and loratadine illegally mixed in herbal preparations. Sample treatment is quick and simple. The analytical method has high specificity, low detection limit, accuracy and precision met the requirements of AOAC 2016. The method has high feasibility in analyzing real samples circulating in the market. The study analyzed and found 17.1% positive samples (6 out of 35 tested samples), detected 4 out of 5 research active pharmaceutical ingredients adulterated in the samples, except promethazine, with concentrations ranging from 0.048 to 3.13 mg/g.

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ANTI-INFLAMMATORY EFFECTS OF CHROMONES AND SESQUITERPENOIDS FROM THE AGARWOOD OF *AQUILARIA CRASSNA*

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Summary

Anti-Inflammatory Effects of Chromones and Sesquiterpenoids from the Agarwood of *Aquilaria crassna*

Aquilaria crassna Pierre ex Lecomte is the most dominant species of the genus *Aquilaria*, which is widely distributed from the south to the north of Vietnam. In Vietnam, agarwood from *A. crassna* has been used in traditional medicine to treat insomnia, anxiety, asthma, pain, and digestive disorders. Our continuous research on the agarwood from *A. crassna* led to the isolation of fourteen compounds, which were identified as 12-hydroxy-4(5),11(13)-eudesmadien-15-al (**1**), 4'-methoxy-2-(2-phenylethyl)chromone (**2**), baimuxinic acid (**3**), 1-hydroxy-1,5-diphenylpentan-3-one (**4**), 5-hydroxy-7,4'-dimethoxyflavone (**5**), 6,7,4'-trimethoxy-2-(2-phenylethyl)chromone (**6**), 6,4'-dimethoxy-3'-hydroxy-2-(2-phenylethyl)chromone (**7**), 6,3'-dimethoxy-4'-hydroxy-2-(2-phenylethyl)chromone (**8**), 6-methoxy-4'-hydroxy-2-(2-phenylethyl)chromone (**9**), 4'-hydroxy-2-(2-phenylethyl)chromone (**10**) 6-methoxy-2-(phenylethyl)chromone (**11**), 5-hydroxy-6,4′-dimethoxy-2-(phenylethyl)chromone (**12**), 6-hydroxy-2-(2-phenylethyl)chromone (**13**), 6-hydroxy-2-[2-(4'-methoxyphenyl)ethyl]chromone (**14**), from the ethyl acetate fraction of agarwood of *A. crassna*. Their structures were determined by means of spectroscopic methods (MS, 1D, and 2D-NMR). The anti-inflammatory effects of extracts and isolated compounds were evaluated against NO production in microglial BV2 cells.

Keywords: *Aquilaria crassna*, *Anti-inflammatory effects, Agarwood, Chromone, Sesquiterpenoid.*

1. Introduction

Agarwood is a fragrant resin produced inside the heartwood of various *Aquilaria* species when injured by insects, physical cuts, microbial infections, or chemical stimulation. In recent studies, sesquiterpenoids and chromone derivatives from agarwood were reported to have anti-neuroinflammatory, antibacterial, α-glucosidase inhibitory, cytotoxic, acetylcholinesterase inhibitory, neuroprotective, and antidepressant activities. *Aquilaria crassna* (Thymelaeaceae) produced a popular type of agarwood, which was found in the rainforests of the Indo-Malesia region [1],[2]. It has also been

used as an analgesic, and sedative for treating digestive disorders [3],[4]. The studies on agarwood from *A. crassna* in Vietnam were focused on cultivation, plantation, agarwood formation [5],[6], its genetic relationship with other *Aquilaria* species [7], and essential oil extraction [8]. To our knowledge, phytochemical and pharmacological studies on the agarwood from *A. crassna* have been limited in Vietnam. Our previous study on the agarwood from *A. crassna* reported the isolation of five chromone derivatives from the ethyl acetate extract [9]. In our ongoing study on agarwood, fourteen compounds were isolated from the ethyl acetate extract of agarwood from *A. crassna*. The present study reported the chemical constituents and anti-inflammatory effects of chromone and sesquiterpenoid derivatives of agarwood from *A. crassna*. Their structures were elucidated by various spectroscopic methods (UV, MS, 1D, and 2D NMR). The anti-inflammatory effects of the extracts and compounds were evaluated using the NO production assay in microglial BV2 cells.

2. Experimental

2.1. Materials and reagents

The agarwood chips of *Aquilaria crassna* (5.0 kg) cultivated in Binh Phuoc province, were provided by Evergreen Forest Joint Stock Co. (Ho Chi Minh City, Vietnam) in December 2019. The voucher specimen (No: 201912Agar) was deposited at the Department of Pharmacognosy, University of Medicine and Pharmacy at Ho Chi Minh City.

The solvents: ethanol 96%, *n*-hexane, ethyl acetate, chloroform, and methanol (Scharlau, Spain) were used for isolation and extraction. Solvents for HPLC were purchased from VWR Chemicals BDH (France).

2.2. General experimental procedures

Silica gel (40-63 μm, Merck) and Sephadex LH-20 (GE Healthcare, Sweden) were used for column chromatography. TLC was performed using normal phase *silica gel* 60 F254 plates (Merck). Spots were detected by UV and a 10% H_2SO_4 solution. ESI-MS spectra were obtained from a Xevo G2-XS QTOF mass spectrometer (Waters, USA). An HPLC system (Shimadzu LC20AR, Japan) with an SPD-20A detector and a Phenomenex RP-C18 (5 µm, 4.6 i.d. × 250 mm) column was used for semi-preparative HPLC separation, flow rate 4 mL/min. NMR spectra were measured on a Bruker Advance Neo 400 using TMS as an internal standard. NMR solvents were purchased from Sigma-Aldrich (USA).

