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Megastigmane and abscisic acid glycosides from the leaves of *Laurus nobilis* L.

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ABSTRACT

Two new megastigmane glycosides, laurusides E-F (**1** and **3**) and three known ones (**2**, **5**, and **7**), together with a new abscisic acid glycoside, lauruside G (**4**) and a known one (**6**), were isolated from a methanolic extract of *Laurus nobilis* L. leaves using various chromatographic techniques. The structures of these compounds were fully characterized using a combination of spectroscopic techniques including multinuclear and multidimensional NMR and mass spectrometry. All of the isolates were evaluated for their inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 cells.

1. Introduction

Laurus nobilis L. (family Lauraceae) was known as a folk medicine for the treatment of antiseptic, asthma, cardiac, diarrhea, digestive disorder diseases, and rheumatic pains (Dall'Acqua et al., 2006; Loi et al., 2004). The various parts of *L. nobilis* have been used for diff ;erent purposes; for example, the leaves and fruits were used as serving against emmenagogue and hysteria. Especially, the Iranian used *L. nobilis* leaves to treat epilepsy (khorasani, 1992). Powdered fruit in the form of infusion exhibited carminative properties and diuretic (Simić et al., 2003). Fatty oil from the fruits is used for treating bruises, furuncles, rheumatism, sprains, and as an insect repellent (Chahal et al., 2017). In addition, this herbal is a famous industrial material used in cosmetics, drugs, foods, or seasoning of meat/fishes products and soups. Recently, phytochemical studies of *L. nobilis* have revealed that additional constituents are alkaloids (Pech and Bruneton, 1982) and sesquiterpenes (Cisero et al., 1992), while other compounds including megastigmanes and phenolics have also been identified (De Marino et al., 2004).

The inflammation is considered to be a major risk factor in the pathogenesis of chronic diseases where the macrophages are important immune cells. It may regulate inflammation producing expression of inflammatory proteins and pro-inflammatory chemokines, cytokines, and nitric oxide (Abarikwu, 2014). Macrophages are highly sensitive to initiators of inflammation as lipopolysaccharide (LPS) which respond by the release of mediators such as interleukins and cytokines interferon gamma. Nitric oxide (NO), inducible NO synthase (iNOS), and inducing the inflammatory gene expression where each is associated somehow with the pathophysiological of the inflammation. Hence, modulation of macrophage activation is a good strategy to prevent these diseases (Valledor et al., 2010).

In the present study, we described the isolation and structural elucidation of seven secondary metabolites, including two new megastigmane derivatives, laurusides E (**1**) and F (**3**), and a new abscisic acid glycoside, lauruside G (**4**, Fig. 1), together with four known compounds (**2** and **5–7**, Supplementary information) from a methanolic extract of *L. nobilis*leaves. Furthermore, all the isolates were screened for their inhibition of NO production in LPS-induced RAW264.7 cells.

2. Results and discussion

The methanolic extract of *L. nobilis* was partitioned into fractions and isolated by multiple chromatographic steps over silica gel, Sephadex LH-20, and YMC RP-18 column chromatography (CC) to yield

