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Anti-inflammatory activity in RAW 264.7 cells by standardized extracts and the isolated compound from *Glinus oppositifolius*

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Abstract
Glinus oppositifolius (GO) is an endemic herbaceous plant found in tropical Asian countries and is native in Vietnam. In this study, the effects of two GO standardized extracts (**Biovip** and **Tox-off**) and their major isolates were evaluated on the anti-inflammatory activity by determining the expression of PGE2 production and COX-2 mRNA in RAW 264.7 cells. We observed the COX-2 mRNA expression, as well as PGE2 production was significantly inhibited by **Biovip** and **Tox-off**. Moreover, five isolated compounds: **TRA-GO1** to **TRA-GO5** showed the inhibition effects on both COX-2 mRNA expression and PGE2 production. Among them, three compounds (**TRA-GO1**, **TRA-GO2**, and **TRA-GO5**) showed the most potent effect. Mechanistically, **Biovip** and **Tox-off**, as well as the major isolated compounds, suppressed NF-κB activation and TNF-α production in a dose-dependent manner in RAW 264.7 cells. Taken together, our results demonstrated that the anti-inflammatory activity of **Biovip**, **Tox-off**, and the major isolated compounds exhibit attenuation of LPS-induced inflammatory response: COX-2 protein, PGE2 production, and NF-κB, TNF-α expression in RAW 264.7 cells.

Keywords: Glinus oppositifolius; **Tox-off** and **Biovip**; Traphanoside GO1 (**TRA-GO1**), Anti-inflammation, COX-2, PGE2, NF-κB, TNF-α.

I. INTRODUCTION

Glinus oppositifolius L. Aug. DC. (Molluginaceae), also known as "Rau đắng đất" in Vietnamese, is a characteristic herbal plant mainly found in tropical Asian countries and is native in Vietnam. It has been traditionally used to treat hepatitis, inflammation, fever, and wounds [1]. People in African, Indian, and Filipino communities consume this bitter herb, which is high in iron and calcium, as part of their daily diet [1]. Recent studies have identified triterpenoid saponins as the main bioactive compounds in *G. oppositifolius* [2, 3], along with pectin polysaccharides [4], flavonoids [5], and steroids [6]. It has potential antioxidant [3], hepatoprotective [4], antiprotozoal [2], and immunomodulating effects [7]. Our recent study demonstrated that traphanoside GO1 (**TRA-GO1**), a triterpenoid saponin from *G. oppositifolius*, suppressed the production of PGE2 induced by LPS in HepG2 cells [8]. In addition, another recent report five major compounds and two GO extracts were evaluated for the FAS and SREBP-1c inhibition *via* AMPK activation in HepG2 cells [9]. Continuing previous studies and based on the folk experience utilization of GO in the treatment of hepatitis, inflammation, fever and wounds, this current study conducted the anti-inflammatory activity to further clarify the scientific evidence of this plant to further product development orientation.

Acute and chronic inflammation are innate and protective immune responses that involve the activation of various immune cells such as macrophages. Macrophages play an important role in the regulation of immune response and inflammation [10, 11]. When macrophages are activated, cytokines, enzymes, growth factors, and inflammatory mediators such as prostaglandins (PG), cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE2), interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α) and leukotrienes are increased [12, 13]. The cyclooxygenase (COX) enzymes are the important key biosynthetic enzyme that catalyzes the synthesis of prostaglandins from arachidonic acid. There are two types of COX: COX-1 and COX-2 [14, 15]. Activation of

COX-2 expression by inflammatory stimuli, infections, toxin,… such as lipopolysaccharide (LPS). LPS, which primarily induces PGE2 synthesis and secretes inflammatory mediators such as cytokines, NO, and TNF-α [16, 17], can induce several pathophysiological diseases such as sepsis, cancer, and neuronal degeneration [18-20].

The nuclear factor NF-κB is one of the most common transcription factors and is primarily in response to IL-1 and TNF-α signaling [21, 22]. The over-expression of NF-κB is involved in acute-phase and inflammatory responses by regulating the several inflammatory mediators and cytokines gene expression. NF-κB activation has an important role in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, and inflammatory bowel disease [23-25].

The LPS-induced RAW 264.7 (mouse macrophage cell lines) is commonly the anti-inflammation model that has been used for anti-inflammation candidate screening *in vitro* [4]. Non-steroidal anti-inflammatory drugs (NSAIDs), which are specific to the COX-2 isozyme are called COX-2 inhibitors and have been used widely but these drugs have side effects such as cardiovascular disease, and gastrointestinal and renal disorders [26-28]. Therefore, new potential drug screenings with better effects and fewer side effects are necessary. This study investigates the effects of GO extracts (**Biovip** and **Tox-off**) and five major compounds on COX-2 expression and PGE2 production in RAW 264.7 cells. In addition, the effects of these extracts and isolates were evaluated on NF-κB activation and TNF-α production in RAW 264.7 cells.