2.3. Extraction and isolation

The agarwood chips of *A. crassna* (5.0 kg) were ground and sonicated at 50 °C with 70% MeOH (170 L). The solvents were evaporated under reduced pressure to obtain 70% MeOH extract (AcT, 500 g). The 70% MeOH extract was successively partitioned with *n*-hexane (2 L × 3), ethyl acetate (2 L × 6), and saturated *n*-BuOH (2 L × 3). The solvents were removed to obtain *n*-hexane (AcH, 2.0 g), ethyl acetate (AcE, 140 g), *n*-BuOH (AcB, 140 g), and water (AcN, 49 g) extracts, respectively. The ethyl acetate extract (59.2 g) was subjected to Sephadex LH-20 column chromatography (CC) to obtain 4 fractions (AcE1-AcE4). Fraction AcE2 (27.4 g) was separated by Sephadex LH-20 CC (MeOH) to yield 4 subfractions (AcE2.1-AcE2.4). Fraction AcE2.2 (7.13 g) was applied to a *silica gel* (180 g, Merck, 40-63 µm) column with *n*-hexane-EtOAc solvent system (9:1-6:4), then with $CHCl_3$-MeOH (10:0-6:4) to obtain 16 subfractions (AcE2.2.1-AcE2.2.16). Subfraction AcE2.2.3 (509.7 mg) was subjected to a Sephadex LH-20 CC (MeOH) to give three subfractions (AcE2.2.3.1-AcE2.2.3.3).

Compound **1** (27.6 mg) was isolated from AcE2.2.3.1 (268.4 mg), while compounds **2** (10.7 mg) and **11** (27.4 mg) were obtained from AcE2.2.3.2 (217.9 mg) by semi-preparative HPLC (ACN:water, 45:55, v/v). Fraction AcE2.3 (8.43 g) was applied to a *silica gel* column with the same conditions as used in the fractionation of AcE2.2 to obtain 12 subfractions (AcE2.3.1-AcE2.3.12). Subfraction AcE2.3.2 (520.7 mg) was continuously separated by Sephadex LH-20 CC (MeOH) to give 7 subfractions (AcE2.3.2.1-AcE2.3.2.7). Compound **3** (8.5 mg) was isolated by semi-preparative HPLC (ACN:water, 45:55, v/v) from AcE2.3.2.1 (68.7 mg), and compounds **3** (35.6 mg) and **4** (10.6 mg) were obtained from AcE2.3.2.2 (74.1 mg) by semi-preparative HPLC (ACN:water, 50:50, v/v). Compound **12** (5.0 mg) was obtained by crystallization in MeOH from subfraction AcE2.3.2.6 (26.2 mg). By semi-preparative HPLC (ACN:water, 45:55, v/v) compounds **5** (2.3 mg), **11** (2.1 mg), and **12** (6.0 mg) were isolated from subfraction AcE2.3.2.7 (22.5 mg). Fraction AcE2.3.3 (204.3 mg) was fractionated by Sephadex LH-20 CC (MeOH) to give 6 fractions (AcE2.3.3.1-AcE2.3.3.6). Compounds **2** (1.7 mg) and **12** (5.1 mg) were isolated from subfraction AcE2.3.3.4 (21.8 mg) and compound **13** (11 mg) was purified from AcE2.3.4 (231.2 mg) by semi-preparative HPLC (ACN:water, 45:55, v/v), respectively. Fraction AcE2.3.5 (204.3 mg) was further subjected to Sephadex LH-20 CC (MeOH) to give 5 subfractions (AcE2.3.3.1-AcE2.3.3.5). By semi-preparative HPLC, compound **6** (84 mg, ACN: water, 50:50, v/v) was purified from subfraction AcE2.3.5.1 (87.2 mg), while **7** (2.5 mg) and **8** (0.8 mg) were separated with the isocratic condition of ACN:water (40:60, v/v) from subfraction

AcE2.3.5.2 (9.6 mg), **9** (6.4 mg), and **10** (9.8 mg) and compounds **14** (6.4 mg) were isolated from subfraction AcE2.3.5.4 (179.1 mg) with the isocratic solvent system of ACN:water (45:55, v/v).

2.3. Cell culture

The microglial BV2 cells (ATCC, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Welgene, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma) and 1% penicillin/streptomycin (Gibco, USA) in a 5% CO_2 incubator at 37°C. To evaluate the percentage of cell viability, cells (5.0×10^4) were seeded into 96-well plates and incubated for 24 h at 37°C. The cells were then treated with two concentrations of extract (10 and 50 μg/mL) in the absence of LPS for 24 h. Cell viability was tested using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay method. The absorbance at 595 nm was measured using a microplate absorbance reader (iMark, Bio-Rad, USA) [10].

2.4. NO measurement

The cells (2.0×10^5) were seeded into 48-well plates and incubated at 37°C for 24 h, then pre-treated with various concentrations of test samples (1, 10, and 50 μg/mL for extracts; and 1, 5, 10, 25, and 50 μM for compounds) for 30 min and stimulated with LPS (100 ng/mL) for 24 h. The nitrite levels in the cultured media were measured using a colorimetric method based on the Griess reaction [11]. In brief, 100 μL supernatant and the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 5% phosphoric acid) were incubated at room temperature for 15 min, and the absorbance was measured at 540 nm using a microplate reader (iMark, Bio-Rad, USA). The concentration of nitrite was determined from the standard curve.

