CHEMICAL CONSTITUENTS AND THEIR INHIBITION ACTIVITY ON NO PRODUCTION IN LPS-STIMULATED RAW264.7 MACROPHAGES OF THE LEAVES OF TINOSPORA SINENSIS

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TÓM TẮT

THÀNH PHẦN HÓA HỌC VÀ HOẠT TÍNH ỨC CHẾ NO TRONG ĐẠI THỰC BÀO RAW264.7 ĐƯỢC KÍCH THÍCH BỞI LPS CỦA LÀ LOÀI DÂY ĐAU XƯƠNG TINOSPORA SINENSIS

*Bằng các phương pháp sắc kí kết hợp, hai hợp chất monoterpene lactone calendin (**1**), (-)-loliolide-β-D-glucopyranoside (**2**), và hai hợp chất lignan syringaresinol-4'-O-β-D-glucopyranoside (**3**), (+)-syringaresinol (**4**) đã được phân lập từ phần lá loài dây đau xương. Cấu trúc của các chất này được xác định dựa trên phân tích các dữ kiện phổ khối lượng, phổ cộng hưởng từ hạt nhân một chiều và hai chiều, và kết hợp so sánh với các dữ liệu phổ đã công bố. Các dịch chiết n-hexane (TSH), dichloromethane (TSD), và phần dịch chiết nước (TSW) được đánh giá hoạt tính kháng viêm thông qua sự ức chế sản sinh NO trong đại thực bào RAW264.7 được kích thích bởi LPS. Kết quả cho thấy các dịch chiết này đều có hoạt tính ức chế tốt với giá trị IC_{50} lần lượt là 19.44±1.69, 15.32±2.22 và 51.48±2.76 µM.*

***Từ khóa**: Tinospora sinensis, dây đau xương, ức chế NO.*

1. INTRODUCTION

The *Tinospora* is known as a genus of deciduous woody vines belonging to the Menispermaceae family and distributed around tropical areas with over 34 species [1-4]. In Southeast Asian regions, this genus is commonly used as a traditional medicine to treat malaria, fever, colds, digestive disorder, rheumatoid arthritis, gout, and diabetes [1, 4, 5]. Previous reports showed that the species of this genus brings a variety of biological activities, especially antioxidant, anti-inflammatory, antidiabetic, anti-microbial, anti-tumor, and cytotoxic activities [1, 4, 5]. Besides, the previous phytochemical research has brought the main chemical classes of this genus as lignans, terpenoids, alkaloids, steroids, phenolics, and glycosides [1, 3, 6]. This paper describes the isolation and structural elucidation of two monoterpene lactones calendin (**1**), (-)-loliolide-β-D-glucopyranoside (**2**), and two lignan syringaresinol-4'-O-β-D-glucopyranoside (**3**), (+)-syringaresinol (**4**) from the leaves of *T. sinensis* (Figure 1). The anti-inflammatory activity of crude extracts and isolated compounds (**1-4**) were evaluated by their ability to inhibit NO production in lipopolysaccharide (LPS)-activated RAW 264.7 cells.

2. EXPERIMENTAL

2.1. Plant materials

The leaves of *Tinospora sinensis* were collected at Phuc Yen, Vinh Phuc, Vietnam in

May 2020 and identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST. A voucher specimen (NCCT-TSL93) was deposited at the Institute of Ecology and Biological Resources, VAST.

2.2. General experimental procedures

All NMR spectra, including ^{1}H-NMR (500 MHz), ^{13}C (125 MHz), HSQC and HMBC were recorded on a Bruker AM500 FT-NMR spectrometer and TMS was used as an internal standard. The HR ESI mass spectra were obtained using an Agilent 6550 iFunnel Q-TOF LC/MS system. All the spectrophotometry were measured at the Institute of Chemistry and the Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST). Optical rotations were determined on a Jasco DIP-370 automatic polarimeter. Column chromatography (CC) was performed using a silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) or RP-18 resins (30-50 µm, Fuji Silysia Chemical Ltd.). Thin layer chromatography (TLC) was done using pre-coated silica gel 60 F_{254} (0.25 mm, Merck) and RP-18 F_{254S} plates (0.25, Merck).

