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ANTIOXIDANT AND ANTI-INFLAMMATORY POTENTIAL FROM *OCHNA INTEGERRIMA* STEM

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Summary

Antioxidant and Anti-Inflammatory Potential from *Ochna integerrima* Stem

The present work aims to study the antioxidant activity, the anti-inflammatory effects of *n*-hexane, ethyl acetate (EtOAc), *n*-BuOH, aqueous, and methanol (MeOH) extracts, and chemical components from *n*-hexane extract. The antioxidant potential was determined through 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays. The total phenolic (TPC) and flavonoid contents (TFC) were measured using the Folin-Ciocalteu (FC) and aluminum chloride assays. The anti-inflammatory activity of the extracts was evaluated as the inhibition of bovine serum albumin (BSA) denaturation. All extracts showed significant antioxidant activity, except for the *n*-hexane extract. Among the extracts, the aqueous extract had the most potent antioxidant activity in DPPH and ABTS assays with IC50 values of 9.57 ± 0.21 µg/mL and 3.6 ± 0.15 µg/mL; and with high TPC and TFC values (263.34 ± 6.83 mg GAE/g) and (5.19 ± 0.16 mg QE/g), respectively. Similarly, it also showed the greatest FRAP value of 304.30 ± 25.15 mg AAE/g. For the anti-inflammatory activity, the inhibition of BSA denaturation was ranked as *n*-hexane extract (57.11%) > aqueous extract (49.80%) > *n*-BuOH extract (31.94%) > MeOH extract (27.96%) > EtOAc extract (15.63%) at the concentration of 125 µg/mL. The two steroids stigmasterol-3-O-*β*-D-glucopyranoside (**1**) and stigmasterol (**2**) were isolated to prove the anti-inflammatory activity of *n*-hexane extract. To sum up, the extracts of *O. integerrima* stem may have therapeutic potential against oxidative stress-related and inflammatory disorders. For the first time, *O. integerrima* was evaluated for its anti-inflammatory activity using heated-induced BSA denaturation.

Keywords: *Antioxidant, Anti-inflammatory, Extracts, O. integerrima stem.*

1. Background

Inflammation and oxidative stress are closely linked by the increase of uncontrolled free radicals. The excessive formation of oxygen species reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by endogenous and exogenous processes. They lead to many diseases including neurodegenerative diseases, metabolic disorders, atherosclerosis, cardiovascular disease, diabetes, cancer, trauma recovery, skin damage, and aging [1],[2]. Antioxidant plays a crucial role to neutralize the production of ROS by donating an electron. The free radicals become stable when they gain an extra electron from an antioxidant agent [3]. Besides these natural resources, butylated hydroanisole (BHA), butylated hydrotoluene (BHT), *n*-propyl gallate (PG) are widely used antioxidant food additives. However, they pose a potential health risk due to chemical contamination, toxic solvents, and hazardous by-products during the synthetic process [4]. Therefore, extracts from plants with antioxidant capacity are increasing interest [5]. In addition, inflammation is a process that protects the body from infection from outside attackers, such as bacteria and viruses. Nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are the most usually used to reduce inflammation and relieve pain induced by inflammatory conditions, but these drugs are responsible for liver and kidney damage [6]. BSA is a globular protein that has been widely used as a model protein to study protein-ligand interactions. The BSA method is a well-established method for screening anti-inflammatory compounds as it mimics the interactions between serum albumin and anti-inflammatory drugs [7].

O. integerrima belongs to the Ochnaceae family and is a symbol of luck in spring in southern Vietnam. It is a flowering shrub native to Southeast Asia such as Vietnam, Laos, Cambodia, and Thailand. The plant is well-known for its attractive bright yellow flowers and glossy green leaves, making it a popular ornamental plant in both residential and commercial landscapes. Studies have shown that the main components isolated from the stem of Ochnaceae family are flavonoids, cyanoglycosides, and sterol glycosides [8],[9]. According to folk medicine, the bark stems and roots of *O. integerrima* are used as a digestive tonic, antidysentery, and antipyretic [10]. This species has been reported to possess various pharmacological activities, including antioxidant

and anti-inflammatory effects [11]. In our previous work, we found that MeOH extracts of stems showed significant antioxidant activity using DPPH assay [12]. In going to find bioactive plant extracts, this study aims to further investigate antioxidant properties using ABTS, DPPH, and FRAP along with TPC and TFC assays from *n*-hexane, EtOAc, *n*-butanol, aqueous, and MeOH extracts. Then, their correlations were evaluated using Pearson analysis. Moreover, the *n*-hexane extract was selected for isolation regarding its anti-inflammatory property using the protein denaturation assay.

