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Original Article

Secondary metabolites from *Alphonsea tonkinensis* A.DC. showing inhibition of nitric oxide production and cytotoxic activity

[Metabolitos secundarios de *Alphonsea tonkinensis* A.DC. muestran inhibición de la producción de óxido nítrico y actividad citotóxica]

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Abstract

Context: Bioactivities of *Alphonsea tonkinensis* A.DC have not been reported previously, while the knowledge about its chemical composition is limited.

Aims: To investigate the phytochemical constituents and bioactivities of the stems and leaves of *Alphonsea tonkinensis* A.DC.

Methods: Combination of various extraction, chromatographic methods and crystallization techniques were performed to obtain pure compounds. Chemical structures of isolated compounds were determined by spectroscopic analyses (1D and 2D NMR). The *in vitro* anti-inflammatory and cytotoxic activities of isolates were evaluated by a Griess assay and a sulforhodamine B assay.

Results: A phytochemical study of the stems and leaves of *Alphonsea tonkinensis* A.DC. resulted in the isolation of liriodenine (**1**), *N-trans*-feruloyltyramin (**2**), corydaldine (**3**), 8-oxopseudopalmatine (**4**), 3-hydroxy-7,8-dehydro-*β*-ionone (**5**), pseudopalmatine (**6**), pseudocolumbamine (**7**), and stigmasterol (**8**). Compound **5** showed potent inhibitory activity for NO production with an IC_{50} value of 20.4 µM, which was comparable to that of positive control. Compound **4** and **5** displayed inhibitions against the HepG2, SK-LU-1 cancer cell lines with IC_{50} values ranging from 54.4 and 69.6 µM.

Conclusions: Among eight compounds isolated from *Alphonsea tonkinensis* A.DC., compounds **3** and **5** were isolated from the genus *Alphonsea* for the first time. Compound **5** was stronger inhibitor of NO production than positive control L-NMMA. In addition, this is the first investigation showing the bioactivities of **5** and cytotoxicity against the HepG2, SK-LU-1 cancer cell lines of **4**.

Keywords: 3-hydroxy-7,8-dehydro-*β*-ionone; *Alphonsea tonkinensis*; cytotoxicity; nitric oxide inhibition.

Resumen

Contexto: Las bioactividades de *Alphonsea tonkinensis* A.DC no se han informado anteriormente, mientras que el conocimiento sobre su composición química es limitado.

Objetivos: Investigar los componentes fitoquímicos y las bioactividades de los tallos y las hojas de *Alphonsea tonkinensis* A.DC.

Métodos: Se realizaron combinaciones de diversas técnicas de extracción, cromatografía y cristalización para obtener compuestos puros. Las estructuras químicas de los compuestos aislados se determinaron mediante análisis espectroscópicos (RMN 1D y 2D). Las actividades antiinflamatorias y citotóxicas *in vitro* de los aislados se evaluaron mediante un ensayo de Griess y un ensayo de sulforhodamina B.

Resultados: un estudio fitoquímico de los tallos y hojas de *Alphonsea tonkinensis* A.DC. resultó en el aislamiento de liriodenina **(1),** *N-trans*-feruloiltyramina **(2),** coridaldina **(3)**, 8-oxopseudopalmatina **(4)**, 3-hidroxi-7,8-deshidro-β-ionona **(5)**, pseudopalmatina **(6)**, pseudocolumbamina **(7)** y estigmasterol **(8)**. El compuesto **5** mostró una potente actividad inhibitoria para la producción de NO con un valor de CI_{50} de 20,4 µM, que fue comparable al del control positivo. Los compuestos **4** y **5** mostraron inhibiciones contra las líneas celulares de cáncer HepG2, SK-LU-1 con valores de CI_{50} entre 54,4 y 69,6 µM.

Conclusiones: Entre ocho compuestos aislados de *Alphonsea tonkinensis* A.DC., los compuestos **3** y **5** se aislaron del género *Alphonsea* por primera vez. El compuesto **5** fue un inhibidor más fuerte de la producción de NO que el control positivo L-NMMA. Además, esta es la primera investigación que muestra las bioactividades de **5** y la citotoxicidad contra las líneas celulares de cáncer HepG2, SK-LU-1 de **4**.