II. MATERIALS AND METHODS

Information of all tested samples including the standardized GO extracts (**Biovip** and **Tox-off**) and five major isolated compounds [traphanoside GO1 (**TRA-GO1)**, spergulacin (**TRA-GO2**), spergulacin A **(TRA-GO3**), 3-*O*-(*β*-D-xylopyranosyl)-spergulagenin A (**TRA-GO4**), and vitexin (**TRA-GO5**)] were reported to our previous publication [9].

2.1 Cell culture

The RAW 264.7 cell line is purchased from ATCC. The cells were culturecontainscco's modified Eagle's medium (DMEM) containing 10% FBS (Fetal bovine serum), 100 µg/mL streptomycin in a 5% CO_2 atmosphere at 37°C. Cells have to be subcultured every three days.

2.2 Cell viability assay

RAW 264.7 cells were grown in 96-well plates at an initially seeded cell density of 2×10^3 cells/well. The cells were incubated with extracts (**Biovip** and **Tox-off** at 1, 3, and 10 µg/mL) and compounds **TRA-GO1** – **TRA-GO5** (1, 3, and 10 µM) for 24 hours. The cytotoxicity of tested samples was determined by adding 40 µL of MTT solution (1 mg/mL) to each well and incubating for 1–2 hours. After that, 100 µL of DMSO was used to dissolve formazan crystals and absorbance was measured at 550 nm using a Microplate Reader. The cell viability percentage was calculated based on the absorbance of the cells treated with the test sample compared with the absorbance of the untreated control cells.

2.3 PGE2 and TNF-α measurements

RAW 264.7 macrophages were subcultured in 24-well plates and pretreated LPS and stimulated extractions or isolated compounds for 24 hours. The accumulated PGE2 and TNF-α production in the medium were determined by using Mouse PGE2 (#MBS766181, Mybiosource) and TNF-α Elisa kits (#AB0477-1KT, sigma) according to the manufacturer's protocols.

2.4 Quantitative real‑ time pcr (qRT‑ PCR)

Total RNA was isolated using Trizol (Takara, Japan) according to the manufacturer's instructions. COX-2 mRNA expression was analyzed by real-time qPCR (StepOnePlus qPCR cycler, Applied Biosystems) using QuantiFast SYBR Green RT-PCR Kit Qiagen, (#204156) and primers (Qiagen): COX-2 (Ptgs2) (Cat: QT00165347), Mm_GADPH (Cat: QT01658692).

2.5 Reporter gene assay

One µg of the plasmid NF-κB or 50 ng of pRL Renilla was transfected into the cells using LipofectAMINE2000 (Invitrogen Corp., Carlsbad, CA) using the Dual-Luciferase Reporter Assay Systems (Promega, Madison, WI, USA). After 6 h, the transfection medium was replaced with the DMEM without serum and the cells were further incubated for 18 h. The firefly and hRenilla luciferase activity was detected using a multilayer counter. The ratio of activity was determined by normalizing the promoter-driven luciferase activity versus hRenilla luciferase.

2.7 Statistical analysis

Quantitative data are presented as the mean ± S.E.M. Data were analyzed by Student's t-tests or one-way analysis of variance (ANOVA) for multiple comparisons with Šídák's multiple comparisons test using GraphPad Prism Software 5. The difference was considered significant when $p < 0.05$.

III. RESULTS

3.1 The effects of go standardized extracts and five major isolated compounds on cox-2 mrna expression and pge2 production in raw 264.7 cells.

RAW 264.7 macrophages have been used as an inflammation *in vitro* model [29, 30]. To investigate the possibility that the cytotoxicity of two standardized extracts of *G. oppositifolius* (**Biovip** and **Tox-off**; 1, 3, 10, and 30 µg/mL) might contribute to its anti-inflammatory effects, the cytotoxicity of **Biovip** and **Tox-off** (Figure 1) on RAW 264.7 cells was performed by using an MTT assay. As shown in Figure 1, the cell survival of RAW 264.7 cells was more than 70% after 24 h incubation with **Biovip** and **Tox-off** extracts at a concentration of 30 µg/mL. Thus, the effects of two extracts (**Biovip** and **Tox-off**) at 30 µg/mL on COX-2 mRNA were evaluated in RAW 264.7 cells.

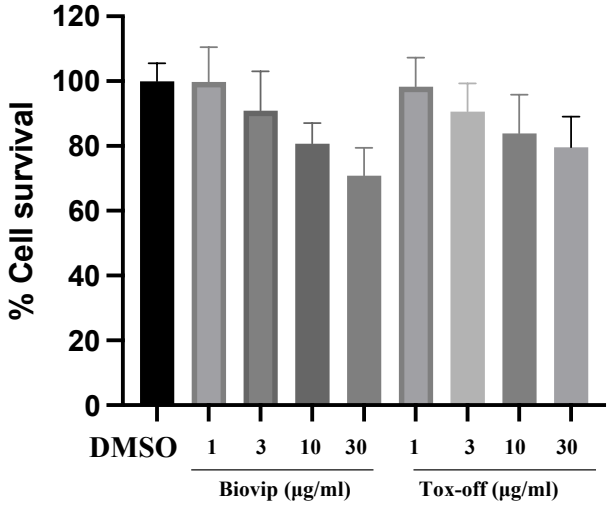

Figure 1: The effects of **Biovip** and **Tox-off**, in dose-dependent on RAW 264.7 cells viability for 24 hours by using MTT assay. *$p < 0.05$; **$p < 0.01$; compared to DMSO control group.