2. Object and research method

2.1. Plant material

The whole stems of *O. integerrima* with a diameter of approximately 24 cm and a lifespan of 9-year-old were collected in Ben Tre province, Vietnam in July 2022, identified by botanist Dr. Van Son Dang at the Institute of Tropical Biology, Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam. A voucher specimen (BTr-OI) was deposited at the Faculty of Pharmacy, Ton Duc Thang, Vietnam.

2.2. General procedures

Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker Avance NEO spectrometers (Bruker, USA) (600 MHz for ^{1}H and 150 MHz for ^{13}C) and (400 MHz for ^{1}H and 100 MHz for ^{13}C). LR-ESI-MS were measured on Agilent 1260 Infinity II HPLC system (USA) equipped with Agilent G6125B single quad MSD. HR-ESI-MS was recorded on Shimazu LC/MS 8040 (Japan). A Thermo Fisher Scientific UV-Vis spectrophotometer was used to measure the absorbance of antioxidant assays. U.S. *Silica gel* (60 N, spherical, neutral, 40-50 µm, Kanto Chemical Co., Inc., Tokyo, Japan) was used for open column chromatography (CC). Spots were visualized by spraying with a solution of 10% H_2SO_4 in ethanol, followed by heating at 100°C. DPPH, FC reagent, TPTZ, ABTS, BSA, quercetin (10230364; 97.0%), gallic acid (0000237403; 98.5%), and ascorbic acid (BCCH6669; 99.0%) were purchased from Sigma Aldrich (USA). Diclofenac sodium was provided by the Institute of Drug Testing, Ho Chi Minh City, Vietnam.

2.3. Antioxidant assays of the extracts

2.3.1. Total phenolic content (TPC):

The TPC was indicated through a redox reaction between phenolic compounds with the Folin-Ciocalteau reagent. Briefly, the protocol was performed according to the FC assay with some minor alterations [13]. Gallic acid prepared in distilled water was used as a positive control at different concentrations (1.5625 to 25 µg/mL). The extracts were dissolved in DMSO solution to obtain a concentration of 20 mg/mL, and then diluted with H_2O to obtain a solution of 50 µg/mL. 2 mL of the extract with a final concentration of 50 µg/mL was added with 0.25 mL of 25% FC reagent. After 3 minutes, 0.25 mL of 10% Na_2CO_3 was added and mixed well. Then, these mixtures were incubated in the dark for 30 mins and measured at 760 nm. All experiments were repeated three times. The TPC was calculated based on the standard curve of gallic acid and expressed as mg gallic acid equivalent (GAE)/g.

2.3.2. Total flavonoid content (TFC):

The TFC was evaluated by the coloration reaction between $AlCl_3$ and flavonoid compounds. The experimental procedure was performed according to the colorimetric method of Chang et al. (2002) with minor changes [14]. The extracts were dissolved in DMSO solution to obtain a concentration of 20 mg/mL, and then diluted with H_2O to obtain a solution of 2.0 mg/mL. 0.5 mL of the extract with a final concentration (2.0 mg/mL) was added to 1.5 mL of methanol. After 5 minutes, 0.1 mL of 10% $AlCl_3$ was added to the mixture above and mixed well. After 6 minutes, the reaction mixture was added to 0.1 mL of 1 M CH_3COOK, and 2.8 mL of distilled water and incubated in the dark for 45 minutes at room temperature. The samples were measured UV-Vis at 415 nm. Quercetin was used as a positive control at different concentrations (3.215 to 100 µg/mL). All experiments were repeated three times. The TFC was calculated as mg quercetin equivalents (QE)/g based on a standard curve of quercetin.