Palabras Clave: 3-hidroxi-7,8-deshidro-β-ionona; *Alphonsea tonkinensis*; citotoxicidad; óxido nítrico inhibición.

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INTRODUCTION

The *Annonaceae* family is one of the largest tropical woody families, which comprises over 124 genera and 2440 species widely distributed in the South and Central America, Australia, Africa and Asia. Some plants of *Annonaceae* have been used as the folk remedy (Leboeuf et al., 1980; Ekundayo, 1989; Couvreur et al., 2011). *Alphonsea* is a small genus of *Annonaceae* family with about 30 species. The species of this genus can be found in the northeastern India, southern China and Southeast Asian countries (Srivastava and Mehrotra, 2013; Bakri et al., 2017). Many species of this genus have cytotoxic, anti-oxidant, anti-fungal, anti-inflammatory and anti-trypanosomal effects. Previous phytochemical studies of *Alphonsea sp.* have introduced alkaloids, steroids, lignan, sesquiterpenes and monoterpenes (Bakri et al., 2017). However, the number of studies published on the chemical and pharmacological properties of this genus has been limited, probably due to the narrow distribution.

Alphonsea tonkinensis A.DC. is one of the species with a single specimen over two sheets, which grows in Viet Nam and Laos (Turner et al., 2018). The study of essential oils of *A. tonkinensis* showed that the main compounds are germacrene D (17.4%), *β*-caryophyllene (9.9%), *α*-pinene (9.5%), *β*-elemene (9.3%) and *β*-pinene (9.2%) in the stem oil, and *β*-caryophyllene (27.8%), *β*-elemene (15.6%) and caryophyllene oxide (14.5%) in the leaf oil (Nguyen et al., 2018a,b). Besides, the knowledge about the chemical composition of *A. tonkinensis* is limited, and its bioactivities have not been reported to date. This work aims to report the isolation, structural determination, NO production inhibitory and cytotoxic activities of compounds **1–8**.

MATERIAL AND METHODS

General experimental procedures

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance 500 spectrometer (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR) (Bruker, Billerica, MA, USA) with tetramethylsilane (TMS) as an internal reference. Column chromatography was conducted using silica gel (60 N, spherical, neutral, 40 - 50 µm, Kanto Chemical Co., Inc., Tokyo, Japan), reversed-phase C_{18} (RP-18) (Fuji Silysia Chemical Ltd., Kasugai, Aichi, Japan), and Sephadex LH-20 (Dowex® 50WX2-100, Sigma–Aldrich, St.Louis, MO, USA). Analytical thin-layer chromatography (TLC) was performed with pre-coated silica gel $60F_{254}$ and RP-18 F_{254} plates (0.25 or 0.50 mm thickness, Merck KGaA, Darmstadt, Germany). Preparative HPLC was carried out on an Agilent 1260 Infinity II system (Agilent, Santa Clara, CA, USA) using a Zorbax SB–C_{18} column (5 µm particle size, 9.4 × 250 mm) and a DAD detector.

Plant material

The stems and leaves of *A. tonkinensis* were collected from Dakrong district, Quang Tri province, Viet Nam (geographical coordinates: 16°28′45.2″N; 107°00′49.9″E) in July 2017. The plant material was identified by Dr. Chinh Tien Vu, Viet Nam National Museum of Nature, VAST, Viet Nam. A voucher specimen under the code AT-01 was deposited in the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Viet Nam.

Extraction and isolation

The dried stems and leaves of *A. tonkinensis* ground into a powder (8.0 kg) were extracted three times with 20 L each of MeOH by maceration at room temperature. The filtrates were combined and evaporated under reduced pressure to yield 0.79 kg of a dark solid extract. The extract was suspended in water (1.0 L) adjusted to pH = 2 with 0.5 M HCl, and extracted with ethyl acetate (5×1.0 L) to obtain the ethyl acetate extract (EA, 455.7 g). The aqueous phase was treated with saturated ammonia solution to pH = 9 and extracted with CH_2Cl_2 (5 × 1.0 L) to yield 19.26 g of CH_2Cl_2 extract. This extract was then partitioned with *n*-hexane (4 × 0.5 L), ethyl acetate (4 × 0.5 L) and MeOH (4 × 0.5 L) to get the *n*-hexane (0.14 g), ethyl acetate (EC, 1.34 g), and MeOH (M, 14.59 g)-soluble portions, after removal of the solvents in vacuo (Fig. 1).

