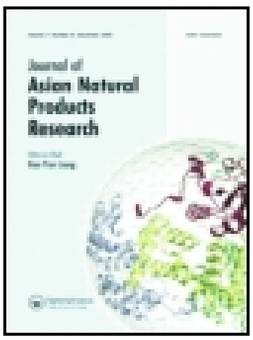

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# Bioactive compounds from *Physalis angulata* and their anti-inflammatory and cytotoxic activities

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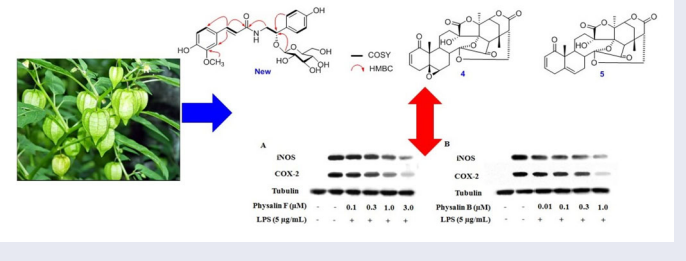
**ABSTRACT**

A new compound, physalucoside A (**1**), together with seven withanolides (**2**–**8**) and three flavonoids (**9**–**11**), were isolated from *Physalis angulata* L. (Solanaceae), a medicinal plant native to Vietnam. The chemical structures of these compounds were elucidated by one- and two-dimensional NMR spectra, high-resolution electrospray ionization mass spectrometry analyses, and chemical reactivity. The anti-inflammatory and cytotoxic activities of isolated compounds were also evaluated. These data suggest that the anti-inflammatory activity of *P. angulata* is due primarily to its withanolide content. This study demonstrates the potential of withanolides as promising candidates for the development of new anti-inflammatory drugs.

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## 1. Introduction

Inflammatory diseases and disorders include autoimmune diseases, cancers, metabolic syndromes, and cardiovascular diseases. Diverse pro-inflammatory cytokines and mediators, such as interleukin 1$\beta$, tumor necrosis factor $\alpha$, interleukin 6, prostaglandins, and nitric oxide (NO), are released mainly by activated macrophages [1] in response to exogenous stress or endogenous signals including external pathogens and damage-associated molecular patterns [2]. The inhibition of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) plays an important role in many inflammatory disease therapies. Hence, selective iNOS and COX-2 inhibitors are potential candidates for new therapeutic agents against inflammation.

The plant *Physalis angulata* L. belongs to the genus *Physalis* (Solanaceae), which includes approximately 120 species distributed primarily within South and North America, with a few species found in Europe and southeastern and central Asia [3]. In Vietnam, four species of *Physalis* are identified with the local name Tam Bop [4]. It is used widely in Vietnamese folk medicines for treating fever, cough, vomiting, hiccups, and diabetes. *Physalis* fruit is eaten in China, Korea, Japan, and Vietnam for its high levels of vitamin C, vitamin B1, and pro-vitamin A [5]. The fruits are also used to prevent urinary tract infections, kidney inflammation, and gout. Interestingly, the branches, fruits, or leaves of *P. angulata* are used as one of the ingredients in a traditional anti-cancer (cervical, throat, lung, and colon) medicine that also includes *Atractylodes macrocephala, Platycodon grandiflorus, Ophiopogon japonicas, Scruphularia buergeriana, Scutellaria baicalensis,* and *Glycyrrhiza uralensis* [4]. As with other *Physalis* sp., *P. angulata* produces withanolide triterpenoids containing a steroidal lactone possessing 28 carbon atoms [6,7]. These compounds have exhibited antimicrobial, anticancer, anti-inflammatory, hepatoprotective, immunomodulatory, and insect-repelling properties [8–11].

In our anti-inflammatory screening program evaluating Vietnamese medicinal and natural plants, an ethanol extract of *P. angulata* exhibited significant inhibition of NO production. This report describes the isolation and structural elucidation of a new alkaloid, physalucoside A (**1**), from the branches of *P. angulata*, along with 10 known compounds comprising withanolides (**2–8**) and flavonoids (**9–11**). The anti-inflammatory and cytotoxic activities of these compounds were also investigated. Moreover, their effects on protein expression were determined by Western blot analysis.