2.5. Statistical analysis

Data are presented as the mean ± standard deviation (SD) of three independent experiments. IC_{50} values were calculated using a non-linear regression using GraphPad Prism 8 (GraphPad Software Inc., CA, USA). Statistical significance was analyzed by one-way analysis of variance (ANOVA). Statistical significance is expressed as *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

12-Hydroxy-4(5),11(13)-eudesmadien-15-al (**1**): colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 10.2 (1H, s, -CHO-15), 5.12 (1H, s, H-13a), 4.99 (1H, s, H-13b), 4.16 (2H, s, H-12), 3.43 (1H, d, J = 10.4 Hz, H-6a), 2.35 (1H, dd, J = 15.6, 4.0 Hz, H-3a), 2.16 (2H, m, H-6b and H-7), 2.09 (1H, m, H-3b), 1.74 (1H, m, H-8), 1.71 (1H, m, H-9a), 1.65 (1H, m, H-2a), 1.57 (1H, m, H-1a), 1.55 (1H, m, H-2b), 1.50 (1H, m, H-9b), 1.37 (1H, m, H-1b), 1.21 (3H, s, H_3-14). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 190.7 (C-15), 163.8 (C-5), 152.5 (C-11), 132.1 (C-4), 109.1 (C-13), 65.2 (C-12), 43.2 (C-7), 41.5 (C-9), 39.4 (C-1), 36.7 (C-10), 29.7 (C-6), 27.7 (C-8), 25.2 (C-14), 23.8 (C-3), 17.8 (C-2).

4'-Methoxy-2-(2-phenylethyl)chromone (**2**): yellowish oil; ESI-MS: *m/z* 281.1186 $[M+H]^+$, $C_{18}H_{16}O_3$. ^{1}H NMR and ^{13}C NMR data: see Table 1 and 2.

Baimuxinic acid (**3**): colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 6.89 (1H, t, J = 3.2 Hz, H-1), 2.53 (1H, m, H-7), 2.20 (1H, m, H-2a), 2.15 (1H, m, H-2b), 2.03 (1H, m, H-9a), 1.73 (1H, m, H-6a), 1.72 (1H, m, H-8a), 1.71 (1H, m, H-3a), 1.68 (1H, m, H-4), 1.66 (1H, m, H-9b), 1.60 (1H, m, H-6b), 1.45 (1H, m, H-3b), 1.39 (1H, m, H-8b), 1.19 (6H, s, H_3-12 and H_3-13), 0.95 (3H, d, J = 6.4, H_3-15). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 171.3 (C-14), 140.1 (C-1), 138.0 (C-10), 72.1 (C-11), 51.9 (C-7), 46.4 (C-5), 39.3 (C-9), 38.4 (C-4), 35.7 (C-6), 28.3 (C-12), 28.3 (C-13), 27.9 (C-8), 26.4 (C-3), 23.4 (C-2), 15.5 (C-15).

1-Hydroxy-1,5-diphenylpentan-3-one (**4**): colorless oil; ESI-MS: *m/z* 277.1081 $[M+Na]^+$, $C_{17}H_{18}O_2$; ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 7.33 (2H, m, H-3′ and H-5′), 7.32 (2H, m, H-2′ and H-6′), 7.27 (2H, m, H-3″ and H-5″), 7.26 (1H, m, H-4′), 7.19 (1H, m, H-4″), 7.15 (2H, m, H-2″ and H-6″). 5.13 (1H, dd, J = 9.6, 3.2 Hz, H-1), 2.90 (2H, t, J = 7.5 Hz, H_2-5), 2.83 (1H, dd, J = 17.6, 9.6 Hz, H-2a), 2.75 (2H, m, H_2-4), 2.74 (1H, m, H-2b). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 210.2 (C-3), 142.7 (C-1′), 140.6 (C-1″), 128.5 (C-3′ and C-5′), 128.5 (C-3″ and C-5″), 128.2 (C-2″ and C-6″), 127.6 (C-4′), 126.2 (C-4″), 125.6 (C-2′ and C-6′), 69.9 (C-1), 51.3 (C-2), 45.1 (C-4), 29.4 (C-5).

5-Hydroxy-7,4'-dimethoxyflavone (**5**): pale yellow amorphous powder; ESI-MS: *m/z* 299.1066 $[M+H]^+$, $C_{17}H_{14}O_5$; ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 12.80 (1H, s, 5-OH), 7.85 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 7.02 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 6.58 (1H, s, H-3), 6.48 (1H, d, J = 2.8 Hz, H-8), 6.37 (1H, d, J = 2.8 Hz, H-6), 3.90 (3H, s, 7-OCH_3), 3.89 (3H, s, 4′-OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 182.5 (C-4), 165.4 (C-7), 164.0 (C-2), 162.6 (C-4′), 162.2 (C-5), 157.7 (C-9), 128.1 (C-2′ and C-6′), 123.6 (C-1′), 114.5 (C-3′ and C-5′), 105.6 (C-10), 104.4 (C-3), 98.0 (C-6), 92.6 (C-8), 55.8 (7-OCH_3), 55.5 (4′-OCH_3).

6,7,4'-Trimethoxy-2-(2-phenylethyl)chromone (**6**): yellowish amorphous powder; ESI-MS: *m/z* 341.1417 $[M+H]^+$, $C_{20}H_{20}O_5$. ^{1}H NMR and ^{13}C NMR data: see Table 1 and 2.

6,4'-Dimethoxy-3'-hydroxy-2-(2-phenylethyl)chromone (**7**): yellowish oil; ESI-MS: *m/z* 327.12897 $[M+H]^+$, $C_{19}H_{18}O_5$. ^{1}H NMR and ^{13}C NMR data: see Table 1 and 2.

6,3'-Dimethoxy-4'-hydroxy-2-(2-phenylethyl) chromone (**8**): yellowish oil; ESI-MS: *m/z* 327.11416 $[M+H]^+$, $C_{19}H_{18}O_5$. ^{1}H NMR and ^{13}C NMR data: see Table 1 and 2.