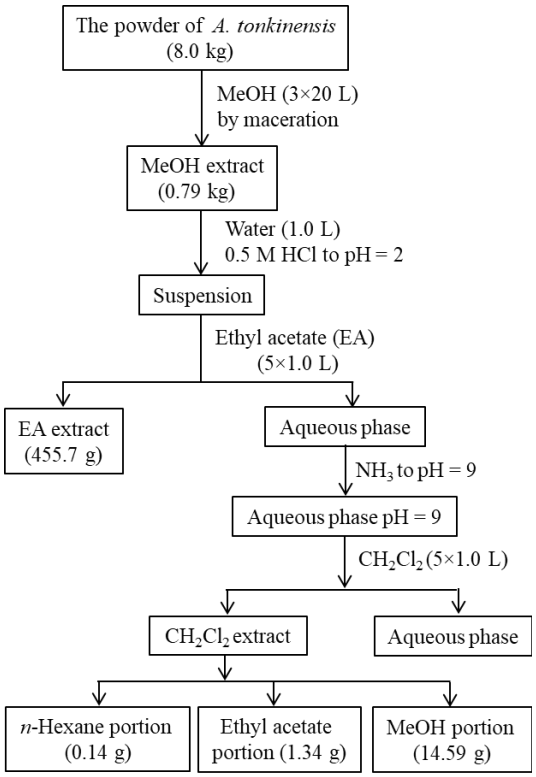

Figure 1. Schematic diagram of the extraction process and the fractions obtained from *A. tonkinensis* A.DC.

The EC extract was applied to a silica gel column and eluted with an CH_2Cl_2-MeOH-NH_3 (25:1:0.05, v/v) to give eleven major fractions (EC1–EC11). Fraction EC2 (78 mg) and EC8 (80 mg) were applied to Sephadex LH-20 columns and eluted with CH_2Cl_2–MeOH (1:1, v/v) to yield the fractions (EC2.1–EC2.3) and (EC8.1–EC8.3) respectively. Fraction EC2.2 was applied to preparative reversed-phase HPLC and eluted with MeOH–H_2O (56:44, v/v) with flow rate 2 mL/min to afford **4** (6.2 mg). Fraction EC8.2 was successively separated by preparative reversed-phase HPLC using the eluent MeOH–H_2O (40:60, v/v) and flow rate 2 mL/min to furnish **2** (5.6 mg) and **3** (5.8 mg). Fraction EC3 (130 mg) was submitted to a silica gel column and eluted with *n*-hexane–CH_2Cl_2–MeOH (20:20:1, v/v) to obtain seven fractions (EC3.1–EC3.7). Fraction EC3.1 was further purified by preparative reversed-phase HPLC using MeOH–ACN–H_2O (30:23:47, v/v) as the eluent and flow rate 2 mL/min to obtain **1** (3.0 mg). Fraction M was followed by fractionation on a silica gel column eluted with CH_2Cl_2–MeOH–$C_2H_5NH_2$ (10:1:0.1, v/v) to obtain eleven fractions (M1–M11). Fraction M4 and M6 gave precipitates. The precipitate of M4 was filtered, and then recrystallized in CH_2Cl_2–MeOH (2:1, v/v) in three times to afford **6** (290 mg) as yellow crystals. The precipitate of M6 was loaded onto Sephadex LH-20 column using CH_2Cl_2–MeOH (1:1, v/v) to give 3 fractions (M6B.1- M6B.3). The precipitate in fraction M6B.2 was recrystallized in MeOH in three times to get **7** (9.0 mg).

The EA extract was fractionated on a silica gel column eluted with an hexane–acetone gradient solvent system (100:0, 90:10, 80:10, 50:10, 20:10, 10:10, 0:100 v/v, each 2.0 L) to obtain 7 major fractions (EA1–EA7). There appeared precipitate in fraction EA5. The white precipitate was dissolved and recrystallized in five times to get **8** (0.83 g). Fraction EA3 (58.2 g) was fractionated on a silica gel column, eluted with *n*-hexane–acetone (15:1, v/v) to obtain 14 fractions (EA3.1– EA3.14). Fraction EA3.6 (3.4 g) was loaded onto a RP-18 column

and eluted with acetone–water (9:1, v/v) to give 8 fractions (EA3.6.1– EA3.6.8). Fraction EA3.6.2 (371 mg) was then separated by on a Sephadex LH-20 column eluted with CH_2Cl_2–MeOH (2:1, v/v) to afford **5** (19.0 mg).