2.3. Extraction and isolation

The dried leaves of *Tinospora sinensis* (3.0 kg) were pulverized and sonicated with 15 liters of methanol (MeOH) three times at room temperature for 10 hours to obtain the MeOH crude extract. After removal of solvent, the MeOH extract residue was suspended in 2 liters of hot water and then sequence partitioned with *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) to give the corresponding n-hexane (**TSH**, 100 g), dichloromethane (**TSD**, 3.8 g), ethyl acetate (TSE, 2.6 g) and water extract residue after evaporation at reduced pressure.

The **TSH** was run on a silica gel CC and eluted with a gradient solvent system including *n*-hexane – acetone gradient (100:0 – 5:1, v/v) and CH_2Cl_2 – MeOH (100:0 – 10:1) to obtain eleven fractions from **TSH16A** to **TSH16K**. Two fractions, **TSH16E** and **TSH16F**, were combined and performed on an RP-18 column chromatography eluting with solvent system MeOH – water (1.5:1, v/v) to yield compound **1** (15.6 mg) and **4** (36.5 mg).

An aqueous residue (**TSW**) was chromatographed on a diaion HP-20 column eluting with the increasing concentration of MeOH in water from 0% MeOH to 100% MeOH to give five fractions (**TSW4A – TSW4E**). Combine two fractions **TSW4B** and **TSW4E** and use solid-liquid extraction on a silica gel gradient CC eluting with CH_2Cl_2 – MeOH (100:0 – 0:100) to obtain six fractions from **TSW5A** to **TSW5F**. The fraction **TSW5C** was subjected to a YMC RP-18 column and eluted with MeOH – water (1:3, v/v) to obtain nineteen fractions (**TSW7A – TSW7S**). The fractions **TSW7G** and **TSW7H** were subjected on a silica gel CC eluting with a solvent system of CH_2Cl_2 – MeOH – water (6:1:0.05, v/v/v) to give four fractions **TSW11A, TSW11B, TSW11C** and **TSW11D**. Compound **2** (10 mg) was refined from the fraction **TSW11A**, and compound **3** (12.4 mg) was yielded from the fraction **TSW7P** on a silica gel CC and eluted with CH_2Cl_2 – MeOH – water (6.5:1:0.05, v/v/v).

Calendin (1): Needles; $[\alpha]_D^{25}$: -95.65° (*c* 0.1, $CHCl_3$). ESI-MS: *m/z* 197.25 $[M+H]^+$, $C_{11}H_{16}O_3$. ^{1}H- and ^{13}C-NMR (CD_3OD) see Table 1.

(-)-loliolide-*β*-*D*-glucopyranoside (2): colorless oil; $[\alpha]_D^{25}$: -34.42° (*c* 0.1, MeOH). ESI-MS: *m/z* 359.16 $[M+H]^+$, $C_{17}H_{26}O_8$. ^{1}H- and ^{13}C-NMR (CD_3OD) see Table 1.

syringaresinol-4'-O-*β*-*D*-glucopyranoside (3): an amorphous powder; $[\alpha]_D^{25}$: -15.68° (*c* 0.1, MeOH). ESI-MS: *m/z* 581.24 $[M+H]^+$, $C_{28}H_{36}O_{13}$. ^{1}H- and ^{13}C-NMR (CD_3OD) see Table 2.

(+)-syringaresinol (4): colorless crystal; $[\alpha]_D^{25}$: +56.71° (*c* 0.05, MeOH). ESI-MS: *m/z* 419.15 $[M+H]^+$, $C_{22}H_{26}O_8$. ^{1}H- and ^{13}C-NMR (CD_3OD) see Table 2.