## 2. Results and discussion

Bioassay-guided separation of the MeOH extract of *P. angulata* resulted in the isolation and structural elucidation of eleven compounds (**1**–**11**) (Scheme S1, and Extraction and isolation), including new compound **1**. The known compounds were identified as physagulin B (**2**), (20*S*,22*R*)-15$\alpha$-acetoxy-5$\alpha$-chloro-6$\beta$,14$\beta$-dihydroxy-1-oxowitha-2,24-dienonide (**3**), physalin F (**4**), physalin B (**5**), physalin G (**6**), and physagulide Q (**7**), physagulin D (**8**) [6,12–15], and three flavonoid glucosides as quercetin 3-*O*-$\beta$-rutinoside (**9**), kaempferol 3-*O*-$\beta$-rutinoside (**10**), and kaempferol 3-*O*-$\beta$-rutinoside-7-*O*-$\beta$-D-glucopyranoside (**11**) [16] by comparison of their NMR spectroscopic data to those previously reported values.

**Table 1.** $^{1}H$ and $^{13}C$ NMR spectral data of new compound **1**.

| No. | $\delta_C$[a,b] | $\delta_H$[a,c] (mult., *J* in Hz) |
|---|---|---|
| 1 | 128.0 | – |
| 2 | 111.7 | 7.03 (d, *J* = 1.5 Hz) |
| 3 | 149.3 | – |
| 4 | 149.9 | – |
| 5 | 116.5 | 6.69 (d, *J* = 8.5 Hz) |
| 6 | 123.3 | 6.93 (dd, *J* = 1.5, 8.5 Hz) |
| 7 | 142.4 | 7.34 (d, *J* = 15.5 Hz) |
| 8 | 118.6 | 6.32 (d, *J* = 15.5 Hz) |
| 9 | 169.3 | – |
| 1′ | 132.3 | – |
| 2′ | 129.2 | 7.15 (d, *J* = 8.5 Hz) |
| 3′ | 115.9 | 6.65 (d, *J* = 8.5 Hz) |
| 4′ | 158.2 | – |
| 5′ | 115.9 | 6.65 (d, *J* = 8.5 Hz) |
| 6′ | 129.2 | 7.15 (d, *J* = 8.5 Hz) |
| 7′ | 81.8 | 4.75-4.77 (m)* |
| 8′ | 47.7 | 3.46 (dd, *J* = 7.0, 13.5 Hz)<br>3.63 (ddd, *J* = 5.5, 13.5 Hz) |
| 3-OMe | 56.4 | 3.79 (s) |
| 1″ | 104.5 | 4.40 (d, *J* = 7.5 Hz) |
| 2″ | 75.5 | 3.20-3.22 (m) |
| 3″ | 78.1 | 3.25 (d, *J* = 9.0 Hz) |
| 4″ | 71.5 | 3.18 (dd, *J* = 5.0, 9.0 Hz) |
| 5″ | 77.9 | 3.09 (dd, *J* = 2.5, 5.5 Hz) |
| 6″ | 62.6 | 3.46-3.48 (m)/3.60-3.62 (m) |

[a]Measured in $CD_3OD$.
[b]125 MHz.
[c]500 MHz.
*This assignment may be interchanged.

Compound **1** was obtained as a white amorphous powder and showed a positive reaction with Dragendorff's reagent. The molecular formula of **1** was $C_{24}H_{29}NO_{10}$, based on positive-mode high-resolution electrospray ionization mass spectrometry, which yielded a protonated molecular ion at *m/z* 492.1838 $[M+H]^+$. Infrared absorption spectrum indicated the presence of hydroxyl (3380 $cm^{-1}$), carboxylic acid (1635 $cm^{-1}$), and aromatic ring (1,60,51,520 $cm^{-1}$) moieties. The $^{1}H$-NMR spectrum of **1** indicated the presence of an ABX proton system with peaks at $\delta$ 7.03 (1H, d, *J* = 1.5 Hz, H-2), 6.69 (1H, d, *J* = 8.5 Hz, H-5), and 6.93 (1H, dd, *J* = 1.5, 8.5 Hz, H-6). An AABB system was also evident, with peaks at $\delta$ 7.15 (2H, d, *J* = 8.5 Hz, H-2′, H-6′) and 6.65 (1H, d, *J* = 8.5 Hz, H-3′, H-5′) indicating the presence of a 1,3,4-trisubstituted aromatic system and a *p*-substituted aromatic ring, respectively. Two doublets proton signals at $\delta$ 7.34 (1H, d, *J* = 15.5 Hz, H-7) and 6.32 (1H, d, *J* = 15.5 Hz, H-8) indicated the presence of a *trans*-substituted double bond. Additional signals indicated the presence of an oxygenated methine proton at $\delta$ 4.75-4.77 (1H, m, H-7′), methylene protons at $\delta$ 3.46 (1H, dd, *J* = 7.0, 13.5 Hz, H-8′a) and 3.63 (1H, dd, *J* = 5.5, 13.5 Hz, H-8′b), a methoxyl at $\delta$ 3.79 (3H, s), in addition to a glucosyl anomeric proton at 4.40 (1H, d, *J* = 7.5 Hz, H-1″). $^{13}C$-NMR and DEPT spectra of **1** revealed 24 carbon signals corresponding to 12 aromatic carbon atoms (for two aromatic rings), one oxymethine group at $\delta$ 81.8 (C-7′), one methylene group at 47.7 (C-8′), one double bond at $\delta$ 142.4 (C-7) and 118.6 (C-8), one carbonyl at $\delta$ 169.3 (C-9), one methoxyl group at $\delta$ 56.4 (3-$OCH_3$), and six carbon atoms belonging to a glucosyl moiety at $\delta$ 62.6 to $\delta$ 78.1 (Table 1). These spectroscopic data suggest that **1**