Liriodenine (**1**): pale yellow solid; ^{1}H NMR (500 MHz, DMSO-d_6): 7.59 (1H, s, H-3), 8.08 (1H, d, *J* = 4.5 Hz, H-4), 8.82 (1H, d, *J* = 4.5 Hz, H-5), 8.37 (1H, d, *J* = 8.0 Hz, H-8), 7.66 (1H, t, *J* = 8.0 Hz, H-9), 7.90 (1H, t, *J* = 8.0 Hz, H-10), 8.64 (1H, d, *J* = 8.0 Hz, H-11), 6.52 (2H, s, -O-CH_2-O-); ^{13}C NMR (125 MHz, DMSO): 148.8 (C-1), 152.0 (C-2), 103.5 (C-3), 143.9 (C-3a), 124.8 (C4), 143.9 (C-5), 135.8 (C-6a), 180.5 (C-7), 130.6 (C-7a), 127.9 (C-8), 128.6 (C-9), 134.3 (C-10), 127.1 (C-11), 132.5 (C-11a), 106.1 (C-11b), 122.5 (C-11c), 103.3 (O-CH_2-O).

N-trans-Feruloyltyramin (**2**): colorless oil; ^{1}H NMR (500 MHz, CD_3OD): 6.42 (1H, d, *J* = 15.5 Hz, H-2), 7.45 (1H, d, *J* = 15.5 Hz, H-3), 7.13 (1H, d, *J* = 1.5 Hz, H-5), 6.82 (1H, d, *J* = 8.0 Hz, H-8), 7.04 (1H, dd, *J* = 8.0, 2.0 Hz, H-9), 3.49 (2H, t, *J* = 7.0 Hz, H-1′), 2.78 (2H, t, *J* = 7.0 Hz, H-2′), 7.08 (2H, d, *J* = 8.5 Hz, H-4′, H-8′), 6.74 (2H, d, *J* = 8.5 Hz, H-5′, H-7′), 3.90 (3H, s, 6-OCH_3); ^{13}C NMR (125 MHz, CD_3OD): 169.2 (C-1), 118.8 (C-2), 142.0 (C-3), 128.3 (C-4), 111.6 (C-5), 149.3 (C-6), 149.9 (C-7), 116.5 (C-8), 123.2 (C-9), 42.5 (C-1′), 35.8 (C-2′), 131.3 (C-3′), 130.7 (C-4′), 116.3 (C-5′), 156.9 (C-6′), 116.3 (C-7′), 130.7 (C-8′), 56.4 (OCH_3-6).

Corydaldine (**3**): white powder; ^{1}H NMR (500 MHz, CD_3OD): 2.93 (2H, t, *J* = 7.0 Hz, H-4), 3.50 (2H, t, *J* = 7.0 Hz, H-3), 3.91 (3H, s, 6-OCH_3), 3.87 (3H, s, 7-OCH_3), 6.89 (1H, s, H-5), 7.50 (1H, s, H-8); ^{13}C NMR (125 MHz, CD_3OD): 28.6 (C-4), 41.1 (C-3), 56.5 (C-6, C-7), 111.4 (C-8), 114.4 (C-5), 122.0 (C-9), 135.1 (C-10), 149.5 (C-7), 154.2 (C-6), 169.0 (C-1).