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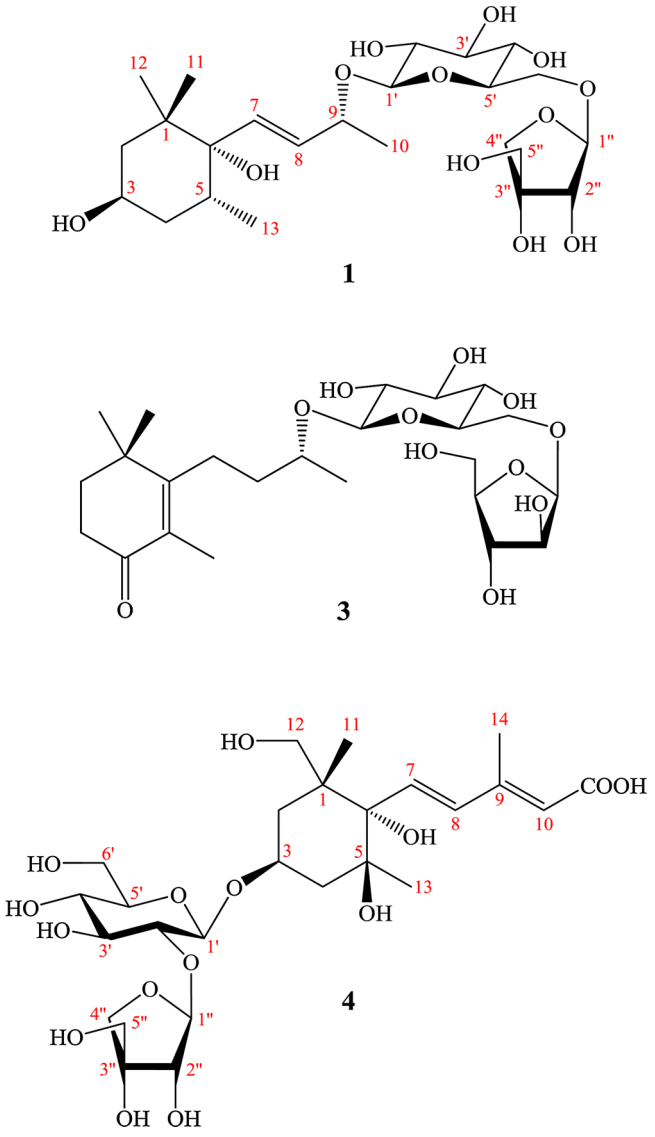

Fig. 1. Structures of new compounds (**1**, **3**, and **4**) isolated from L. *nobilis*.

Table 1
^{1}H and ^{13}C NMR spectroscopic data for laurusides E–G (in CD_3OD).

Position	Lauruside E (**1**)		Lauruside F (**3**)		Lauruside G (**4**)	
	δ_C[a]	δ_H[b] mult. (*J* in Hz)	δ_C[a]	δ_H[b] mult. (*J* in Hz)	δ_C[a]	δ_H[b] mult. (*J* in Hz)
1	43.9	–	37.7	–	49.8	–
2	52.4	2.88 d (13.5) 1.83 dd (2.8, 13.5)	38.4	1.79 m	43.0	1.94 m/ 1.75 m
3	78.0	3.93 ddd (2.8, 10.5, 13.5)	35.1	2.43 t (6.7)	74.2	4.21 m
4	46.1	2.45 brd (13.5) 2.13 m	201.6	–	43.0	2.15 dd (7.1, 16.6) 1.83 m
5	37.9	2.29 m	131.5	–	87.6	–
6	78.1	–	168.9	–	83.1	–
7	134.2	5.75 d (15.8)	28.0	2.53 m	130.8	6.26 d (15.8)
8	134.6	5.90 dd (6.3, 15.8)	37.2	1.64 dd (6.3, 15.8)	133.0	7.75 d (15.8)
9	77.6	4.44 dq (6.3, 12.4)	76.0	3.90 m	142.2	–
10	21.4	1.33 d (6.3)	20.0	1.21 d (6.3)	127.2	5.82 s
11	25.1	0.93 s	27.2	1.18 s	16.4	0.92 s
12	25.3	1.00 s	27.3	1.18 s	77.1	3.78 m/ 3.72 m
13	16.5	0.91 d (6.7)	11.8	1.74 s	19.8	1.16 s
14					20.8	1.97 s
COOH					175.1	–
9-Glucose						
1'	102.6	4.35 d (7.8)	102.3	4.33 d (7.8)	102.1	4.41 d (7.5)
2'	75.3	3.19 dd (7.8, 9.0)	75.1	3.15 dd (7.8, 9.0)	79.3	3.46 dd (7.5, 9.0)
3'	78.0	3.91*	78.0	3.34*	77.8	3.26 dd (9.0, 9.0)
4'	71.5	3.33*	72.1	3.25*	71.6	3.27*
5'	76.9	3.34 m	76.7	3.43 m	77.9	3.94 m
6'	68.5	3.96 dd (2.4, 11.5) 3.59 dd (5.4, 11.5)	68.2	3.99 dd (2.4, 11.5) 3.58 dd (5.4, 11.5)	62.7	3.83 dd (2.4, 11.5) 3.66 dd (5.4, 11.5)
	6'-Apiose		*6'-Arabinose*		*2'-Apiose*	
1"	111.0	4.99 d (2.4)	109.9	4.97 brs	110.8	5.35 brs
2"	78.0	3.92*	83.1	3.96*	78.5	3.29*
3"	80.5	–	78.9	3.79*	80.6	–
4"	75.3	3.68 dd (9.0) 4.01 dd (9.0)	85.8	3.93 m	75.3	3.69 dd (9.0) 4.02 dd (9.0)
5"	65.6	3.59 dd (10.8) 3.54 dd (10.8)	63.0	3.69 m/ 3.61 m	66.0	3.60 dd (10.8) 3.55 dd (10.8)