6-Methoxy-4'-hydroxy-2-(2-phenylethyl) chromone (**9**): yellowish amorphous powder; ESI-MS: *m/z* 297.12082 $[M+H]^+$, $C_{18}H_{16}O_4$. ^1H NMR and ^{13}C NMR data: see Table 1 and 2.

4'-Hydroxy-2-(2-phenylethyl)chromone (**10**): yellowish oil, ESI-MS: *m/z* 267.0721 $[M+H]^+$, $C_{17}H_{14}O_3$. ^1H NMR and ^{13}C NMR data: see Table 1 and 2.

3. Results and Discussion

3.1. Evaluation of the cell viability and anti-inflammatory activity of the extracts

AcT, AcH, AcE, and AcB extracts were evaluated for cell viability and anti-inflammatory effects in BV2 microglial cells. As a result, the methanol extract displayed no cytotoxicity at the tested concentrations. At a concentration of 50 μg/mL, the total extract showed strong effect against NO production on LPS-stimulated BV2 cells (Fig. 1). The *n*-hexane and ethyl acetate extracts showed significant NO inhibitory activity with IC_{50} values of 5.67 ± 0.9 and 26.46 ± 6.12 μg/mL, respectively. Both *n*-hexane and ethyl acetate extracts showed percentages of cell survival of 7.8% and 14.1% at a concentration of 50 μg/mL and 96.5% and 95.3% at a concentration of 10 μg/mL, respectively, indicating that these extracts showed insignificant cytotoxicity at low concentrations. The *n*-BuOH extract showed neither cytotoxicity nor NO inhibitory activity on LPS-induced BV2 cells. Thus, the ethyl acetate extract was used for further isolation of pure compounds based on the activity test results.

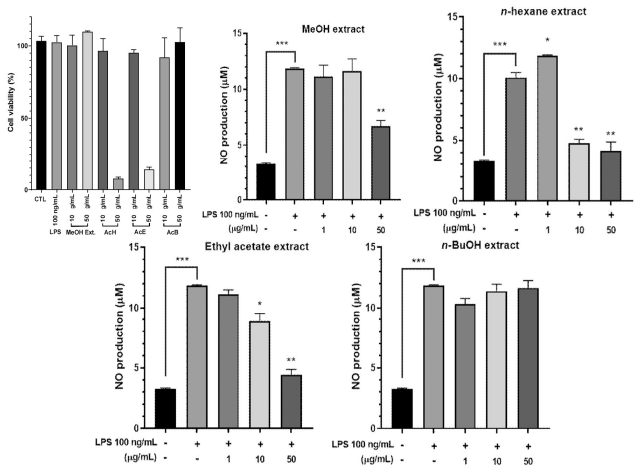

Fig. 1. Cell viability and anti-inflammatory effects of extracts and fractions on BV2 cells

3.2. Structure determination of isolated compounds

Compound **1** was isolated as a colorless oil. The ^{13}C NMR spectra of **1** showed 15 carbon signals, including one formyl group (δ_C 190.7, C-15), one olefinic carbon (δ_C 109.1, C-13), four non-protonated carbons (δ_C 163.8 (C-5), 152.5 (C-11), 132.1 (C-4), and 36.7 (C-10)), one aliphatic methine carbon (δ_C 43.2, C-7), seven methylene carbons (δ_C 65.2 (C-12), 41.5 (C-9), 39.4 (C-1), 29.7 (C-6), 27.7 (C-8), 23.8 (C-3), and 17.8 (C-2)), and one methyl group (δ_C 25.2, C-14). The ^1H NMR spectrum showed the singlet signals of a formyl group at δ_H 10.2 (-CHO-15), a terminal double bond at δ_H 5.12 and 4.99 (H_2-13), and an oxymethylene at δ_H 4.16 (H_2-12). The ^1H-^1H COSY revealed the correlations of two consecutive aliphatic protons of H_2-1/H_2-2/H_2-3 and H_2-6/H-7/H_2-8/H_2-9. In addition, the HMBC spectrum showed the correlations from H_2-1 (δ_H 1.57 and 1.37) to C-3/C-5/C-6/C-14, from H_2-2 and C-3, from H_2-6 to C-8/C-10, from H_2-8 to C-6/C-7/C-10, from H_2-9 to C-7/C-10, from H_2-12 to C-7/C-11/C-13, from H_2-13 to C-7/C-11/C-12, from H_3-14 to C-1/C-5/C-9/C-10, and from -CHO-15 to C-3/C-4. The aforementioned data demonstrated that **1** was a sesquiterpenoid derivative. In comparison with reported data in the literature [12], compound **1** was identified as 12-hydroxy-4(5),11(13)-eudesmadien-15-al.

Compound **2** was obtained as a yellowish oil. The molecular formula was $C_{18}H_{16}O_3$ based on the positive ESI-MS spectrum with an ion peak at *m/z* 281.1186 $[M+H]^+$. The ^{13}C NMR showed signals of 18 carbons, comprising one carbonyl group (δ_C 178.3), three oxygenated carbons (δ_C 168.6, 158.3, and 156.5), two non-protonated carbons (δ_C 131.7 and 123.7), nine olefinic carbons (δ_C 133.5, 129,3 (C-2′/C-6′), 125.7, 125.0, 117.8, 114.1 (C-3′/C-6′), and 110.3), two aliphatic methylene carbons (δ_C 36.4 and 32.1), and one methoxy group (δ_C 55.3). The carbon signals appeared in the downfield region in the range of δ_C 110-178, together with two methylene groups of the typical region of the phenylethylchromone framework. In addition, the ^{13}C NMR spectrum of **2** showed the presence of a 1,4-disubstituted benzene ring, which demonstrated that one substituent was attached to C-4'. The ^1H NMR spectrum showed the signals of four consecutive aromatic protons at δ_H 8.18 (1H, d, J = 7.2 Hz, H-5), 7.38 (1H, d, J = 7.2 Hz, H-6), 7.65 (1H, d, J = 7.2 Hz, H-7), and 7.44 (1H, d, J = 7.2 Hz, H-8). The signals of one pair of aromatic symmetric protons at δ_H 7.11 (2H, d, J = 8.0 Hz, H-2' and H-6') and 6.83 (2H, d, J = 8.0 Hz, H-3' and H-5') were assigned to the 1',4'-disubstituted structure of the phenethyl group. Based on the MS, NMR spectroscopic data, and comparison with the published data [13], compound **2** was identified as 4'-methoxy-2-(2-phenylethyl)chromone.