8-Oxopseudopalmatine (**4**): Amorphous yellow powder; ^{1}H NMR (500 MHz, CD_3OD): 7.43 (1H, s, H-1), 6.93 (1H, s, H-4), 2.98 (2H, t, *J* = 6.0 Hz, H-5), 4.33 (2H, t, *J* = 6.0 Hz, H-6), 7.72 (1H, s, H-9), 7.20 (1H, s, H-12), 7.72 (1H, s, H-12a), 7.19 (1H, s, H-13), 3.96 (3H, s, OCH_3-2), 3.97 (3H, OCH_3-3), 3.92 (3H, s, OCH_3-10), 4.00 (3H, s, OCH_3-11); ^{13}C NMR (125 MHz, CD_3OD): 109.7 (C-1), 150.0 (C-2), 152.0 (C-3), 112.1 (C-4), 138.0 (C-4a), 30.7 (C-5), 41.3 (C-6), 163.5 (C-8), 119.0 (C-8a), 108.2 (C-9), 151.0 (C-10), 155.5 (C-11), 107.7 (C-12), 108.2 (C-12a), 103.5 (C-13), 129.5 (C-13a), 123.0 (C-13b), 56.9 (OCH_3-2), 56.5 (OCH_3-3), 56.6 (OCH_3-10), 56.6 (OCH_3-11).

3-Hydroxy-7,8-dehydro-*β*-ionone (**5**): reddish-brown oil; ^{1}H-NM (500 MHz, CD_3OD): 1.16 (3H, s), 1.21 (3H, s), 1.44 (1H, t, *J* = 12.0 Hz), 1.85 (1H, m), 2.00 (3H, s), 2.10 (1H, m), 2.40 (3H, s), 2.48 (1H, ddd, *J* = 1.5, 5.5, 17.0 Hz), 3.94 (1H, m); ^{13}C NMR (125 MHz, CD_3OD): 22.9, 28.9, 30.6, 32.8, 37.2, 42.3, 47.0, 64.7, 91.4, 94.7, 123.2, 148.8, 186.3.

Pseudopalmatine (**6**): amorphous yellow powder; ^{1}H NMR (500 MHz, DMSO): 7.68 (1H, s, H-1), 7.09 (1H, s, H-4), 3.24 (2H, t, *J* = 6.0 Hz, H-5), 4.80 (2H, t, *J* = 6.0 Hz, H-6), 9.49 (1H, s, H-8), 7.70 (1H, s, H-9), 7.63 (1H, s, H-12), 8.79 (1H, s, H-13), 3.95 (3H, s, OCH_3-2), 3.87 (3H, s, OCH_3-3), 4.02 (3H, s, OCH_3-10), 4.09 (3H, s, OCH_3-11); ^{13}C NMR (125 MHz, DMSO): 109.1 (C-1), 148.8 (C-2), 151.7 (C-3), 111.5 (C-4), 128.5 (C-4a), 25.9 (C-5), 54.5 (C-6), 145.2 (C-8), 121.9 (C-8a), 106.5 (C-9), 152.2 (C-10), 157.5 (C-11), 105.1 (C-12), 136.5 (C-12a), 117.7 (C-13), 138.2 (C-13a), 118.8 (C-13b), 56.1 (OCH_3-10 and OCH_3-2), 56.1 (OCH_3-3), 56.4 (OCH_3-11).

Pseudocolumbamine (**7**): amorphous yellow powder; ^{1}H NMR (500 MHz, CD_3OD): 7.56 (1H, s, H-1), 7.03 (1H, s, H-4), 3.26 (2H, t, *J* = 6.5 Hz, H-5), 4.83 (2H, H-6), 9.32 (1H, s, H-8), 7.62 (1H, s, H-9), 7.61 (1H, H-12), 8.50 (1H, s, H-13), 3.99 (3H, s, OCH_3-3), 4.08 (3H, s, OCH_3-10), 4.15 (3H, s, OCH_3-11); ^{13}C NMR (125 MHz, CD_3OD): 113.2 (C-1), 148.2 (C-2), 152.5 (C-3), 112.1 (C-4), 128.6 (C-4a), 27.9 (C-5), 56.7 (C-6), 146.1 (C-8), 124.1 (C-8a), 107.2 (C-9), 154.6 (C-10), 160.0 (C-11), 106.3 (C-12), 139.0 (C-12a), 119.1 (C-13), 140.7 (C-13a), 120.8 (C-13b), 56.6 (OCH_3-3), 57.0 (OCH_3-10), 57.5 (OCH_3-11).