2.4. Nitric Oxide Assay

Inhibition of NO production in LPS-stimulated RAW 264.7 cell assay, see reference [7]

3. RESULTS AND DISCUSSION

3.1. Structural elucidation

Compound **1** was obtained as needles. Its molecular formula was determined to be $C_{11}H_{16}O_3$ based on a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 197.25 (calcd. for $C_{11}H_{17}O_3$) in the ESI-MS spectra. The ^{1}H-NMR spectral of compound **1** showed the

singlet proton signals including the characteristic proton signals of *gem*-dimethyl at δ_H 1.27, 1.47 and one methyl group at δ_H 1.78; one olefinic proton at δ_H 5.69; four proton signals of two methylene groups at δ_H 1.53 (dd, J = 4.0, 15.0 Hz), 1.74 (dd, J = 4.0, 13.5 Hz), 1.97 (dt, J = 2.5, 14.5 Hz) and 2.46 (dt, J = 2.5, 14.0 Hz). The ^{13}C-NMR and HSQC spectrum of **1** defined the signals of eleven carbons comprising three methyl groups at δ_C 26.5, 27.0 and 30.7; two methylenes at δ_C 45.7 and 47.4; an *α,β*-unsaturated ketone group at δ_C 113.0, 171.9 and 182.4; one oxymethine at δ_C 66.9, and two non-protonated carbons at δ_C 35.9 and 86.6. Analysis of the ^{1}H-NMR, ^{13}C-NMR spectrum data and combined with the literature suggested that **1** was a monoterpene lactone and similar to calendin [8]. The HMBC correlations between an olefinic proton H-3 (δ_H 5.69) and C-2 (δ_C 171.9)/C-3a (δ_C 182.4)/C-7a (δ_C 86.6) indicated the structure signal of an *α,β*-unsaturated lactone ring. In addition, the HMBC correlations from H-8 (δ_H 1.27) to C-4 (δ_C 35.9)/C-5 (δ_C 47.4)/C-9 (δ_C 26.5) and H-9 (δ_H 1.47) to C-4 (δ_C 35.9)/C-5 (δ_C 47.4)/C-8 (δ_C 30.7) and H-10 (δ_H 1.78) to C-7 (δ_C 45.7); from δ_H 1.53 and 1.97 to C-7 (δ_C 45.7) suggested the presence of a 4-dimethyl-7a-methylcyclohexane that were directly bonded to a lactone ring at C-3a and C-7a. Moreover, the HMBC correlations between H-5 (δ_H 1.53 and 1.97)/ H-7 (δ_H 1.74 and 2.46) and C-6 (δ_C 66.9); between H-6 (δ_H 4.33) and C-4 (δ_C 35.9)/C-5 (δ_C 47.4)/C-7 (δ_C 45.7) /C-7a (δ_C 86.6) affirmed the position of a hydroxyl group at C-6. Consequently, the structure of **1** was elucidated to be calendin.