Compound **3** was obtained as a colorless oil. The ^{13}C NMR spectrum of **3** indicated signals of 15 carbons, including one carbonyl carbon (δ_C 171.3, C-14), one olefinic carbon (δ_C 1140.165.5, C-1), three non-protonated carbons (δ_C 138.0 (C-10), 72.1 (C-11), and 46.4 (C-5)), two aliphatic methine carbons (δ_C 51.9, C-7 and 38.4, C-4), five methylene carbons (δ_C 39.3 (C-9), 35.7 (C-6), 27.9 (C-8), 26.4 (C-3), and 23.4 (C-2)), and three methyl groups (δ_C 28.3 (C-12 and C-13), and 15.5 (C-15)). The ^1H NMR spectrum of **3** showed five methylene, three methine, and three methoxy groups. The ^1H-^1H COSY spectrum revealed the correlations of two-spin systems of H-1/H_2-2/H_2-3/H-4/H_3-15 and H_2-6/H-7/H_2-8/H_2-9. These data suggested that compound **3** was a sesquiterpenoid. The HMBC spectrum showed correlations from H-1 (δ_H 6.89) to C-2 (δ_C 23.4)/C-3 (δ_C 26.4)/C-5 (δ_C 46.4)/C-10 (δ_C 138.0)/C-14 (δ_C 171.3), from H_2-2 (δ_H 2.20 and 2.15) to C-3/C-10/C-14, from H_2-3 (δ_H 1.71 and 1.45) to C-1 (δ_C 140.1)/C-4 (δ_C 38.4)/C-10, from H-4 (δ_H 1.68) to C-3/C-5/C-6 (δ_C 35.7)/C-8 (δ_C 27.9)/C-10, from H_2-6 (δ_H 1.73 and 1.60) to C-4/C-5/C-8/C-11 (δ_C 72.1), from H-7 (δ_H 2.53) to C-6/C-8/C-12/C-13 (δ_C 28.3), from H_2-8 (δ_H 1.72 and 1.39) to C-7/C-9/C-11, from H_2-9 to C-4/C-5/C-8/C-10, from H_3-12 to C-7/C-11/C-13, H_3-13 to C-7/C-11/C-12, and from H_3-15 to C-3/C-4/C-5. Compared to the reported spectroscopic data in the literature [14], compound **3** was identified as baimuxinic acid, which was first reported from the agarwood of *A. crassna*.

Table 1. ^1H NMR (400 MHz) spectroscopic data of compounds **2** and **6-10**.

No.	**2**[a]	**6**[a]	**7**[a]	**8**[a]	**9**[b]	**10**[a]
	δ_H mult (J in Hz)	δ_H mult (J in Hz)	δ_H mult (J in Hz)	δ_H mult (J in Hz)	δ_H mult (J in Hz)	δ_H mult (J in Hz)
H-3	6.14 s	6.10 s	6.11 s	6.14 s	6.15 s	6.15 s
H-5	8.18 d (7.2)	7.51 s	7.54 d (2.0)	7.54 d (2.4)	7.56 d (2.8)	7.18 d (7.6)
H-6	7.38 t (7.2)					7.38 t (7.6)
H-7	7.65 t (7.2)		7.24 dd (9.2, 2.0)	7.24 dd (8.8, 2.4)	7.27 dd (9.2, 2.8)	7.67 t (7.6)
H-8	7.44 d (7.2)	6.86 s	7.37 d (9.2)	7.37 d (8.8)	7.40 d (9.2)	7.44 d (7.6)
H-2′	7.11 d (8.0)	7.11 d (8.0)	6.79 d (2.0)	6.66 d (2.0)	7.06 d (8.0)	7.06 d (7.2)
H-3′	6.83 d (8.0)	6.83 d (8.0)			6.78 d (8.0)	6.78 d (7.2)
H-5′	6.83 d (8.0)	6.83 d (8.0)	6.75 d (8.4)	6.84 d (8.4)	6.78 d (8.0)	6.78 d (7.2)
H-6′	7.11 d (8.0)	7.11 d (8.0)	7.64 dd (8.4, 2.0)	7.70 dd (8.4, 2.0)	7.06 d (8.0)	7.06 d (7.2)
H_2-7′	3.01 m	3.01 m	2.96 m	2.99 m	3.01 m	3.00 m
H_2-8′	2.89 m	2.89 m	2.89 m	2.90 m	2.91 m	2.90 m
6-OCH_3		3.97 s	3.89 s	3.89 s	3.91 s	
7-OCH_3		3.99 s				
3′-OCH_3				3.82 s		
4′-OCH_3	3.78 s	3.78 s	3.86 s			
3′-OH			5.58 s			
4′-OH				5.48		

[a] Data were measured in $CDCl_3$. [b] Data were measured in DMSO-d_6.