Assignments were confirmed by HMQC, HMBC, and COSY experiments.
[a] 00 MHz.
[b] 400 MHz.
* Overlapped signals.

seven compounds (**1–7**).

Lauruside E (**1**) was obtained as a white, amorphous powder with an optical rotation $[\alpha]_D^{21}$ − 22.0° (*c* 0.25, MeOH). The HR-QTOF-MS indicated a basic peak at *m/z* 545.2567 $[M+Na]^+$, confirming the molecular formula of $C_{24}H_{42}O_{12}$. The infrared (IR) spectrum of compound **1** showed absorption peaks characteristic of hydroxyl (3387 cm^{-1}) and substituted double bond (1678 cm^{-1}) groups. The ^{1}H NMR spectrum of **1** exhibited signals for four methyl protons [δ_H 1.33 (3H, d, *J* = 6.3 Hz, H-10), 0.93 (3H, s, H-11), 1.00 (3H, s, H-12), and 0.91 (3H, d, *J* = 6.7 Hz, H-13)], a pair of methylene protons [δ_H 2.88 (1H, d, *J* = 13.5 Hz, H-2a) and 1.83 (1H, dd, *J* = 2.8, 13.5 Hz, H-2b)], two olefinic protons [δ_H 5.75 (1H, d, *J* = 15.8 Hz, H-7) and 5.90 (1H, dd, *J* = 6.3, 15.8 Hz, H-8)], and two anomeric [an *β*-glycoside δ_H 4.35 (1H, d, *J* = 7.8 Hz, H-1′) and an *α*-glycoside δ_H 4.99 (1H, d, *J* = 2.4 Hz, H-1″)]. The ^{13}C NMR and HSQC spectra of **1** (Table 1) showed 24 carbon signals with characteristic signals of a megastigmane skeleton including a substituted double bond with two olefinic methine [δ_C 134.2 (C-7) and 134.6 (C-8)], two none-protonated carbons [δ_C 43.9 (C-1) and 78.1 (C-6)], three methines [78.0 (C-3), 37.9 (C-5), and 77.6 (C-9)], two methylenes [δ_C 52.4 (C-2) and 46.1 (C-4)], four methyls [δ_C 21.4 (C-10), 25.1 (C-11), 25.3 (C-12), and 16.5 (C-13)], and 11 aliphatic carbons [δ_C 102.6 (CH, C-1′), 75.3 (CH, C-2′), 78.0 (CH, C-3′), 71.5 (CH, C-4′), 76.9 (CH, C-5′), 68.5 (CH_2, C-6′), and 111.0 (CH, C-1″), 78.0 (CH, C-2″), 80.5 (C, C-3″), 75.3 (CH_2, C-4″), 65.6 (CH_2, C-5″)]. Two sugar units including a glucopyranoside and an apiofuranoside moiety were suggested. Indeed, the acid hydrolysis of **1** yielded D-glucose and D-apiose products which were detected by comparison the retention time of their hydrolysis products ($t_{R\text{-}D\text{-}api}$ = 22.6 min and $t_{R\text{-}D\text{-}glc}$ = 27.5 min) with authentic samples ($t_{R\text{-}D\text{-}api}$ = 22.9 min and $t_{R\text{-}D\text{-}glc}$ = 27.7 min) using analytical HPLC, respectively.