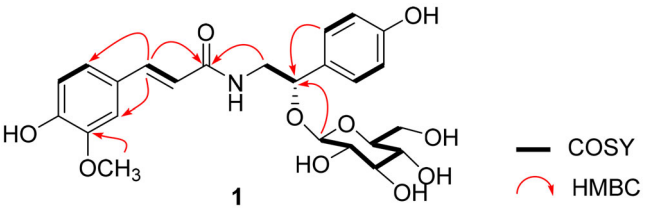


**Figure 1.** Selected HMBC and COSY correlations of **1**.

contains a feruloyl group and a 2-amino ethanoyl side chain, indicating that the main skeleton is cinnamic acid amide with a glycoside moiety. Acid hydrolysis of **1** in 1 N methanolic hydrochloric acid (HCl), together with the observed coupling constant of the anomeric proton ($J = 7.6$ Hz), confirmed the presence of D-(+)-glucose. Further confirmation was provided by direct comparisons with a reference sample in co-spotted (co)-TLC and GC analyses (Supplementary data). COSY, HSQC, and HMBC spectra confirmed the planar structure of **1** (Figure 1). The COSY spectrum revealed couplings between the oxymethine proton H-7′ and the two nitrogenated methylene protons H-8′, and between the two olefinic protons H-7 and H-8. The HMBC spectrum of **1** showed long-range correlations between H-7 and the carbonyl carbon C-9 and methine carbons C-2 and C-6, indicating the presence of a feruloyl group. Interestingly, long-range correlations between H-8′ protons and the carbonyl carbon C-9 and oxygenated methine carbon C-7′ confirmed the presence of a 2-hydroxy-2-(4-hydroxyphenyl) ethyl amine group (octopamine) linked to C-9 of the ferulic unit. A single methoxyl group at C-3 was evidenced by an HMBC correlation between $\delta_H$ 3.79 and $\delta_C$ 150.9 (C-3). HMBC correlations between $\delta_H$ 4.40 (H-1″) and $\delta_C$ 81.8 indicated that the glucopyranosyl unit was linked to the oxygen atom at C-7′. In addition, the optical rotation value of aglycon of **1** was determined as negative ($\alpha_D$ −3.0), indicating the absolute configuration of *S* at the chiral center C-7′ [17]. Based on all of the above evidence, the structure of **1** was characterized as physalucoside A.

NO is a key mediator of inflammatory responses and pathogenesis. The inhibition of NO by each of the isolated compounds (**1**-**11**) was evaluated in RAW246.7 cells by measuring NO production after LPS stimulation (1 $\mu$g/ml). The data in Table 2 show that physagulin B (**2**), (20*S*,22*R*)-15$\alpha$-acetoxy-5$\alpha$-chloro-6$\beta$,14$\beta$-dihydroxy-1-oxowitha-2,24-dienonide (**3**), physalin B (**5**), and physagunin R (**7**) strongly inhibited NO production, with $IC_{50}$ values less than 1.0 $\mu$M, followed by physalin F (**4**, $IC_{50}$ 1.06 ± 0.68 $\mu$M) and physalin G (**6**, $IC_{50}$ 3.74 ± 0.29 $\mu$M). The new compound, physalucoside A, also exhibited significant inhibitory activity, with an $IC_{50}$ value of 2.69 ± 0.17 $\mu$M, and the isolated flavonoid glucosides showed weak inhibition, with $IC_{50}$ values greater than 30 $\mu$M. The positive control, cardamonin, showed anti-NO production activity, with an $IC_{50}$ value of 1.17 ± 0.10 $\mu$M. These results indicate that withanolides play an active role in the anti-inflammatory activity of *P. angulata*.