Stigmasterol (**8**): white powder; ^{1}H NMR (500 MHz, $CDCl_3$): 5.35 (1H, brd, *J* = 4.5 Hz, H-6), 5.15 (dd, *J* = 15.5, 3.5 Hz, H-22), 5.03 (1H, dd, *J* = 15.5; 9.0 Hz, H-23), 3.52 (m, H-3), 0.70 (1H, s, H_3-18), 1.01 (1H, s, H_3-19), 0.82 (3H, d, *J* = 7.0 Hz, H_3-27), 0.85 (3H, d, *J* = 7.0 Hz, H_3-26), 0.93 (3H, d, *J* = 6.5 Hz, H_3-21) 0.85 (3H, t, *J* = 7.5 Hz, H_3-29).

The documents that support this data can be seen in the supplementary data.

Anti-inflammatory assay

The RAW 264.7 cells were obtained from the Institute of Biotechnology, the Viet Nam Academy of Science and Technology (Hanoi, Viet Nam). The cells were seeded at a concentration of 2×10^5 cells/well in 96-well plate and incubated at 37°C and 5% CO_2 for 24 h. The culture medium was then replaced with DMEM containing 10.0 mM HEPES, 2.0 mM L-glutamine and 1.0 mM sodium pyruvate without FBS for 3 hours. After stimulating with LPS (1 µg/mL) for 24 h in incubator, the cells were then treated with compounds at various concentrations for two hours. By using a Griess reaction, a nitrite concentration in the supernatant was measured as an indicator for NO creation. Briefly, 100 µL the cell supernatant was reacted with the same volume of a Griess reagent [1:1 mixture of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) N-1-naphthylethylenediamine dihydrochloride in water], and further incubated at room temperature for 10 min. The absorbance of the mixture was read using a microplate reader (Bio-Rad, Hercules, CA, USA) at 540 nm. The nitrite concentration in each sample was calculated with sodium nitrite as a standard. A MTT-based colorimetric assay was used to evaluate cell viability of the remaining cells (Nguyen et al., 2020).

Cytotoxicity assay

The cytotoxic activity of isolated compounds from *A. tonkinensis* were tested using a sulforhodamine B assay against the growth of human cancer cells including HepG2 and SK-LU-1 (Monks et al., 1991). Stock cultures were grown in Dulbecco's modified eagle medium (DMEM) consisting of 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, 10.0 mM HEPES and 10% fetal bovine serum - FBS (GIBCO, New York, NY, USA). The solution of 0.05% trypsin - EDTA was applied for the dissociation of cells. The subculture was performed every 3-5 days with the ratio of (1:3) by incubating at 37 ^{0}C under a humidified 5% CO_2 atmosphere. Tumor cells were seeded in a humidified atmosphere of 5% CO_2 at 37°C for two days. The sulforhodamine B (SRB) method based on the estimation of cellular protein content was used for examination of cell viability in a cell population. Viable cells were cultivated for overnight (4×10^4 cells per well) in 96-well microplates (CLS3635, Corning®, Sigma, Santa Clara, CA, USA) containing 180 µL of growth medium. Test samples with various concentrations of compounds were added into each well. The growth of cells was maintained under the same conditions for another 72 hours. After removing the medium, the remaining cell monolayers were fixed with the cold 20% (w/v) trichloroacetic acid for 1 h at 4°C and stained by 1X SRB staining solution at room temperature for 30 min. Then, 1% (v/v) acetic acid was used in three times for removing an unbound dye. The absorbance measurement was performed using an ELISA Plate Reader (Bio-Rad, Hercules, CA, USA) at 515 nm for the protein-bound dye dissolved in 10 mM Tris base solution. DMSO 10% and ellipticine were used as a negative control and a positive control, respectively. All experiments were set up in triplicate (Ho et al., 2020).

Statistical analysis

All results were presented as mean ± standard deviation. The statistical significance of the data was evaluated by the *t*-test at $p<0.05$ and $p<0.001$ using statistical software (GraphPad InStat, Version 3.10, GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Isolation and structural determination

Using combined chromatographic separation techniques, eight pure compounds (**1–8**), including six alkaloids were isolated from an MeOH extract of the stems and leaves of *A. tonkinensis* (Fig. 2). The structures of these compounds were identified as liriodenine (**1**) (Kristanti et al., 2015), *N-trans*-feruloyltyramin (**2**) (Kanada et al., 2012), corydaldine (**3**) (Atan et al., 2011), 8-oxopseudopalmatine (**4**) (Costa et al., 2010), 3-hydroxy-7,8-dehydro-*β*-ionone (**5**) (Sannai et al., 1984), pseudopalmatine (**6**) (Stubba et al., 2015), pseudocolumbamine (**7**) (Moulis et al., 1977), and stigmasterol (**8**) (Forgo and Kövér, 2004) by means of 1D and 2D NMR spectroscopy, and by comparisons to the reported data in the literature.