The NMR data of **1** (Table 1) was similar to those of alangioside B (**2**) (Otsuka et al., 1994) except for the downfield shift at C-3 (δ_C 78.0) in **1** respect to its chemical shift (δ_C 67.5) in **2**. This information suggested for the difference of geometry at C-3. Additionally, the HMBC cross-peaks of other anomeric H-1″ (δ_H 4.99) to C-6′ (δ_C 68.5) of glucose indicated the connection of second sugar moiety to its megastigmane glucopyranoside framework via C-1″→C-6′. This information was clearly expressed the downfield shift of C-6′ (δ_C 68.5) in

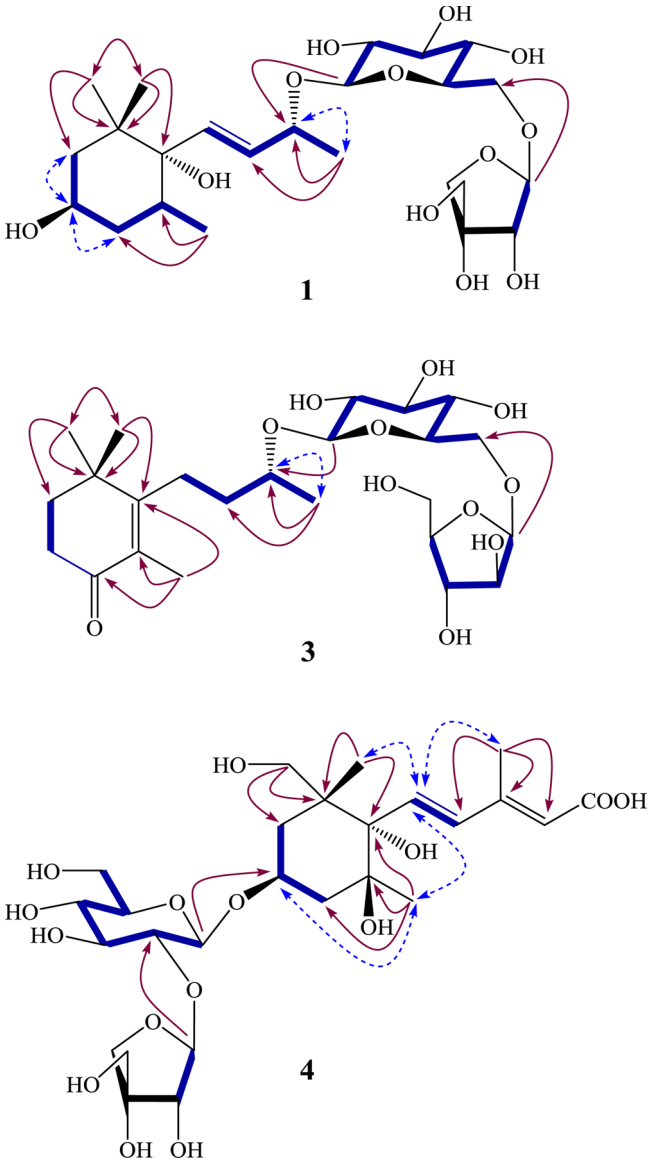

Fig. 2. Key HMBC (⟷), COSY (▬), and NOESY (⇢) correlations of **1**, **3**, and **4**.

glucopyranoside part. Moreover, the HMBC cross-peak of an anomeric proton at δ_H 4.35 (H-1′) to C-9 (δ_C 77.6) was also observed. Hence, an apiofuranosyl-(1″→6′)-glucopyranosyl moiety as the disaccharide sugar chain was allocated to C-9 of an ionol moiety. Further assignments for COSY, HMQC and HMBC correlations of **1** were displayed in Fig. 2.

The relative configuration of **1** was determined by analyzing proton-proton coupling constants and spectroscopic data as well as NOESY correlations. The hydroxyl group at C-3 (δ_C 78.0) was assigned to *R*-orientation by comparing its chemical shift value to respect of reported data [δ_C 67.4 (*S*-form, C-3) and 78.1 (*R*-form, C-3)] (Shu et al., 2013; Zhang et al., 2010) as well as large coupling constant (J = 13.5 Hz) between H-3 and H-2α/H-2β, H-4. In the same manner, the Me-13 group was suggested to *S*-orientation with the similar chemical shift value [δ_C 16.8–17.5 ppm (*S*-form, Me-13) and 13.1–13.4 ppm (*R*-form, Me-13)] (Shu et al., 2013). The appearance of C-9 (δ_C 77.6) was similar to that of alangioside B (Otsuka et al., 1994) and different from *R*-form (δ_C 74.0–75.5) (Marino et al., 2004), confirming the configuration of C-9 as *S*-form. Moreover, the 3-hydroxy-1-butenyl side chain at C-6 was determined to be β-oriented based on the NOESY cross-peaks of H-7 to H-5 and Me-12β. The larger coupling constant (J = 15.8 Hz) between H-7 and H-8 indicates that the geometry of the $\Delta^{7(8)}$ double bond is *E*. Therefore, the structure of lauruside E (**1**) was established as (3*R*,6*S*,9*S*,7*E*)-megastigman-7-en-3,6,9-triol-9-*O*-β-D-apiofuranosyl-(1″→6′)-*O*-β-D-glucopyranoside.