2.3.3. Ferric reducing antioxidant power assay (FRAP):

The FRAP was determined by reducing $Fe^{3+}([Fe(TPTZ)_2]^{3+})$ to a dark blue $Fe^{2+}([Fe(TPTZ)_2]^{2+})$. The experimental procedure was performed according to the method of Benzie and Strain (1996) with some slight modifications [15]. Firstly, the TPTZ solution was prepared by mixing the following reagents at a ratio of 10:1:1 (300 mM acetate buffer, 10 mM TPTZ prepared in 40 mM HCl, 20 mM $FeCl_3.6H_2O$). The extracts were dissolved in DMSO solution to obtain a concentration of 20 mg/mL, and then diluted with H_2O to obtain a solution of 50 µg/mL. Then, 1.5 mL of TPTZ solution was added to 0.5 mL of the extract at a final concentration (50 µg/mL) and mixed well. The test tubes were incubated at 37ºC for 15 min and stabilized at room temperature for 5 min. Finally, the absorbances were measured at 593 nm. Ascorbic acid was used as a positive control at different concentrations (3.215 to 50 µg/mL). All experiments were repeated three times. The iron-

reducing capacity was calculated based on the standard curve of ascorbic acid and expressed as mg ascorbic acid equivalents (AAE)/g.

2.3.4. DPPH radical scavenging assay:

The DPPH assay was evaluated by the color change from purple to yellow. The free electron of DPPH is reduced by receiving a hydrogen atom from the antioxidant to form DPPH-H. As a result, its absorbance decreases [16]. The protocol was carried out by the method of Xie J. et al. (2014) with some minor modifications [17]. The extracts were dissolved in DMSO solution to obtain a concentration of 10 mg/mL, and then diluted with 0.1 mM DPPH solution to obtain different concentrations (3.125 to 100 µg/mL). The mixture was incubated in the dark for 30 min. The absorbance was read at 517 nm. The positive control (ascorbic acid) was prepared under the same condition as the extracts. The control sample was prepared as above without any sample. All experiments were repeated three times. The results are shown as the IC_{50} values (the concentration of extracts to inhibit 50% of DPPH radical scavenging). Its radical scavenging capacity was calculated according to the following formula:

$$\text{DPPH inhibition (\%)} = [(A_c - A_s)/A_c] \times 100$$

Where A_c and A_s are absorbances of control and extract, respectively.

2.3.5. ABTS radical scavenging assay:

The ABTS assay was evaluated by the color change from dark blue to light blue. The protocol was performed according to the method of Re et. al. (1999) with some slight modifications [18]. Firstly, $ABTS^{\bullet+}$ cation is formed by mixing 7 mM ABTS and 2.45 mM $K_2S_2O_8$ at a ratio of 1:1. This mixture was incubated for 12-16 hours at room temperature. It was further diluted with acetate buffer (pH = 4.5) to obtain an absorbance of 0.74 ± 0.03. The extracts were dissolved in DMSO solution to obtain a concentration of 10 mg/mL, and then diluted in this ABTS solution (1.5625 to 25 µg/mL). The extract solution was incubated in the dark for 7 min at room temperature and measured UV-Vis at 734 nm. Ascorbic acid (1.5625 to 25 µg/mL) was used as a positive control. All experiments were repeated three times. The control sample was prepared as above without any sample. The results are shown as the IC_{50} values. Its radical scavenging capacity was calculated according to the following formula:

$$\text{ABTS inhibition (\%)} = [(A_c - A_s)/A_c] \times 100$$

Where A_c and A_s are absorbances of control and extract.

2.4. Anti-inflammatory activity

Inhibition of BSA denaturation: The capacity to inhibit the denaturation of BSA was evaluated by the method of Sakat et al. (2010) with some minor modifications [19]. The extracts were dissolved in DMSO solution to obtain a concentration of 4 mg/mL and then diluted in distilled water to achieve different concentrations (31.25 to 125 µg/mL). 500 µL of the extract was added to 200 µL of 5% BSA, 1300 µL of phosphate saline buffer (pH = 6.3), and mixed well. The reaction mixtures were incubated at 37ºC for 10 min and 100ºC for 3 min. Finally, the samples were cooled and measured UV-Vis at 660 nm. Diclofenac was used as a positive control at different concentrations (31.25 to 125 µg/mL). All experiments were repeated three times. The control sample was prepared as above without any sample. The results are shown as the IC_{50} values (the concentration of extracts to inhibit 50% of BSA denaturation). Its denaturation inhibition capacity was calculated according to the following formula:

$$\text{BSA denaturation inhibition (\%)} = [(A_c - A_s)/A_c] \times 100$$

Where A_c and A_s are absorbances of control and extract.