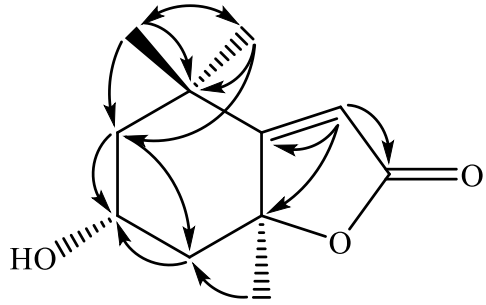

*Figure 2. The key HMBC correlations of compound **1***

*Table 1. The ^{1}H- and ^{13}C-NMR data of compounds **1-2***

C	1		2	
	[a,b]δ_C	[a,c]δ_H (mult., J = Hz)	[a,b]δ_C	[a,c]δ_H (mult., J = Hz)
2	171.9	-	174.4	-
3	113.0	5.69 (s)	113.1	5.76 (s)
3a	182.4	-	185.8	-
4	35.9	-	37.2	-
5	47.4	1.53 (dd, 4.0, 15.0)	46.9	1.59 (dd, 14.5, 4.0)
		1.97 (dt, 2.5, 14.5)		2.25 (dt, 14.0, 2.5)
6	66.9	4.33 (m)	74.3	4.32 (m)
7	45.7	1.74 (dd, 4.0, 13.5)	43.0	1.69 (dd, 14.5, 4.0)
		2.46 (dt, 2.5, 14.0)		2.68 (dt, 14.0, 2.5)
7a	86.6	-	88.9	-
8	30.7	1.27 (s)	30.9	1.30 (s)
9	26.5	1.47 (s)	26.6	1.47 (s)
10	27.0	1.78 (s)	27.1	1.78 (s)
1'			103.4	4.40 (d, 8.0)
2'			75.4	3.20 (dd, 8.0, 9.0)
3'			78.0	3.29 (t, 9.0)
4'			71.8	3.30 (m)
5'			78.5	3.38 (m)
6'			62.9	3.69 (dd, 11.5, 5.5)
				3.89 (dd, 11.5, 2.0)

Measured in [a] CD_3OD, [b] 125MHz, [c] 500MHz

Compound **2** was isolated as a colorless oil. It was determined a molecular formula of $C_{17}H_{26}O_8$ based on analysis of the ESI-MS data showing a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 359.16 (calcd. for $C_{17}H_{27}O_8$). Comparison of the NMR spectral data of **1** and **2** showed the similarity except for the addition of one sugar moiety. The ^{1}H-NMR spectra of **2** indicated the appearance of the characteristic proton signals of a sugar moiety at δ_H 4.40 (d, *J* = 8.0 Hz, 1H), 3.20 (dd, *J* = 8.0, 9.0 Hz, 1H), 3.29 (t, *J* = 9.0 Hz, 1H), 3.30 (m, 1H), 3.38 (m, 1H), 3.69 (dd, *J* = 11.5, 5.5 Hz, 1H), 3.89 (dd, *J* = 11.5, 2.0 Hz, 1H). The ^{13}C-NMR and the HSQC spectra of **2** showed the signals of 17 carbons. Of these, carbon signals of a sugar residue appeared at δ_C 103.4 (CH), 75.4 (CH), 78.0 (CH), 71.8 (CH), 78.5 (CH), 62.9 (CH_2) and the multiplicity of the anomeric proton in the ^{1}H-NMR spectrum [δ_H 4.40 (d, *J* = 8.0 Hz)] confirmed that the sugar moiety was *β*-D-glucopyranosyl. As a result, the NMR analysis afforded that **2** was a glycoside derivative of **1**. Hence, combined analysis of the NMR spectral data and comparision with the literature, the structure of **2** was elucidated as (-)-loliolide-*β-D*-glucopyranoside [9].