Lauruside F (**3**) was found to be a white, amorphous powder, and the molecular formula $C_{24}H_{40}O_{11}$ was determined by HR-QTOF-MS, with a sodium adduct molecular ion peak at *m/z* 527.2464 $[M + Na]^+$. The NMR spectroscopic data of **3** exhibited for a megastigmane glycoside framework including 13 carbon signals for megastigmane skeleton, six carbon signals attributed to a β-glucopyranosyl [δ_H 4.33 (d, J = 7.8 Hz, H-1′)] moiety, and five carbon signals for an α-arabinofuranosyl [δ_H 4.97 (brs, H-1′)] moiety. Further examination of the 2D NMR data of **3** (Supplementary information) indicated that it is structurally similar to those of platanionoside G (Otsuka and Tamaki, 2002), except for difference from an α-arabinofuranoside in **3** instead of an β-xylopyranoside in platanionoside G, and the configuration of C-9 position. These conclusions were confirmed via the HMBC cross-peaks from H-2 (δ_H 1.79) and H-3 (δ_H 2.43) to ketone group at δ_C 201.6, and those of δ_H 1.74 (H-13) to C-4/C-5/C-6, clearly identified the ketone group at C-4 and Δ^5 at C-5/C-6, respectively. The HMBC correlations of an anomeric proton at δ_H 4.33 (H-1′) to C-9 (δ_C 76.0) and secondary anomeric proton at δ_H 4.97 (H-1″) to C-6′ (δ_C 68.2) indicated a arabinofuranosyl-(1″→6′)-glucopyranosyl moiety as the disaccharide sugar chain which was allocated to C-9 of its megastigmane skeleton. Furthermore, acid hydrolysis of **3** yielded glucose and arabinose products which were detected by comparison the retention time of their hydrolysis products ($t_{R\text{-}L\text{-}ara}$ = 24.4 min and $t_{R\text{-}D\text{-}glc}$ = 27.3 min) with authentic samples ($t_{R\text{-}L\text{-}ara}$ = 24.4 min and $t_{R\text{-}D\text{-}glc}$ = 27.6 min) using analytical HPLC, respectively. Further assignments by COSY, HMQC and HMBC correlations of **3** were displayed in Fig. 2. The appearance of C-9 (δ_C 76.0) relatively confirmed the *S*-form of C-9 position (Otsuka et al., 1994). According to the above evidence, the structure of **3** was established as a new compound as (9*S*)-9-hydroxymegastigman-5-en-4-one-9-ol-*O*-β-D-(α-L-arabinofuranosyl-(1″→6′)-glucopyranoside.

Lauruside G (**4**) was obtained as a white, amorphous powder {$[\alpha]_D^{21}$ −12.6° (*c* 0.25, MeOH)}. The molecular formula of **4** was established as $C_{26}H_{42}O_{15}$ according to the HR-QTOF-MS peak at *m/z* 617.2420 $[M + Na]^+$. The ^1H NMR spectrum of **4** exhibited the presence of characteristic signals of a *trans* olefilic protons [δ_H 6.26 (1H, d, J = 15.8 Hz, H-7), 7.75 (1H, d, J = 15.8 Hz, H-8)], a vinyl group [δ_H 5.82 (1H, s, H-10), and 1.97 (3H, s, H-14)], two anomeric protons [δ_H 5.35 (1H, brs, H-1″) and 4.41 (1H, d, J = 7.5 Hz, H-1′)], an oxymethine [δ_H 4.21 (1H, m, H-3)], two oxymethylenes [δ_H 3.78 (1H, m, H-12a) and 3.72 (1H, m, H-12b)], and two singlet methyl protons [δ_H 0.92 (3H, H-11) and 1.16 (3H, H-13)]. The ^{13}C NMR data of **4** exhibited 26 carbons including one carboxyl [δ_C 175.1 (COOH)], a substituted double bond [δ_C 130.8 (C-7) and 133.0 (C-8)], a vinyl [δ_C 142.2 (C-9), 127.2 (C-10), and 20.8 (C-14)], an oxymethine [δ_C 74.2 (C-3)], three none-protonated carbons [δ_C 49.8 (C-1), 87.6 (C-5), and 83.1 (C-6)], one oxymethylene [δ_C 77.1 (C-12)], two methyl carbons [δ_C 16.4 (C-11) and 19.8 (C-13)], and 11 carbons of two sugar moieties (Table 1). Compound **4** was deducted with a monocyclofarnesane sesquiterpene (Fraga, 2013) containing a conjugated carbonyl group.