2.5. Extraction and isolation

The fresh stem (0.72 kg) of *O. integerrima* was dried under shade for 3 days to obtain the dried sample (0.50 kg). It was grounded and macerated using MeOH (3 times × 3.0 L, each) for one time 24 hours. This crude extract was partitioned with increasing polarities of solvents to give *n*-hexane (5.7 g), EtOAc (4.6 g), *n*-butanol (2.9 g), and aqueous extracts (3.2 g), respectively. 0.8 g of *n*-hexane extract was used for bioactivity assays. The remaining *n*-hexane extract (4.9 g) was subjected to a normal phase-open column (NP-OP), eluted with *n*-hexane-EtOAc (80:20; 50:50; 0:100) to yield four sub-fractions (Fr. 1-Fr. 4). Fr. 2 (1.4 g) was re-crystallized with $CHCl_3$-MeOH (1:1) to yield **2** (5.1 mg). Fr. 3 (1.2 g) was further purified with $CHCl_3$-MeOH (95:0.5) using a NP-OP to obtain **1** (5.4 mg).

2.6. Statistical analysis

The data are expressed as mean ± standard deviation (n=3). The statistical analyses were performed using GraphPad Prism 9.3.1 (San Diego, CA, USA).

3. Results and discussion

3.1. Antioxidant activity of the extracts

3.1.1. Total polyphenolic, flavonoid contents and ferric reducing antioxidant power assay:

TPC was determined by the FC method constructing a calibration curve ($y = 0.0671x + 0.0245$, $R^2 = 0.9998$) based on different concentrations of gallic acid. The aqueous extract had the highest TPC value (263.34 ± 6.83 mg GAE/g), while the *n*-hexane had the lowest one (27.59 ± 1.52 mg GAE/g).

TFC was calculated from the regression equation of the calibration curve (y = 0.0079x-

0.0266, R^2 = 0.9968) and expressed as mg QE per gram of sample in dry weight (mg/g). By using GraphPad calculation, the *p*-value of an intercept (0.063735) is statistically non-significant. Therefore, the equation becomes y= 0.0079x. The results showed that the EtOAc extract had the highest TFC with a value of 19.7 ± 0.50 mg QE/g, followed by aqueous, MeOH, and *n*-hexane extracts, respectively. Meanwhile, the lowest TFC value was *n*-BuOH extract (0.78 ± 0.13 mg QE/g).

FRAP was determined by the calibration curve of ascorbic acid (y = 0.009x + 0.1764, R^2 = 0.999), expressed as ascorbic acid equivalents (mg AAE/g) of the dried sample. The highest value of FRAP was 307.26 ± 19.23 mg AAE/g for *n*-BuOH extract, and the lowest 29.48 ± 4.57 mg AAE/g was for *n*-hexane extract (Fig. 1 and Table 1).

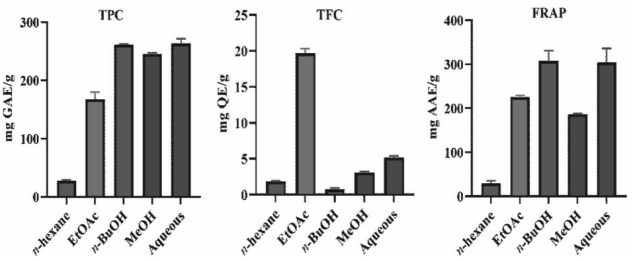

Fig. 1. TPC, TFC and FRAP of the extracts

Table 1. TPC, TFC and FRAP values of the extracts

Extracts	TPC ± SD (mg GAE/g)	TFC ± SD (mg QE/g)	FRAP ± SD (µg/mL)
n-hexane	27.59 ± 1.52	1.88 ± 0.08	29.48 ± 4.57
EtOAc	167.36 ± 10	19.7 ± 0.50	224.86 ± 3.45
n-BuOH	261.35 ± 0.98	0.78 ± 0.13	307.26 ± 19.23
MeOH	245.15 ± 1.52	3.12 ± 0.11	186.52 ± 1.05
Aqueous	263.34 ± 6.83	5.19 ± 0.16	304.30 ± 25.75
Data were presented as the mean ± SD (n=3).			