Compound **4** was obtained as a pale-yellow amorphous powder. The ESI-MS spectrum of **4** showed a positive ion peak at *m/z* 277.1081 $[M+Na]^+$, giving the molecular formula of $C_{17}H_{18}O_2$. The signals of seventeen carbons, including one carbonyl carbon (δ_C 210.2), two oxygenated carbons (δ_C 170.2, 158.3, 150.5, 149.5, and 143.3), two non-protonated carbons (δ_C 142.7, C-1″ and 140.6, C-1″), eleven methine groups (δ_C 128.5 (C-3′ and C-5′), 128.5 (C-3″ and C-5″), 128.2 (C-2″ and C-6″), 127.6 (C-4′), 126.2 (C-4″), 125.6 (C-2′ and C-6′), and 69.9 (C-1)), and three methylene carbons (δ_C 51.3 (C-2), 45.1 (C-4), and 29.4 (C-5)) were observed in the ^{13}C NMR spectrum. The ^1H NMR spectrum of **4** showed ten aromatic methines, one aliphatic methine, and three methylene groups. The ^1H-^1H COSY spectrum showed correlations between H-1 and H_2-2, and between H_2-4 and H_2-5. The HMBC correlations were observed from H-1 (δ_H 5.13) to C-2/C-3/C-1′/C-2′/C-6′, from H_2-2 (δ_H 2.83 and 2.74) to C-1/C-3/C-1′, from H_2-4 (δ_H 2.75) to C-3/C-5/C-1″, from H_2-5 (δ_H 2.90) to C-3/C-4/C-1″/C-2″/C-6″, from H-2′/H-6′ (δ_H 7.32) to C-1 (δ_C 69.9), and from H-2″/H-6″ (δ_H 7.15) to C-5 (δ_C 29.4). Based on the above evidence and the spectroscopic data in the literature [15], **4** was identified as 1-hydroxy-1,5-diphenylpentan-3-one.

Table 2. ^{13}C NMR (100 MHz) spectroscopic data of compounds **2** and **6-10**.

No	**2**[a]	**6**[a]	**7**[b]	**8**[a]	**9**[b]	**10**[a]
	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
2	168.6	167.7	168.2	168.3	168.4	168.6
3	110.3	109.7	109.5	109.6	109.5	110.3

No	2[a]	6[a]	7[b]	8[a]	9[b]	10[a]
4	178.3	177.6	178.2	178.2	178.3	178.4
5	125.7	104.4	104.8	104.9	104.8	125.7
6	125.0	147.4	156.7	156.8	156.8	125.0
7	133.5	154.3	123.5	123.6	123.7	133.6
8	117.8	99.6	119.7	119.2	119.3	117.9
9	156.5	152.5	151.3	151.3	151.4	156.5
10	123.7	117.0	124.3	124.3	124.2	123.7
1′	131.7	131.9	133.0	131.7	131.7	131.8
2′	129.3	129.3	114.4	114.5	129.4	129.4
3′	114.1	114.1	145.7	144.3	115.5	115.5
4′	158.3	158.3	145.2	146.5	154.4	154.3
5′	114.1	114.1	110.7	110.8	115.5	115.5
6′	129.3	129.3	119.3	120.9	129.4	129.4
7′	32.1	32.1	32.4	32.9	32.2	32.1
8′	36.4	36.4	36.2	36.5	36.4	36.4
6-OCH_3		56.4	55.9	55.94	55.9	
7-OCH_3		56.4				
3′-OCH_3				55.87		
4′-OCH_3	55.3	55.3	56.0			

[a] Data were measured in $CDCl_3$ [b] Data were measured in DMSO-d_6

Compound **5** was obtained as a pale-yellow amorphous powder, and its molecular formula was determined as $C_{17}H_{14}O_5$ from the positive ESI-MS by an ion peak at *m/z* 299.1066 $[M+H]^+$. The ^{13}C NMR spectrum of **5** indicated signals for 17 carbons, including seven olefinic carbons (δ_C 128.1 (C-2′ and C-6′), 114.5 (C-3′ and C-5′), 104.4, 98.0, and 92.6), five oxygenated carbons (δ_C 164.0, 165.4, 162.6, 162.2, and 157.7), two non-protonated carbons (δ_C 123.6 and 105.6), one carbonyl group (δ_C 182.5), and two methoxy groups at δ_C 55.8 (7′-OCH_3) and 55.5 (4′-OCH_3). The ^1H NMR spectrum of **5** showed seven methine and two methoxy groups. It revealed the correlations of two *meta*-coupled proton signals at δ_H 6.37 (1H, d, J = 2.8 Hz, H-6) and 6.48 (1H, d, J = 2.8 Hz, H-8), and the correlations between a pair of symmetric protons H-3′/H-5′ and H-2′/H-6′. The singlet signal at δ_H 12.80 was allocated to the hydroxyl group at C-5. These data suggested that **5** was a flavonoid. Compared to the previously reported spectroscopic data of 5-hydroxy-7,4'-dimethoxyflavone[9,16], compound **5** was identified as 5-hydroxy-7,3',4'-trimethoxyflavone.