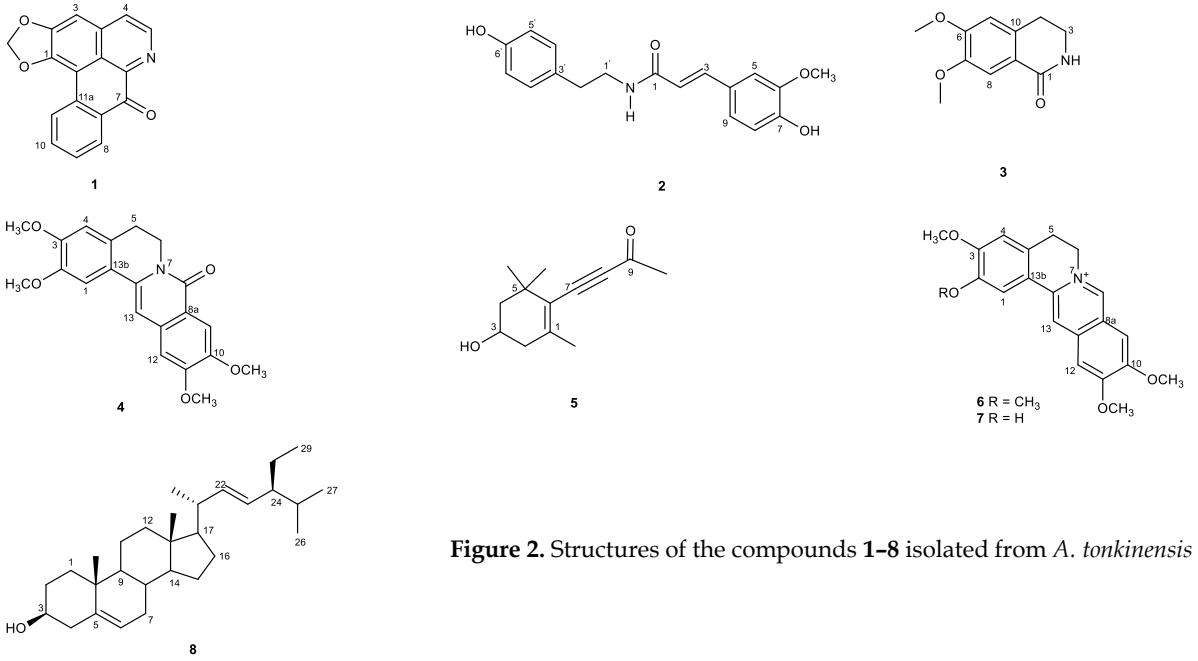

Figure 2. Structures of the compounds **1–8** isolated from *A. tonkinensis* A.DC.

To the best of our knowledge, this is the first report on the isolation of compounds **3** and **5** from the genus *Alphonsea*. The documents that support this data can be seen in the supplementary data.

Bioassays

Anti-inflammatory activities of all isolates were evaluated by a Griess assay (Kiem et al., 2016) and assessed for NO production inhibitory activities against LPS stimulated RAW264.7 cells. The dose-dependent response of compounds **1–8** was investigated to determine the IC_{50} values. The results of inhibition of NO production are summarized in Table 1. Among the isolated compounds, compound **5** exhibited the most potent NO production inhibitory activity with an IC_{50} value of 20.4 µM. Noticeable, its activity was significantly less than that of positive control L-NMMA. Based on our results, the compound **5** indicating strong anti-inflammatory activity could be selected for further studies.