Interestingly, all of the withanolide compounds (**1**–**7**) were good inhibitors of NO production. iNOS and COX-2 are two main targets typically evaluated in anti-inflammation studies [18]. Physalin F (**4**) and physalin B (**5**) were selected for use in our subsequent experiments. RAW 264.7 cells were pretreated with the indicated concentrations of physalin F (**4**) and physalin G (**5**) for 2 h and then incubated with LPS

**Table 2.** Inhibition of NO production in macrophage RAW264.7 cells by **1–11**.

| Compounds | $IC_{50}$ (μM)[a] |
|---|---|
| **1** | 2.69 ± 0.17 |
| **2** | 0.24 ± 0.09 |
| **3** | 0.68 ± 0.02 |
| **4** | 1.06 ± 0.68 |
| **5** | 0.28 ± 0.10 |
| **6** | 3.74 ± 0.29 |
| **7** | 0.57 ± 0.18 |
| **8** | 12.5 ± 1.70 |
| **9** | > 30 |
| **10** | > 30 |
| **11** | > 30 |
| Cardamonin[b] | 1.17 ± 0.10 |

[a]The inhibitory effects are represented as giving 50% inhibition ($IC_{50}$) relative to the vehicle control. The values are expressed as the means ± S.D. of three individual experiments.
[b]Positive control for NO production.

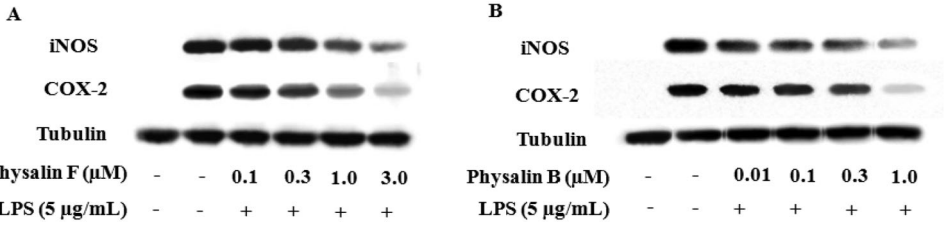


**Figure 2.** Effect of physalin F (**A**) and physalin B (**B**) on LPS-induced iNOS and COX-2 expressions in RAW 264.7 macrophages. Expressions of iNOS and COX-2 proteins were determined by Western blot analysis and tubulin was chosen as positive control.

(1 μg/ml) for 24 h. Equal amounts of total protein were subjected to SDS-PAGE, and the levels of iNOS and COX-2 proteins were determined by Western blot analyses. The data in Figure 2 show that iNOS and COX-2 protein and mRNA expression were almost undetectable in unstimulated (no LPS activation) RAW 264.7 cells. However, after LPS treatment, the protein levels of iNOS and COX-2 increased markedly. Pretreating the cells with physalin F (**4**, 0.1–3.0 μM) and physalin G (**5**, 0.01–0.3 μM) attenuated LPS-induced iNOS and COX-2 protein production in a concentration-dependent manner. These data suggest that both of these compounds, along with the other withanolides, may downregulate LPS-induced iNOS and COX-2 expression at the transcription level. These results are consistent with those of previous studies [19,20] and provide evidence for the anti-inflammatory properties of *P. angulata*. Furthermore, experiments to confirm the anti-inflammation mechanism of these compounds are now in process.

The potentially cytotoxic activities of compounds **1–11** were investigated using three human cancer cell lines: A-549 (human lung carcinoma cells), HeLa (human cervical cancer cells), and Panc-1 (human pancreatic cancer cells). Compounds **3**, **4**, **5,** and **6** exhibited particularly strong cytotoxic activities against A-549 cancer cell lines, with $IC_{50}$ values of 1.03 ± 0.09, 0.68 ± 0.05, 0.95 ± 0.04, and 6.88 ± 2.41 μM, respectively. These results are comparable with that for the positive control, etoposide ($IC_{50}$ 2.68 ± 0.89 μM) (Table 3). Therefore, the withanolide constituents (**3–6**) of *P. angulata* are potentially candidate anticancer therapeutic agents.