Compound **6** was isolated as colorless crystals. The ESI-MS spectrum of **6** revealed a positive ion peak at *m/z* 341.1417 $[M+H]^+$, consistent with the molecular formula of $C_{20}H_{20}O_5$. The ^{13}C NMR data showed 20 carbons signals, including one carbonyl (δ_C 177.6), seven aromatic carbons (δ_C 129.3 (C-2′ and C-6′), 114.1 (C-3′ and C-5′), 109.7 (C-3), 104.5 (C-5), and 99.6 (C-8)), five oxygenated carbons (δ_C 167.7 (C-2), 158.3 (C-4′), 154.3 (C-7), 152.5 (C-9), 147.4 (C-6), two non-protonated carbons (δ_C 131.9 (C-1′) and 117.0 (C-10)), three methoxy groups at δ_C 56.4 (6-OCH_3 and 7-OCH_3) and 55.3 (4′-OCH_3), and two aliphatic methyl groups (δ_C 36.54 and 32.1). The carbon signals appeared in the downfield region in the range of δ_C 109-179, together with two methylene groups, which are the typical region of the phenylethylchromone structure. The ^1H NMR spectrum of **6** showed two singlet proton signals at δ_H 6.86 (H-8) and 6.10 (H-3), and the signal of one pair of symmetric aromatic protons at δ_H 7.11 (2H, d, J = 8.0 Hz, H-2' and H-6') and 6.83 (2H, d, J = 8.0 Hz, H-3' and H-5'), which were assigned to the structure of the phenethyl group. The data demonstrated three methoxy groups were attached to C-6, C-7, and C-4′. According to the reference data [17], the structure of compound **6** was identified to be 6,7,4'-trimethoxy-2-(2-phenylethyl)chromone.

Compound **7** was isolated as a yellowish oil. The ESI-MS spectrum of **7** revealed a positive ion peak at *m/z* 327.12897 $[M+H]^+$, consistent with the molecular formula of $C_{19}H_{18}O_5$. The ^{13}C NMR data of **7** were similar to those of compound **12** [9]. The hydroxyl group at C-5 in **12** was repositioned to C-3' in **7** as evidenced by the *ortho*- and *meta*-coupled proton and carbon signals on A-ring and B-ring in **7**. The planar structure of **7** was further interpreted from HMBC and ^1H-^1H COSY correlations. Therefore, the structure of compound **7** was elucidated to be 6,4'-dimethoxy-3'-hydroxy-2-(2-phenylethyl)chromone [18].

Compound **8** was isolated as a yellowish oil. The ESI-MS spectrum of **8** showed a positive ion peak at *m/z* 327.11416 $[M+H]^+$, consistent with the molecular formula of $C_{19}H_{18}O_5$. The ^{13}C NMR data of **8** were similar to those of **7**, except that the carbon resonances of C-4′ at δ_C 145.2 in **7** were downfield-shifted in **8** to δ_C 146.5, and the carbon resonances of C-3′ at δ_C 145.7 in **7** were upfield-shifted in **8** to δ_C 144.3. The hydroxyl group at C-4′ in **7** was repositioned by a methoxy group (δ_C 55.87, δ_H 3.81) in **8**. The location of methoxy group was supported by the HMBC and ^1H-^1H COSY

correlations of **8**. Accordingly, the structure of compound **8** was elucidated to be 6,3'-dimethoxy-4'-hydroxy-2-(2-phenylethyl)chromone [19].

Compound **9** was isolated as a yellowish amorphous powder. The ESI-MS spectrum of **9** showed a positive ion peak at *m/z* 297.12082 $[M+H]^+$, consistent with the molecular formula of $C_{18}H_{16}O_3$. The ^{13}C NMR data of **9** were similar to those of **8**. The main difference was that a methoxy group at C-3′ (δ_C 144.3) in **8** was replaced by a methine group in **9** (δ_C 115.5). The 2D NMR spectra of compound **9** were further interpreted from the detailed analysis of HMBC and ^1H-^1H COSY spectroscopic data. Thus, the structure of compound **9** was elucidated to be 6-methoxy-4'-hydroxy-2-(2-phenylethyl)chromone [18].

Compound **10** was obtained as a yellowish oil. The ESI-MS spectrum of **10** showed a positive ion peak at *m/z* 267.0721 $[M+H]^+$, consistent with the molecular formula of $C_{17}H_{14}O_3$. The ^1H and ^{13}C NMR data of **10** were similar to those of **2** and the difference between **10** and **2** was the replacement of the methoxy group in **2** by the hydroxyl group in **10**. The ^1H NMR spectrum data further confirmed the structure of compound **10**. Therefore, the structure of compound **10** was elucidated to be 4'-hydroxy-2-(2-phenylethyl)chromone [20].

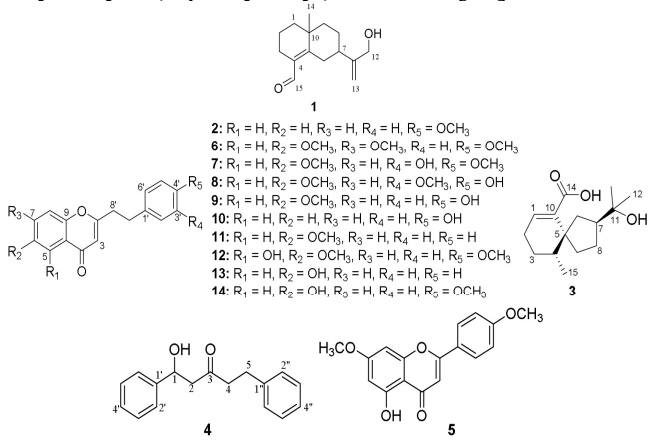

Fig. 2. Chemical structures of the isolated compounds **1-14**

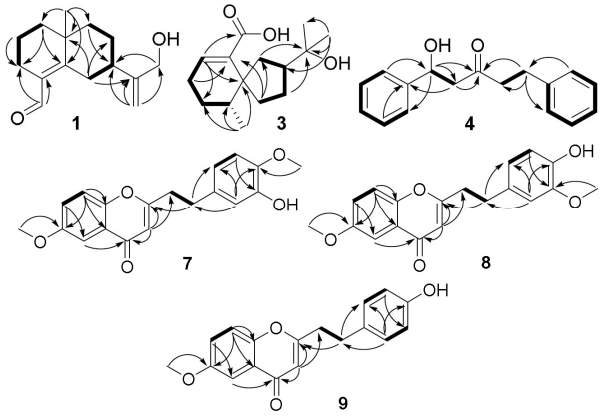

Fig. 3. The key HMBC (⟶) and COSY (▬) correlations of compounds **1**, **3**, **4**, and **7-9**.