Lung carcinoma is the leading cause and hepatocellular carcinoma is the third most cause of cancer-related death worldwide (Forner et al., 2012; Pratap et al., 2018). Therefore, the isolated compounds **1–8** were evaluated for their cytotoxic activity against human hepatocellular carcinoma (HepG2) and human lung carcinoma (SK-LU-1) cell lines. As shown in Table 1, among all isolates, the compound **4** and **5** exhibited an inhibitory effect towards the two tested cancer cell lines, with IC_{50} values ranging from 54.4 and 69.6 µM. To our best knowledge, this is the first study reporting the inhibition of NO production and cytotoxic activity of compound **5**. In addition, compound **4** was isolated from *Stephanla suberosa* for the first time in 1985 (Patra et al., 1987). However, the cytotoxic activity against HepG2 and SK-LU-1 has not been reported with this compound. Nonetheless, except for compound **1** having an insufficient amount for testing cytotoxicity activity, the remaining compounds did not show any inhibitions against the tested cell lines as well as inhibition of NO-production activities. Interestingly, a comparison of the cytotoxicity of compound **4** and that of compound **6** and **7** clearly indicated that the presence of carbonyl functional group (in **4**) may have a significant effect on cytotoxic activity. Previously, the 3,5-di-O-5-hydroxytigloyl-2-O-2-methyl-butyroyl-6-O-tigloyl-chiro-inositol isolated from leaves of *Chisocheton* paniculatus displayed the potent inhibition of NO with an IC_{50} value of 7.1 µM (Nguyen et al., 2020). The pogostemonons A-C

Table 1. Inhibition of NO-production in LPS-stimulated RAW 274.7 cells and cytotoxic activities of the compounds **1–8**.

Compound	IC_{50} (μM) ± SD		
	Inhibition of NO-production activities	Cytotoxicity	
		HepG2	SK-LU-1
1	>100	N.T	N.T
2	>100	>100	>100
3	>100	>100	>100
4	>100	56.6 ± 1.5*	69.6 ± 1.3*
5	20.4 ± 0.4*	56.2 ± 0.5*	54.0 ± 0.8*
6	>100	>100	>100
7	>100	>100	>100
8	>100	>100	>100
L-NMMA	25.0 ± 0.8		
Ellipticine		1.5 ± 0.1	1.5 ± 0.1

IC_{50}: The half-maximal inhibitory concentration; HepG2: human hepatocellular carcinoma cell lines; and SK-LU-1: human lung carcinoma cell lines. N.T: the compounds were not tested. L-NMMA was used as a positive control for nnhibition of NO-production activities; and Ellipticine was used as a positive control for cytotoxicity evaluation. Values represent mean ± SD of three parallel measurements. *$p<0.05$ indicates significant differences when compared to a positive control.

from the aerial parts of *Pogostemon auricularius* showed anticancer activity with an IC_{50} value of 39.80 - 98.32 μM (Tran et al., 2018) and pogostemin A from the same plant showed significant cytotoxicities against the human colon adenocarcinoma SW-480, epidermoid carcinoma KB, gastric cancer AGS, hepatoma cancer Hep-G2, and lung cancer LU-1 cell lines with IC_{50} values of 7.21, 8.49, 9.44, 11.75, and 12.76 μg/mL, respectively (Nguyen et al., 2018). So, the anticancer activity of compounds **1-8** studied was relatively weak but the NO production, with an IC_{50} value of 20.4 μM, was comparable to that of positive control.

CONCLUSIONS

Eight compounds comprising of six alkaloids were successfully isolated from *A. tonkinensis*. In addition, compounds **3** and **5** are for the first time reported from the genus *Alphonsea*. Compound **5** exhibits strong inhibitory activity for NO production, and its IC_{50} value is less than that of positive control L-NMMA. Compound **4** and **5** show a moderate inhibition against the HepG2, SK-LU-1 cancer cell lines. Such bioactivity of **4** and **5** are reported for the first time.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found at http://jppres.com/jppres/pdf/vol9/jppres20.900_9.1.24.suppl.pdf

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Nguyen KV	Ho DV	Le AT	Heinämäki J	Raal A	Nguyen HT
Concepts or ideas	x			x	x	x
Design	x	x	x	x	x	x
Definition of intellectual content	x	x		x	x	x
Literature search	x	x	x	x	x	x
Experimental studies	x	x				
Data acquisition	x	x	x			
Data analysis	x	x	x			
Statistical analysis	x					
Manuscript preparation	x	x				
Manuscript editing	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x

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