**Table 3.** Cytotoxic effect of selected compounds.

| Compounds | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | A-549 | Hela | Panc-1 |
| **3** | 1.03 ± 0.09 | 29.89 ± 1.15 | 11.53 ± 0.36 |
| **4** | 0.68 ± 0.05 | 0.23 ± 0.03 | >30 |
| **5** | 0.95 ± 0.04 | 13.84 ± 1.27 | 12.77 ± 1.07 |
| **6** | 6.88 ± 2.41 | >30 | >30 |
| Etoposide[a] | 2.68 ± 0.89 | 3.29 ± 0.05 | 0.08 ± 0.11 |

[a]Positive control. The values are presented as the means ± S.D (n = 3).

Natural products are a consistent source of active pharmacological compounds. Previously, withanolides isolated from *P. angulata* exhibited remarkable anti-inflammatory, antinociceptive, and anti-cancer activities [21,22]. In this research, 11 compounds (**1–11**), including the new compound physalucoside A, were isolated from a methanol extract of *P. angulata* via combined column chromatography. Compounds **3**, **4**, **5,** and **6** exhibited significant anti-inflammatory and cytotoxic effects. Among these, compounds **5** and **6** exhibited iNOS and COX-2 expression in Western blot analyses. Our results indicate that the withanolides in *P. angulata* may potentially be used to treat inflammation, cancer, and other related diseases.

## 3. Experimental

### 3.1. General experimental procedures

The optical rotation values were recorded with a JASCO P-1020 polarimeter (JASCO, Oklahoma, OK, US). FT-IR spectra were performed on a Bruker TENSOR 37 FT-IR spectrometer (Bruker, Billerica, MS, USA). 1 D and 2 D NMR spectra were carried out on a Bruker Avance III 500 spectrometers (MA, USA) with TMS as the internal standard. HR-ESI-MS results were afforded by Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent, Santa Clara, CA, USA). Silica gel (Kieselgel 60, 70-230, and 230-400 mesh, Merck, Darmstadt, Germany), YMC*GEL (ODS-A, 12 nm S-150 μm, YMC Co., Ltd., Kyoto, Japan), and Sephadex LH-20 (Sigma-Aldrich, MO, USA), and Diaion HP-20 (Supelco, PA, USA) were chosen for the column chromatography by similarly described [23]. All solvents were supplied by SK chemical Company, Korea. Sigma-Aldrich provided the chemical reagents, and tubulin antibodies while iNOS, and COX-2 were afforded from Cell Signaling Technology (Beverly, MA, USA).

### 3.2. Plant material

The plant material of *Physalis angulata* was obtained from Thai Binh province, Vietnam, in June 2018. Dr. Tran Thi Phuong Anh and Prof. Young Ho Kim taxonomically identified the plant species. A voucher specimen (QTB 16 2018) was stored at the herbarium of the Natural Product Laboratory, Chungnam National University.

### 3.3. Extraction and isolation

The dried and powdered form of whole plants of *P. angulata* (2.0 kg) were extracted by methanol at 50 °C using ultrasonic for three times (4.0 L each). After removal of