The NMR spectroscopic data of **4** was similar to monaspilosuslin (Cheng et al., 2010), except for differences with more two sugar units and an 12-oxymethylene in **4**. The HMBC correlations of δ_H 0.92 (H-11) to C-1 (δ_C 49.8)/C-6 (δ_C 83.1)/C-12 (δ_C 77.1) as well as those of δ_H 1.16 (H-13) to C-4 (δ_C 43.0)/C-5 (δ_C 87.6)/C-6 (δ_C 83.1), indicated the CH_2OH-12 and OH-5 positions, respectively. Moreover, the anomeric proton at δ_H 4.41 (d, J = 7.5 Hz, H-1′) correlated to C-3 (δ_C 74.2) confirmed the glucopyranosyl moiety was connected to its skeleton through C-3. Furthermore, acid hydrolysis of **4** yielded glucose and apiose products which were detected by comparison the retention time of its hydrolysis product with authentic samples using analytical HPLC. Additionally, the HMBC cross-peaks of the second anomeric proton at δ_H 5.35 (brs, H-1″) to C-2′ (δ_C 79.3) was also observed. This finding was

reasonable to resonate to the downfield shift of C-2′. Thus, an apiofuranosyl-(1″→2′)-glucopyranosyl moiety of the sugar side chain was allocated to C-3 of the skeleton.

The NOESY cross-peaks of δ_H 1.16 (H_3-13) to δ_H 4.21 (H-3) and 6.26 (H-7) as well as those of δ_H 6.26 (H-7) to δ_H 5.82 (H-10) and 0.92 (H_3-11), suggested the *axial* positions of H-3, H-7, H_3-11, and H_3-13, respectively (Cheng et al., 2010). This finding and the chemical shift of C-14 (δ_C 20.8) was also expressed the *Z*-configuration of $\Delta^{9(10)}$. Whereas, the appearance of C-5 (δ_C 87.6) and C-6 (δ_C 83.1) were similar to those of monaspilosuslin (Cheng et al., 2010), allowed to assign *R*-form of OH-5 and *S*-form of OH-6, respectively. Therefore, the structure of **4** was established as lauruside G.

The other compounds were identified as alangionoside B (**2**) (Otsuka et al., 1994), (6*S*,9*R*)-vomifoliol-9-*O*-*β*-D-apiofuranosyl-(1″→6′)-*O*-*β*-D-glucopyranoside (**5**) (Ito et al., 2001), (1′*S*,6′*R*)-8′-hydroxyabscisic acid *O*-*β*-D-glucoside (**6**) (del Refugio Ramos et al., 2004), and (6*S*,9*R*)-roseoside (**7**) (Yajima et al., 2009) (Supplementary information) by comparing their spectroscopic data with published literature values.

NO is a multifunctional molecule in the regulation of host defense mechanisms, apoptosis, neurotransmission, acute and chronic inflammation. The enhanced NO levels associated with acute or chronic inflammatory diseases indicate that NO is related to pro-inflammatory agents for inflammation-mediated pathogenesis. To assess the anti-inflammatory activity, all the isolated compounds (**1–7**) were evaluated for NO production inhibitory activity in LPS-stimulated RAW 264.7 cells. Unfortunately, all the isolated compounds showed the weak inhibitory effects (IC_{50S} > 50 μM).