Four compounds 6-methoxy-2-(phenylethyl)chromone (**11**), 5-hydroxy-6,4′-dimethoxy-2-(phenylethyl)chromone (**12**), 6-hydroxy-2-(2-phenylethyl)chromone (**13**), and 6-hydroxy-2-[2-(4'-methoxyphenyl)ethyl]chromone (**14**) were reported in our previous work [9].

*3.3. Evaluation of the cell viability and anti-inflammatory activity of compounds **4, 6, 12-14***

Five compounds (**4**, **6**, and **12-14**) were tested for their anti-inflammatory activity on the BV2 cells. The other compounds were not tested due to the scarcity of samples. The positive control is quercetin, and the test concentrations were 1, 5, 25, and 50 μM to determine IC_{50} values of the tested samples. As a result, compound **4** showed NO inhibitory activity with an IC_{50} value of 43.46 ± 2.26 (μM), whereas compounds **12-14** showed strong activity against NO production with IC_{50} values of 41.16 ± 5.76, 39.88 ± 12.58, and 29.88 ± 8.20 μM, respectively, when compared with quercetin (IC_{50} 7.28 ± 1.32 μM).

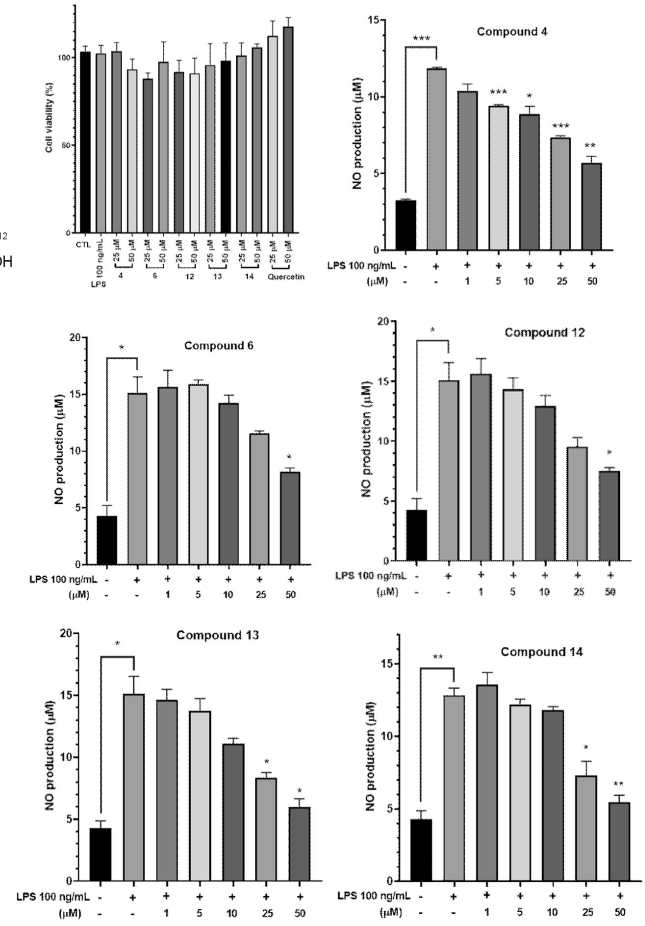

Fig. 4. Cell viability and anti-inflammatory effects of compounds on BV2 cells

Table 3. IC_{50} value of extracts and compounds against NO production in BV2 cells

No.	Samples	IC_{50} value ± SD
1	AcT	> 50 (μg/mL)
2	AcH	5.67 ± 0.9 (μg/mL)
3	AcE	26.46 ± 6.12 (μg/mL)
4	Compound **4**	43.46 ± 2.26 (μM)
5	Compound **6**	> 50 (μM)
6	Compound **12**	41.16 ± 5.76 (μM)
7	Compound **13**	39.88 ± 12.58 (μM)
8	Compound **14**	29.88 ± 8.20 (μM)
9	Quercetin	7.28 ± 1.32 (μM)

4. Conclusion

In summary, fourteen compounds were isolated from the agarwood of *Aquilaria crassna*. Their structures were identified as 12-hydroxy-4(5),11(13)-eudesmadien-15-al (**1**), 4'-methoxy-2-(2-phenylethyl)chromone (**2**), baimuxinic acid (**3**), 1-hydroxy-1,5-diphenylpentan-3-one (**4**), 5-hydroxy-7,4'-dimethoxyflavone (**5**), 6,7,4'-trimethoxy-2-(2-phenylethyl)chromone (**6**), 6,4'-dimethoxy-3'-hydroxy-2-(2-phenylethyl)chromone (**7**), 6,3'-dimethoxy-4'-hydroxy-2-(2-phenylethyl)chromone (**8**), 6-methoxy-4'-hydroxy-2-(2-phenylethyl)chromone (**9**), 4'-hydroxy-2-(2-phenylethyl)chromone (**10**), 6-methoxy-2-(phenylethyl)chromone (**11**), 5-hydroxy-6,4′-dimethoxy-2-(phenylethyl)chromone (**12**), 6-hydroxy-2-(2-phenylethyl)chromone (**13**), and 6-hydroxy-2-[2-(4'-methoxyphenyl)ethyl]chromone (**14**). The tested compounds showed moderate anti-inflammatory effects against NO production in BV2 microglial cells. To our best knowledge, compounds **1-10** were first reported from the agarwood of *A. crassna*.

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