colorless needle. The ^{1}H- and ^{13}C-NMR spectroscopic data of **5** were similar to those of 4. The main difference between the two compounds is the addition of desoxyribosyl moiety in the structure of **5** which was assumed from the signal of anomeric proton at δ_H 6.30 (1H, t, J = 7.0 Hz, H-1') in the ^{1}H NMR spectrum together with a set of characteristic signals at δ_C 86.3 (C-1'), 41.2 (C-2'), 72.2 (C-3'), 88.8 (C-4'), and 62.8 (C-5') in the ^{13}C-NMR spectrum. Thus, compound **5** was determined as thymine 2-desoxyriboside (Thymidine).

Compound **6** was obtained as a pale brown oil. The ^{1}H-NMR spectrum exhibited an aldehyde proton at δ_H 9.52 (1H, s, CHO), two aromatic protons at δ_H 7.24 (1H, d, J = 3.0 Hz, H-3), and 6.52 (1H, d, J = 3.0 Hz, H-4), and an oxygenated methylene at δ_H 4.69 (2H, s, OCH_2). The ^{13}C-NMR spectrum showed signals of six carbons, including an aldehyde carbon at δ_C 177.9 (CHO), four aromatic carbons at δ_C 161.3 (C-5), 152.1 (C-2), 123.6 (C-3), and 110.0 (C-4), an oxymethylene carbon at δ_C 57.2 (OCH_2), which suggested that a 2,5-disubstituted pyrrole ring. The ^{1}H- and ^{13}C-NMR data were in good agreement with those of 5-(hydroxymethyl)-2-furfural. Thus, the structure of **6** was determined as 5-(hydroxymethyl)-2-furfural.

3.2. The inhibition of NO production and enzyme AchE

As results are shown in Table 1 for the biological activities, the inhibitory activities of NO production and enzyme AchE of compounds **1**-**6** were evaluated with L-NMMA and galantamine served as the positive controls.

All isolated compounds have tested the inhibition of NO production in LPS-induced RAW 264.7 cells only compound **2** (emodin) showed a strong inhibitory effect on NO production with an IC_{50} value of 5.13±0.42 (positive control IC_{50} 8.13±0.46) without the cytotoxicity. Our result was also consistent with the previous study reporting that emodin can effectively inhibit the excessive production of NO in RAW 264.7 [16]. In addition, these other compounds were inactive. Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), a natural anthraquinone derivative, displays anti-inflammatory, antidiabetic, antimicrobial, antioxidant and anti-angiogenic effects, both *in vitro* and *in vivo* [17]. This study further confirmed the anti-inflammatory effects of emodin through the inhibition of NO production and suggested that emodin may be used as an inhibitor of inflammatory diseases from natural sources.

Furthermore, all isolates were also evaluated for their AchE inhibitory activities. They weakly inhibited AchE activities with IC_{50} range from 69.86±4.06 to 237.63±13.80 μM when compared to the positive control galantamine (IC_{50} 2.40±0.45 μM). Pickhardt reported that emodin and its derivatives possess the potential anti-alzheimer's disease (AD) activity and hold great promise for the treatment of AD and suggested that these compounds have great value in the development of therapeutic and preventive agents for AD [18]. However, further *in vivo* studies should be conducted to clarify the detailed mechanism of action of these compounds.

Table 1. Inhibition of NO production and AchE activity by compounds **1**-**6**

Compound	IC_{50} (μM)		
	NO inhibition	**MTT**	**AchE inhibition**
1	> 100	-	119.20±14.61
2	5.13±0.42	> 100	69.86±4.06
3	> 100	-	82.98±9.19
4	> 100	-	210.57±13.77
5	> 100	-	88.16±8.92
6	> 100	-	237.63±13.80
L-NMMA	8.13±0.46	> 100	-
Galantamine	-		2.40±0.45

4. CONCLUSION

Six compounds, including chrysophanol (**1**), emodin (**2**), physcion (**3**), thymine (**4**), thymidine (**5**), 5-(hydroxymethyl)-2-furfural (**6**) were isolated and identified from the *C. asiaticum* by the extensive spectroscopic analysis and by comparison of spectral data with those of previously reported data. This is the first report of compounds **1**-**6** from *C. asiaticum*. In addition, all compounds were evaluated for the inhibition of NO production and AchE activity. As result, compound **2** showed a strong inhibitory effect on NO production with an IC_{50} value of 5.13±0.42 (positive control IC_{50} 8.13±0.46). And they have weakly inhibited AchE with IC_{50} range from 69.86±4.06 to 237.63±13.80 μM. The findings of the present study suggest the potential of emodin (**2**) for use in the development of therapeutic or preventive agents for inflammatory diseases. In addition, further studies of anti-AD activities through inhibition of AChE activities by isolates (**1**-**6**) should be more conducted.

Acknowledgments: *This research was supported by the Ministry of Science and Technology under grant number DTDL.CN-128/21.*

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SUMMARY

Crinum asiaticum L. (locally known Nang hoa trang), belonging to the *Crinum* genus of the Amaryllidaceae family, has been widely used in oriental and traditional medicine around the world for the treatment of various diseases and exerts diverse promising pharmacological effects. To find out new inhibitors for the treatment of inflammatory and Alzheimer's diseases, a methanol extract of the aerial part of *C. asiaticum* was investigated to afford six compounds, chrysophanol (**1**), emodin (**2**), physcion (**3**), thymine (**4**), thymidine (**5**), and 5-(hydroxymethyl)-2-furfural (**6**) by using various chromatographic separations. Their structures were identified by the extensive analysis of ^{1}H- and ^{13}C-NMR data as well as a comparison with those of previously reported data. This is the first report of these compounds (**1**-**6**) from *C. asiaticum*. In addition, all compounds evaluated the inhibitory activities of NO production and acetylcholinesterase (AChE). Among them, emodin (**2**) significantly inhibited NO production with an IC_{50} of 5.13±0.42 μM. This result indicated that emodin (**2**) may be beneficial in the treatment of anti-inflammatory diseases. Besides, all compounds (**1-6**) also showed weak inhibitory effects on the AChE enzyme with a range of IC_{50} values from 82.98±9.19 to 237.63±13.80 μM.

Keywords: *Crinum asiaticum* L., anti-inflammation, acetylcholinesterase (AChE), kháng viêm.

Nhận bài ngày 01 tháng 8 năm 2022
Phản biện xong ngày 22 tháng 09 năm 2022
Hoàn thiện ngày 31 tháng 10 năm 2022

(1) Center for Research and Technology Transfer
(2) Thaibinh University of Medicine and Pharmacy
(3) Hanoi University of Mining and Geology
(4) Institute of Technology-General Department for Defence Industry
(5) Central Ethnic Minority College Preparatory School

Corresponding author: ***Hoang Le Tuan Anh***

Center for Research and Technology Transfer, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam

Tel.: 84-948151838; E-mail: hltanh@ctctt.vast.vn