the solvent, the methanol extract (90 g) was suspended in distilled water (1.5 L) and successively partitioned with dichloromethane and ethyl acetate (three times, 1.5 L each) to give corresponding soluble extracts, dichloromethane (PAD, 24.5 g), ethyl acetate (PAE, 4.6 g), and water-soluble layers (PAW). The PAD fraction was then subjected through a silica gel column and eluted with dichloromethane/methanol (0%, 25%, 50%, 75%, and 100% volume of methanol, each 1.5 L, stepwise) to give five fractions PAD1 - PAD5. Fraction PAD3 (1.8 g) was chromatographed on a silica gel column, eluting with dichloromethane/methanol (15:1) to give five sub-fractions PAD3.1-PDA3.5. Fraction PAD3.2 was purified on an RP-18 reverse phase column and eluted with methanol/water (1:1) to yield compound **6** (5.3 mg). Fraction PAD3.3 was further separated on a silica gel column, eluting with dichloromethane/acetone (15:1 and 15:2) to obtain compounds **1** (5.6 mg) and **2** (6.5 mg). Sub-fraction PAD3.5 was further chromatographed on a silica gel column, eluting with dichloromethane/methanol (10:1, and 10:2), and then further purified on a silica gel column, eluting with dichloromethane/acetone (8:1) to give compounds **4** (12.0 mg) and **5** (10.7 mg). Fraction PAD5 (4.5 g) was separated on an RP18 reversed phase column using methanol/water (2:1) to give three smaller fractions (PAD5.1 - PAD5.3). Fraction PAD5.2 was purified on a silica gel column using dichloromethane/acetone (6:1 and 3:1) to yield **3** (7.0 mg) and **7** (4.5 mg). The water-soluble layers were passed through the Diaion column HP-20, washed with distilled water, then eluted with increasing volume of methanol in water (25%, 50%, 75%, and 100% of methanol) to obtain four fractions PAW1 - PAW4. The fraction PAW2 (3.8 g) was chromatographed on a silica gel column, eluting with dichloromethane/methanol/water (3:1:0.01) to yield two sub-fractions PAW 2.1 and PAW2.2. The sub-fractions PAW2.2 (0.8 g) was continuously separated on an RP18 reversed phase column using methanol/water (1:2); then further purified on a Sephadex LH-20 column, eluting with methanol/water (3:1) to yield compound **8** (10 mg). Fraction PAW4 (3.1 g) was subjected to a silica gel column and eluted with dichloromethane/methanol/water (2:1:0.01) to give two smaller fractions PAW4.1 and PAW4.2. Fraction PAW4.1 (1.1 g) was passed through an RP18 reversed phase column using acetone/water (1:2); and then purified on a silica gel column, eluting with ethyl acetate/methanol/water (3:1:0.05) to give compounds **9** (6.2 mg) and **10** (7.5 mg). Fraction PAW4.2 (0.6 g) was purified on a silica gel column using dichloromethane/methanol/water (2:1:0.01) as eluent to afford compound **11** (10.1 mg).

#### 3.3.1. Physalucoside A (1)

White amorphous powder; $[\alpha]_D^{20}$ −0.080 (c 0.25, $CHCl_3$); IR (KBr) $\nu_{max}$ 3380, 1635, 1605, and 1520 $cm^{-1}$; $^1H$ (500 MHz in $CD_3OD$) and $^{13}C$-NMR (125 MHz in $CD_3OD$) spectral data, see Table 1; HR-ESI-MS: *m/z* 492.1838 $[M+H]^+$ (calcd for $C_{24}H_{30}NO_{10}$, 492.1870).

### 3.4. Anti-inflammatory effects

The anti-inflammatory assay was carried out by previously reported protocol with small modification [24]. RAW264.7 cells were obtained from American Type Culture Collection (ATCC, TIB-71, Rockville, MD, USA) and cultured in DMEM

supplemented with 10% fetal bovine serum (FBS, 16000-044, Gibco, Grand Island, NY, USA) and 100 U/ml penicillin-streptomycin (Gibco) at 37 °C in incubator containing 5% $CO_2$. Cells were plated at $5 \times 10^4$ cells/well in 96-well culture plates and then were incubated with compounds for 1 h before LPS (0.1 μg/ml) stimulation for 18 h. Cell supernatant (100 μl) was mixed with an equal amount of Griess reagent consisting of 1% sulfanilamide (Sigma, St. Louis, MO, USA) and 0.1% N-(1-naphthyl)ethylenediamine (Sigma) in 5% phosphoric acid. Its absorbance was measured at 550 nm using a microplate reader (Varioskan LUX, Thermo Fisher Scientific Inc., Waltham, MA, USA).

### 3.5. Cytotoxic activity

Cytotoxic activity was performed using MTT assay by previously reported method [25]. Cell viability was evaluated by the MTT assay. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) was added to the cells for 2 h. The supernatant was removed and then the MTT formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Sigma). Absorbance was recorded at 570 nm using a microplate reader (Thermo Fisher Scientific Inc.).

### 3.6. Western blotting analysis

According to our previous protocol with minor modification, Western blot analysis was carried out to identify iNOS, and COX-2 protein expression in the RAW264.7 cells [18,23]. Briefly, RAW264.7 cells ($2 \times 10^6$ cells/well) were treated with LPS (0.1 μg/ml) for 16 h after pre-incubated with compounds **4** and **5** for 1 h. The cells lysate was extracted using Cell Lysis Buffer (Cell Signaling Technology) according to the manufacturer's protocol.

### 3.7. Statistical analysis

The results were calculated by using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). The significant difference between compounds treated group and LPS alone group was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test ($^*p < 0.05$).

## Disclosure statement

No potential conflict of interest was reported by the authors.

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