3.1.2. DPPH and ABTS radical scavenging assays:

The antioxidant potential using DPPH assay was found to be relatively strong compared to ascorbic acid with an IC_{50} 3.51 µg/mL (1 µg/mL ≈ 5.68 µM). The free radical scavenging ability was in the following order aqueous extract (IC_{50} 9.57 ± 0.21 µg/mL) > MeOH extract (IC_{50} 10.05 ± 0.07 µg/mL) > *n*-BuOH extract (IC_{50} 11.52 ± 0.65 µg/mL) > EtOAc extract (IC_{50} 45.32 ± 0.21 µg/mL). However, the *n*-hexane extract expressed a very poor antioxidant activity with a value of IC_{50} more than 100 µg/mL.

Similarly to DPPH assay, the results of the ABTS radical scavenging assay can be ranked as aqueous extract (IC_{50} 3.6 ± 0.15 µg/mL) > MeOH extract (IC_{50} 4.21 ± 0.09 µg/mL) > *n*-BuOH extract (IC_{50} 4.88 ± 0.25 µg/mL) > EtOAc extract (IC_{50} 5.49 ± 0.07 µg/mL) > *n*-hexane extract (IC_{50} >25 µg/mL) compared to ascorbic acid (1.61 ± 0.01 µg/mL) (1 µg/mL ≈ 5.68 µM). Meanwhile, *n*-hexane extract revealed the lowest antioxidant activity (Fig. 2 and Table 2).

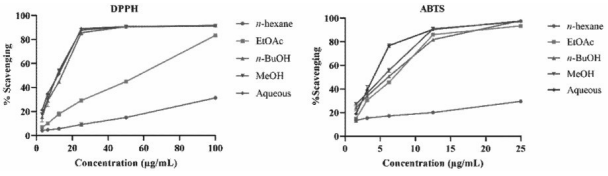

Fig. 2. DPPH and ABTS free radical inhibiting activities (%) of the extracts

Table 2. The DPPH and ABTS assays of the extracts

Extracts	IC_{50} ± SD (µg/mL)	IC_{50} ± SD (µg/mL)
n-hexane	>100	>25
EtOAc	45.32 ± 0.21	5.49 ± 0.07
n-BuOH	11.52 ± 0.65	4.88 ± 0.25
MeOH	10.05 ± 0.07	4.21 ± 0.09
Aqueous	9.57 ± 0.21	3.6 ± 0.15
Ascorbic acid*	3.51 ± 0.10	1.61 ± 0.01
Data were presented as the mean ± SD (n=3). *positive control		

Pearson's correlation coefficient

Due to the low activity of *n*-hexane extract, its IC_{50} values were not calculated. The relationships between antioxidant activities (FRAP, ABTS, and DPPH assays) and the TPC and TFC of the EtOAc, *n*-butanol, aqueous, and MeOH extracts were evaluated using the Pearson method (Table 3). The TPC had strong negative correlations with antioxidant results observed for DPPH (r=-0.969, p<0.01) and ABTS (r=-0.728, p<0.01). Thus, the antioxidant activity is characterized by the presence of phenolic compounds. However, TFC showed strong positive correlations for ABTS and DPPH. Therefore, the flavonoids with a certain structure and the position of -OH group are responsible for antioxidant activity.

Table 3. Pearson's correlation coefficient of TPC and TFC with antioxidant activities

	TPC	TFC	FRAP	ABTS	DPPH
TPC	1	-	-	-	-
TFC	-0.944**	1	-	-	-
FRAP	0.465^{ns}	-0.333^{ns}	1	-	-
ABTS	-0.728**	0.605*	-0.191^{ns}	1	-
DPPH	-0.969****	0.966****	-0.317^{ns}	0.778**	1

ns: not significant ; **, **, *: significant at the 0.0001, 0.01 and 0.05 levels (2-tailed), respectively**

3.2. Anti-inflammatory activity

The anti-inflammatory activity of all tested extracts was indicated by the inhibition of protein denaturation assay. The inhibition of the extracts was relatively moderate compared to its diclofenac sodium at 125 µg/mL (71.76%). In detail, percentage inhibition BSA denaturation was ranked as *n*-hexane extract (57.11%) > aqueous extract (49.80%) > *n*-BuOH extract (31.94%) > MeOH extract (27.96%) > EtOAc extract (15.63%) (Fig. 3 and Table 4).