3. Experimental

3.1. General experimental procedures

Optical rotations were determined on a JASCO P-2000 polarimeter. The UV spectrum was recorded on a JASCO V-630 spectrophotometer. IR spectra were obtained on a Bruker TENSOR 37 FT-IR spectrometer. The NMR spectra were recorded on a JEOL JNM-AL 400 MHz spectrometer, chemical shift (δ) are expressed in ppm with reference to the TMS signals. The HR-QTOF-MS was acquired on an Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Emeryville, CA, USA). Preparative HPLC was performed on an Agilent 1260 apparatus (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1315D Variable Wavelength Detector on collumn of Kinetex C18 (250 × 4.6 mm, 5 μm). Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck), porous polymer gel (Diaion® HP-20, 20–60 mesh, Mitsubishi Chemical, Tokyo, Japan), Sephadex™ LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), octadecyl silica (ODS, 50 μm, Cosmosil 140 C18-OPN, Nacalai Tesque), and YMC RP-18 resins (30–50 μm, Fuji Silysia Chemical). Thin layer chromatography (TLC) used pre-coated silica gel 60 F_{254} (1.05554.0001, Merck) and RP-18 F_{254S} plates (1.15685.0001, Merck) and compounds were visualized by spraying with aqueous 10% H_2SO_4 and heating for 1.5—2 min.

3.2. Plant material

The leaves of *L. nobilis* were collected in July 2016 at Sin Ho, Lai Chau province, Vietnam, and identified by Prof. Tran Huy Thai, Institute of Ecology and Biological Resources, VAST, Vietnam. A voucher specimen (LN-1606) was deposited at the Herbarium of Institute of Ecology and Biological Resources, VAST, Vietnam.

3.3. Extraction and isolation

Fresh leaves of *L. nobilis* was dried under shiny for a dried sample. The dried leaves of *L. nobilis* (2.5 kg) were well ground and extracted three times with 95% MeOH at room temperature for 2 days each to give a MeOH residue (120 g) after removal of the solvent under reduced pressure. This residue was suspended in water (1.5 L) and partitioned in turn with CH_2Cl_2 (3 × 1.5 L) and EtOAc (3 × 1.5 L) to furnish corresponding extracts: CH_2Cl_2 (B, 51 g), EtOAc (C, 8 g) and water layer (D, 1 L).

The water layer has removed the solvent and subjected to Dianion HP-20 open CC using concentration of MeOH in H_2O (from 0% to 100%) to give four fractions (D1-D4). These fractions were collected according to their TLC profiles. Fraction D2 (1.5 g) was separated on a silica gel CC using stepwise elution with CH_2Cl_2-MeOH-H_2O (15/1/0.01–1/1/0.01, v/v/v) and further purified by silica gel CC, YMC RP-18 columm eluting with solvent system of MeOH-H_2O (1/2.5, v/v), and Sephadex LH-20 eluting with solvent system of MeOH-H_2O (1/1, v/v) to obtain **1** (7.2 mg), **3** (6.5 mg), and **5** (8.5 mg) from subfraction D2A (0.7 g) and D2D (0.3 g), respectively. Next, fraction D3 (1.2 g) afforded **4** (9.0 mg) and **6** (10.2 mg) after subject it to YMC RP-18 CC with MeOH-H_2O (1/2, v/v) and acetone-H_2O (1/3, v/v), respectively. Finally, subfraction D4 (0.6 g) were purified on YMC RP-18 CC eluting with acetonitrile-H_2O (1/1.8, v/v) to obtain compound **7** (9.5 mg).

3.4. Physical and spectroscopic data of new compounds

3.4.1. Lauruside E (1)

White, amorphous powder; $[\alpha]_D^{21}$ − 22.0 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ε) nm: 205 (3.56) and 267 (3.03); IR (KBr) ν_{max}: 3387, 2942, 2835, 1678, 1424, and 1011 cm^{-1}; positive-ion HR-QTOF-MS *m/z* 545.2567 $[M + Na]^+$ (calcd. for $C_{24}H_{42}NaO_{12}$, 545.2574); ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) data, see Table 1.

3.4.2. Lauruside F (3)

White, amorphous powder; $[\alpha]_D^{21}$ − 15.6 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε) nm: 191 (3.66) and 266 (2.97); IR (KBr) ν_{max}: 3329, 2940, 2841, 1687, and 1022 cm^{-1}; positive-ion HR-QTOF-MS *m/z* 527.2464 $[M + Na]^+$ (calcd. for $C_{24}H_{40}NaO_{11}$, 527.2468); ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) data, see Table 1.