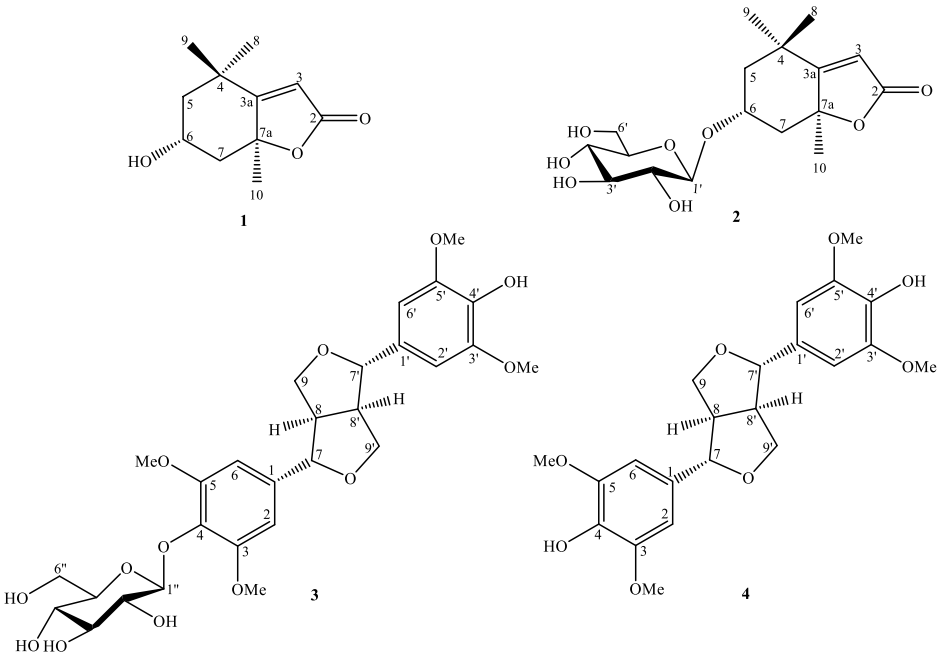

Figure 1. Chemical structures of isolated compounds from T. sinensis

Compound **3** was achieved as an amorphous powder. The molecular formula of **3** was a $C_{28}H_{36}O_{13}$ as affirmed by the ESI-MS spectral at *m/z* 581.24 $[M+H]^+$ (calcd. for $C_{28}H_{37}O_{13}$). Analysis of the ^{1}H-NMR spectra of **3** showed the proton signals of a sugar moiety at δ_H 4.87 (d, *J* = 7.5 Hz, 1H), 3.50 (dd, *J* = 9.0, 7.5 Hz, 1H), 3.44

(t, *J* = 9.0Hz, 2H), 3.22 (m, 1H), 3.68 (1H, dd, *J* = 12.0, 6.0 Hz), 3.80 (dd, *J* = 12.0, 2.0 Hz, 1H); the proton signals of four methoxy groups at δ_H 3.88 (s, 6H) and 3.86 (s, 6H); two singlet signals of four aromatic protons at δ_H 6.74 (s, 2H) and 6.67 (s, 2H). Furthermore, analysis of the ^{13}C-NMR spectrum and the HSQC correlations indicated the signals of 28 carbons including four methoxy groups at δ_C 56.8 (2xO<u>C</u>H$_3$) and 57.1 (2xO<u>C</u>H$_3$); of two aromatic rings at δ_C 139.6, 104.9, 154.4, 135.6, 133.1, 104.6, 149.4 and 136.3; six carbons of a sugar moiety at δ_C 105.4, 75.7, 77.8, 71.4, 78.3 and 62.6; and the signals of six sp^3 hybridized carbons at δ_C 55.5, 55.7, 72.9, 87.2 and 87.6. Combined analysis of the ^{1}H-, ^{13}C-NMR and

HSQC spectra suggested that **3** was a lignan compound with a symmetric structure. Moreover, a comparison of the NMR spectra data of **3** to the references led to determine the structure of **3** to be syringaresinol-4'-O-*β*-*D*-glucopyranoside [10], a compound isolated first time from *Scutellaria lateriflora* in 2012 [11].

Compound **4** was obtained as a colorless crystal. The NMR spectra of **4** were similar to the corresponding spectra of **3**, except for the loss of the sugar moiety signals in the NMR spectra of **4**. The ^{1}H-NMR spectrum of **4** exhibited the following proton signals: two *meta*-aromatic protons at δ_H 6.67 (s), one oxymethine group at δ_H 4.73 (d, J = 4.0 Hz), one oxymethylene group at δ_H 3.89 (m) and δ_H 4.28 (m), one methine at δ_H 3.15 (m), and two methoxy groups at δ_H 3.88 (s). The ^{13}C-NMR and DEPT spectra of **4** showed *tetra*-substituted aromatic signals at δ_C 104.6 (2xCH), 133.2 (C), 136.3 (C), and 149.4 (2xC), and the bis-tetrahydrofuran ring signals at δ_C 56.9 (2xOCH_3), 55.5 (CH), 87.6 (CH-O), 72.8 (CH_2-O). The ESI-MS mass spectrometry of **4** exhibited the ion peak at *m/z* 419.2 $[M+H]^+$, corresponding to the molecular formula of $C_{22}H_{26}O_8$. This result showed that the molecule of compound **4** was symmetrical with the second axis and was completely consistent with NMR spectra. Moreover, the NMR spectra data of **4** was consistency with the corresponding data in the literature [12]. Thus, the structure of **4** was elucidated as (+)-syringaresinol, a compound previously isolated from unrip Japanese apricot and showed antibacterial activity against the *Helicobacter pylori* bacteria [13].