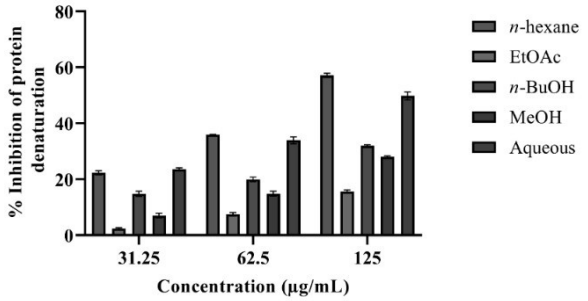

Fig. 3. BSA denaturation inhibition of the extracts

Table 4. Percentage BSA denaturation inhibition of the extracts

Extracts (% inhibition)	Concentration (µg/mL)		
	125	**62.5**	**31.25**
***n*-hexane**	57.11±0.53	35.98±0.02	22.32±0.51
EtOAc	15.63±0.42	7.47±0.53	2.38±0.28
***n*-BuOH**	31.94±0.36	19.95±0.64	14.79±0.75
MeOH	27.95±0.31	14.83±0.72	6.99±0.70
Aqueous	49.80±1.41	33.94±1.23	23.56±0.56
Diclofenac sodium*	71.76±0.43	44.37±0.8	27.84±0.83

Data were presented as the mean ± SD (n=3). *positive control

*3.3. The elucidation of isolated compounds **1-2***

To find anti-inflammatory secondary metabolites, the *n*-hexane extract was selected for isolation. Two known compounds including stigmasterol-3-O-*β*-D-glucopyranoside (**1**) and stigmasterol (**2**) were isolated from the *n*-hexane extract of *O. integerrima* stem. The structures of isolated compounds (**1-2**) were determined by comparing their spectroscopic data with those reported in the literature (Fig. 4). Previously, these steroids **1-2** were known to be active against inflammation [20],[21]. It indicated that the *n*-hexane extract potentially had anti-inflammatory property.

Compound **1**: White powder; LR-ESI-MS *m/z* $[M-H-H_2O]^-$ 555; ^{1}H NMR [600 MHz, $CDCl_3$ &CD_3OD] data showed the typical signals of six methyl groups at δ_H 0.67 (3H, s, H-18), 0.99 (3H, s, H-19), 0.91 (3H, d, J= 6.6, H-21), 0.79 (3H, m, overlapped, H-26), 0.82 (3H, m, overlapped, H-27), 0.83 (3H, m, overlapped, H-29), three olefinic resonances at δ_H 5.34 (1H, br, H-6), 5.13 (1H, dd, J= 9.0, 15.6 Hz, H-22), 5.00 (1H, dd, J= 9.0, 15.6 Hz, H-23), suggesting a stigmastane-type steroid. One anomeric proton at δ_H 4.37 (1H, d, J= 7.8; H-1') and the oxygenated methylene and methines of H-2′ to H-6′ were assigned to a monosaccharide unit. These corresponded to the ^{13}C-NMR data. It revealed the resonances of 35 carbons consisting of the olefinic carbons at δ_C 121.8 (C-6), 138.3 (C-22), 129.2 (C-23), a quarternary carbon at δ_C 140.4 (C-5), a signal of one glucose unit. Based on the data above and comparison with previous publications [22],[23], compound **1** was named stigmasterol-3-O-*β*-D-glucopyranoside ($C_{35}H_{58}O_6$).