3.4.3. Lauruside G (4)

White, amorphous powder; $[\alpha]_D^{21}$ − 12.6 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ε) nm: 202 (3.21) and 266 (2.98); IR (KBr) ν_{max}: 3366, 2939, 2831, 1715, 1419, and 1025 cm^{-1}; positive-ion HR-QTOF-MS *m/z* 617.2420 $[M + Na]^+$ (calcd. for $C_{26}H_{42}NaO_{15}$, 617.2421); ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) data, see Table 1.

3.5. Acid hydrolysis of compounds

Compounds **1**, **3** (2.5 mg, each), and **4** (2 mg) were hydrolyzed by heating in 10% H_2SO_4 aqueous solution at 80 °C for 3 h, neutralized with barium carbonate, and then filtered, and the filtrate was extracted with ethyl acetate (3 × 4 mL) to remove aglycone. The aqueous layer was concentrated to obtain the sugar residue, which was dissolved in 1 mL of pyridine heated with 6 mg L-cysteinemethyl ester at 60 °C for 60 min, then phenylisothiocyanate (0.1 mL) was added to the reaction mixture and further reacted at 60 °C for 60 min then the reaction mixture was evaporated on a water bath, and then 1 mg of the product was dissolved in acetonitrile and analyzed by standard C18 HPLC by standard C18 HPLC(Kinetex C18: 4.6 mm × 250 mm, 5 μm) at a flow rate of 0.8 mL/min with an ultra-violet (UV) detector at wavelength of 250 nm. The The mobile phase was consisted as a gradient solvent system of phase A (H_2O) and phase B (ACN) as following the analytical condition: 20–80% (B) for 0–50 min.

3.6. In vitro NO assay

3.6.1. Cell culture

The RAW264.7 cells were obtained from Korean Cell Line Bank (KCLB, Chongno-gu, Seoul, Korea) and maintained in Dulbecco's

Modified Essential (DMEM) supplemented with 10% fetal bovine serum (FBS), (100 units/mL) penicillin, and streptomycin sulphate (100 μg/mL) at 37 °C in humidified incubator containing 5% CO_2. After pre-incubator of RAW264.7 for 4 h, each compound at concentration of 3–60 μM were added.

3.6.2. Cell viability

Cell viability was evaluated by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) methods (Carmichael et al., 1987), in which the effects of tested compounds (**1–7**) were evaluated by determining mitochondrial reductase function base on the reduction of MTT into formazan. Briefly, MTT was added to the cell suspension (1×10^4 cells/well) for 4 h before treated with vehicle or tested compounds for 24 h. Viability of the macrophages treated with vehicle (0.5% DMSO) only was defined as 100% viable. Survival of macrophage cells after treatment with compounds was calculated using the following formula:

Viable cell number (%) = OD_{570} (treated cell culture)/OD_{570} (vehicle control) × 100

The percentage of dead cells was determined relative to the control group.

3.6.3. Nitric oxide assay

The nitrite, which accumulated in the culture medium, was measured by using Griess reaction as an indicator of NO production (Green et al., 1982). Briefly, RAW264.7 cells (1×10^5 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 tested compounds at different concentration of 3, 10, 30 and 60 μM for 1 h. Neither LPS nor samples were added to control group. Then cell culture medium was mixed with an equal volume of Griess reagent, incubated at room temperature for 4 h, and then the absorbance was measured at 570 nm using a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was obtained by means of the $NaNO_2$ serial dilution standard curve and the nitrite production was measured.

3.6.4. Statistical analysis

All data represent the mean ± SD of at least three independent experiments performed in triplicate. Statistical significance is determined by using the software SPSS 19.0 according to introductions of manufacture.

4. Conclusions

Three new compounds, laurusides E-G (**1**, **3**, and **4**) and four known compounds (**2** and **5–7**) were isolated from a methanolic extract of *L. nobilis* leaves. The structures of these isolates were accomplished using comprehensive spectroscopic methods. This is the first report for NO production inhibitory effects of constituents from this plant. The presence of these compounds may support the interesting metabolism of compounds in nature.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2019.06.011.

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