*Table 2. The ^{1}H- and ^{13}C-NMR data of compounds **3-4***

C	3		4	
	[a,b]δ_C	[a,c]δ_H (mult., J = Hz)	[a,b]δ_C	[a,c]δ_H (mult., J = Hz)
1	139.6	-	133.2	-
2, 6	104.9	6.74 (s)	104.6	6.67 (s)
3, 5	154.4	-	149.4	-
4	135.6	-	136.3	-
7	87.2	4.79 (d, 4.5)	87.6	4.73 (d, 4.0)
8	55.5	3.15 (m)	55.5	3.15 (m)
9	72.9	3.94 (m)/4.29 (m)	72.8	3.89 (m)/4.28 (m)
3, 5 - OMe	56.8	3.88 (s)	56.9	3.88 (s)
1'	133.1	-	133.2	-
2', 6'	104.6	6.67 (s)	104.6	6.67 (s)
3', 5'	149.4	-	149.4	-
4'	136.3	-	136.3	-
7'	87.6	4.73 (d, 4.5)	87.6	4.73 (d, 4.0)
8'	55.7	3.15 (m)	55.5	3.15 (m)
9'	72.9	3.94 (m)/4.29 (m)	72.8	3.89 (m)/4.28 (m)
3', 5' - OMe	57.1	3.86 (s)	56.9	3.88 (s)
4-*O*-*β*-*D*-Glc				
1"	105.4	4.87 (d, 7.5)		
2"	75.7	3.50 (dd, 9.0, 7.5)		
3"	77.8	3.44 (t, 9.0)		
4"	71.4	3.44 (t, 9.0)		
5"	78.3	3.22 (m)		
6"	62.6	3.68 (dd, 12.0, 6.0) 3.80 (dd, 12.0, 2.0)		

Measured in [a] CD_3OD, [b]125MHz, [c]500MHz

3.2. Anti-inflammatory Assay

The *n*-hexane (**TSH**), dichloromethane (**TSD**), water (**TSW**) extracts, and compounds **1-4** was screened for inhibitory NO ability; the L-NMMA was used as a positive control. As a result, the **TSH**, **TSD**, and **TSW** extracts showed moderate inhibitory NO production with the IC_{50} value of 19.44±1.69, 15.32±2.22 and 51.48±2.76 μM, respectively. While **TSE** extract and compounds **1-4** did not inhibit NO production in LPS-stimulated RAW264.7 macrophages. All tested samples exhibited NO effective inhibition without obvious cytotoxicities.

*Table 3. The NO inhibition activity of the crude extracts and compounds **1-4***

Compounds	Cell viability (% of control) at 100 µM		IC_{50} µM[a)]
	% NO inhibition	% living cells	
TSH	77.24	99.98	19.44±1.69
TSD	75.40	98.45	15.32±2.22
TSE	38.67	100.76	>100
TSW	57.15	100.16	51.48±2.76
1	34.78	97.41	>100
2	30.34	98.64	>100
3	<30	>100	>100
4	<30	>100	>100
L-NMMA[b)]	103.28	89.94	8.83±0.75

[a)] The concentration that inhibits 50% of cell growth was calculated (IC_{50}). Data are means of three experiments.

[b)] L-NMMA (N^G-Methyl-L-arginine-acetate) was used as the reference compound.

4. CONCLUSION

Four known compounds: calendin (**1**), (-)-loliolide-*β-D*-glucopyranoside (**2**), syringaresinol-4'-O-*β-D*-glucopyranoside (**3**), (+)-syringaresinol (**4**) were isolated from the dried leaves of *Tinospora sinensis* by mean of chromatography methods. The *n*-hexane (**TSH**), dichloromethane (**TSD**), and water (**TSW**) extracts of *T. sinensis* exhibited NO inhibitory activities in LPS-stimulated RAW264.7 macrophages with the IC_{50} value of 19.44±1.69, 15.32±2.22 and 51.48±2.76 μM, respectively.

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