^{1}H NMR [600 MHz, $CDCl_3$ &CD_3OD]: 1.83 (2H, m, H-1), 1.50, 1.63 (2H, m, H-2), 3.83 (1H, m, H-3), 2.38, 1.83 (2H, m, H-4), 5.34 (1H, br, H-6), 1.85; 1.49 (2H, m, H-7), 1.51 (1H, m, H-8), 1.48 (1H, m, H-9), 1.25, 1.48 (2H, m, H-11), 1.94, 1.14 (1H, m, H-12), 1.50 (1H, m, H-14), 1.94 (2H, m, H-15), 1.89 (2H, m, H-16), 1.03 (1H, m, H-17), 0.67 (3H, s, H-18), 0.99 (3H, s, H-19), 1.98 (1H, m, H-20), 0.91 (3H, d, J= 6.6, H-21), 5.13 (1H, dd, J= 9.0, 15.6 Hz, H-22), 5.00 (1H, dd, J= 9.0, 15.6 Hz, H-23), 2.24 (1H, m, H-24), 2.01 (1H, m, H-25), 0.79 (3H, m, overlapped, H-26), 0.82 (3H, m, overlapped, H-27), 1.46 (2H, m, H-28), 0.83 (3H, m, overlapped, H-29), 4.37 (1H, d, J= 7.8; H-1'), 3.17-3.70 (1H, m, H-2'- H-6').

^{13}C NMR [150 MHz, $CDCl_3$ &CD_3OD]: δ_C 37.2 (C-1), 29.4 (C-2), 78.8 (C-3), 39.7 (C-4), 140.4 (C-5), 121.8 (C-6), 31.8 (C-7), 31.7 (C-8), 50.2 (C-9), 36.6 (C-10), 20.9 (C-11), 39.7 (C-12), 42.2 (C-13), 56.7 (C-14), 24.1 (C-15), 29.0 (C-16), 56.0 (C-17), 11.5 (C-18), 19.3 (C-19), 40.5 (C-20), 18.9 (C-21), 138.3 (C-22), 129.2 (C-23), 51.3 (C-24), 31.8 (C-25), 20.9 (C-26), 19.3 (C-27), 25.9 (C-28), 11.5 (C-29), 101.1 (C-1'), 73.6 (C-2'), 76.6 (C-3'), 70.2 (C-4'), 76.2 (C-5'), 61.6 (C-6').

Compound **2**: White crystal; ν_{max} (KBr) cm^{-1}: 3435 (OH), 2930 (aliphatic, CH), 1626 (C=C), 1457 (cyclic, CH_2), 1053 (C-O). HR-ESI-MS m/z 435.3627 [M + Na]$^+$ (calcd. for $C_{29}H_{48}ONa$, 435.3603). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.53 (1H, m, H-3), 5.35 (1H, m, H-6), 0.70 (3H, s, H-18), 1.01 (3H, s, H-19), 0.94 (3H, d, J= 7.0 Hz, H-21), 5.16 (1H, dd, J= 8.8 and 15.2 Hz, H-22), 5.02 (1H, dd, J= 8.8, 15.2 Hz, H-23), 0.77 (3H, d, J= 7.6 Hz, H-26), 0.85 (3H, d, J= 7.6 Hz, H-27), 0.81 (3H, t, J= 7.2 Hz, H-29). NMR data of **2** was similar to that of **1**, except for the lack of sugar moiety. Compound **2** was assigned as stigmasterol by comparing with published data [22],[24].

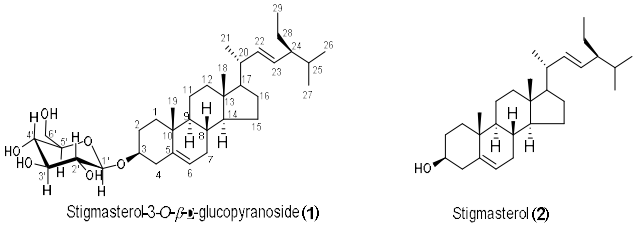

Fig. 4. Structures of isolated compounds **1** and **2**

4. Conclusion

All the extracts (EtOAc, *n*-butanol, aqueous, and MeOH extracts), except for the *n*-hexane extract showed significant antioxidant activity using ABTS and DPPH radical scavenging with IC_{50} values ranging from 3.6 ± 0.15 to 5.49 ± 0.07 μg/mL and 9.57 ± 0.21 to 45.32 ± 0.21 μg/mL, respectively. Moreover, the *n*-BuOH extract had a high TPC value (261.35 ± 0.98 mg GAE/g). The pattern of *n*-BuOH extract using FRAP assay (307.26 ± 19.23 mg AAE/g) was similar to that of TPC. The highest TFC value (19.7 ± 0.50 mg QE/g) was EtOAc extract. Regarding anti-inflammatory activity, the non-polar extract (*n*-hexane extract) displayed the strongest activities using protein denaturation assay. To the best of our knowledge, the anti-inflammatory activity of *O. intergerrima* extracts was analyzed for the first time. Taken together, the polar extracts of *O. integerrima* species were good candidates for antioxidant activity while the non-polar extract favored anti-inflammatory activity. The two compounds stigmasterol-3-O-*β*-D-glucopyranoside (**1**) and stigmasterol (**2**) were isolated from *n*-hexane extract to support its anti-inflammatory effect. Moreover, the other fractions should be performed to find further anti-inflammatory secondary metabolites.

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REGULATORY POTENTIAL OF RHIZOME EXTRACT FROM *PARIS POLYPHYLLA* VAR. *YUNANENSIS* ON PRO-APOPTOTIC PROTEINS IN A549 LUNG CANCER CELLS

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Summary

Regulatory Potential of Rhizome Extract from *Paris polyphylla* var. *yunanensis* on Pro-Apoptotic Proteins in A549 Lung Cancer Cells

Paris polyphylla var. *yunanensis* has been shown to affect against various types of cancer cells. The aim of this study was to investigate the effects of 80% ethanol extract from *Paris polyphylla* var. *yunanensis* rhizomes (PPRE) on cell proliferation and apoptosis in a non-small cell lung cancer cell line (A549) *in vitro via* expression of apoptosis-related proteins using western blot. Our result showed that PPRE at 2.5, 5, and 10 µg/mL concentrations significantly inhibited A549 cell proliferation in a dose-dependent manner after 24 and 48 h of treatment. Furthermore, A549 cells were treated with the PPRE at the concentrations of 4.5, 5.0, and 5.5 µg/mL for 24 h showed markedly higher Bcl-2-associated X (Bax) protein expression levels, significantly lower Bcl-2 expression levels, and obviously notably higher Bax/Bcl-2 ratio than the control. Meanwhile, the p53 protein expression levels were not significantly reduced compared to the control by the PPRE. The findings of this study implicate that *Paris polyphylla* var. *yunanensis* rhizome extract may be a potential candidate to inhibit the proliferation of A549 cells and promote apoptosis by the Bcl-2/Bax pathways. Studying the effect of PPRE on some other molecular markers involved in programmed cell death should be done to elucidate the mechanism of A549 human lung cancer cell apoptosis of the PPRE.

Keywords: *Paris polyphylla* var. *yunanensis*, *Rhizome extract, Apoptosis, Cytotoxicity, A549.*

1. Introduction

Apoptosis is the process of programmed cell death in which cells die in response to self-generated signals. This process is momentous for cellular homeostasis and plays an important role in manipulation in cancer prevention [1]. Apoptosis is a complex mechanism and involves multiple pathways including caspase activation, altered mitochondrial permeability, pro- and anti-apoptotic protein balance, and enhanced p53 protein expression. Among them, the mitochondrial-dependent intrinsic apoptosis pathway plays an important role, especially the persistence of Bcl-2 family members. The Bcl-2 family proteins are further subdivided into anti-apoptotic proteins such as Bcl-XL, Bcl-w, Mcl-1 and pro-apoptotic proteins such as Bax, Bak and Bok. The activation of Bax and Bak regulates cytochrome c to cytosol release from mitochondria through mitochondrial outer membrane permeabilization [2]. Released cytochrome c induces apoptosis by activating the caspase terminal agent. On the other hand, p53 plays an important role in the mechanism of apoptosis in cancer and activates both intrinsic pathways (Bcl-2, Puma, and Noxa protein families) and extrinsic pathways of a programmatically dead process. Thus, upregulation of p53 expression could be an effective way to treat cancer through the induction of apoptosis. Taken together, targeting the induction of cancer cell death by apoptosis may have potential in cancer therapy.

Lung cancer is increasingly common worldwide with increasing rates of poor prognosis and mortality [3]. According to World Cancer Research Fund International, there were more than 2.2 million new cases and almost 1.8