To examine the inhibitory effect of **Biovip** and **Tox-off** on the expression COX-2, RAW 264.7 cells were pre-treated with LPS 5 ng/mL and then stimulated with **Biovip**/**Tox-off** (10 µg/ml) for 24 h. The positive control experiment was treated with Dexamethasone (100 nM). mRNA COX-2 expression was measured by RT-PCR. As shown in Figure 2A, both **Biovip** and **Tox-off** (10 µg/ml) significantly inhibited COX-2 mRNA expression compared to LPS-treated cells. In addition, both **Biovip** and **Tox-off** (1-10 µg/mL) showed the reduction of PGE2 production in a dose-dependent manner in RAW 264.7 cells (Figure 2B).

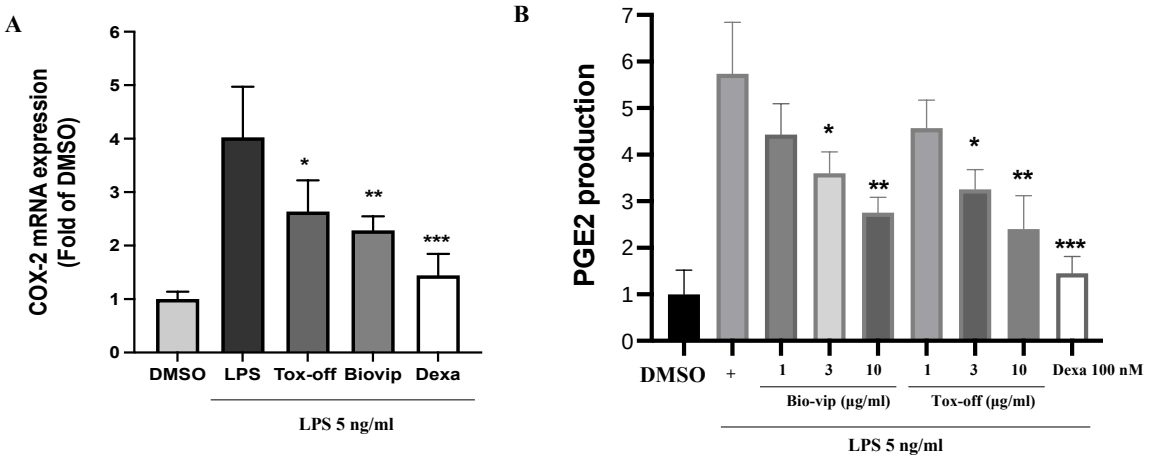

Figure 2. Effect of **Biovip** and **Tox-off** on COX-2 expression and PGE2 production. (**A**) The effect of **Biovip** and **Tox-off** on LPS-induced COX-2 was analyzed by qPCR. RAW 264.7 cells were treated with 5 ng/mL LPS for 24 h with or without **Biovip** or **Tox-off** 10 µg/mL and then harvested and lysated for COX-2 mRNA level by qPCR. Relative changes in the COX-2 mRNA expression (*significant as compared to LPS, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, n=3). Dexamethasone 100 nM was used as a control. (**B**) Effect of **Biovip** and **Tox-off** in a dose-dependent manner (1-10 µg/mL) on PGE2 production. RAW 264.7 cells were incubated with 5 ng/mL LPS for

24 h with or without **Biovip** or **Tox-off** and amounts of PGE2 in the medium were determined using PGE2-specific ELISA assays. (*significant as compared to LPS, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, n=3).

In the previous study, we isolated five major compounds (**TRA-GO1** – **TRA-GO5**) from the aerial part of *G. oppositifolius* [8] including traphanoside GO1 (**TRA-GO1**), spergulacin (**TRA-GO2**), spergulacin A **(TRA-GO3)**, 3-*O*-(*β*-D-xylopyranosyl)-spergulagenin A (**TRA-GO4**), and vitexin (**TRA-GO5**) (Figure 3). Results demonstrated significant effects of five major isolates (**TRA-GO1−TRA-GO5**) on SREBP-1c and FAS suppression in HepG2, suggesting that GO may be potential candidates for the prevention and treatment of obesity and related metabolic disorders *via* activation of the AMPK [9]. In order to carry out the effects of these major isolated compounds on COX-2 mRNA expression and PGE2 production in RAW 264.7 cells, these compounds were determined by the cytotoxic effects in RAW 264.7 cells by using MTT assay. The cell survival was 80–100% after incubation with 10 µM these compounds for 24 h (Figure 4). Therefore, we use the isolated compound at the concentration of 1–30 µM within 24 h of incubation in the subsequent experiments.

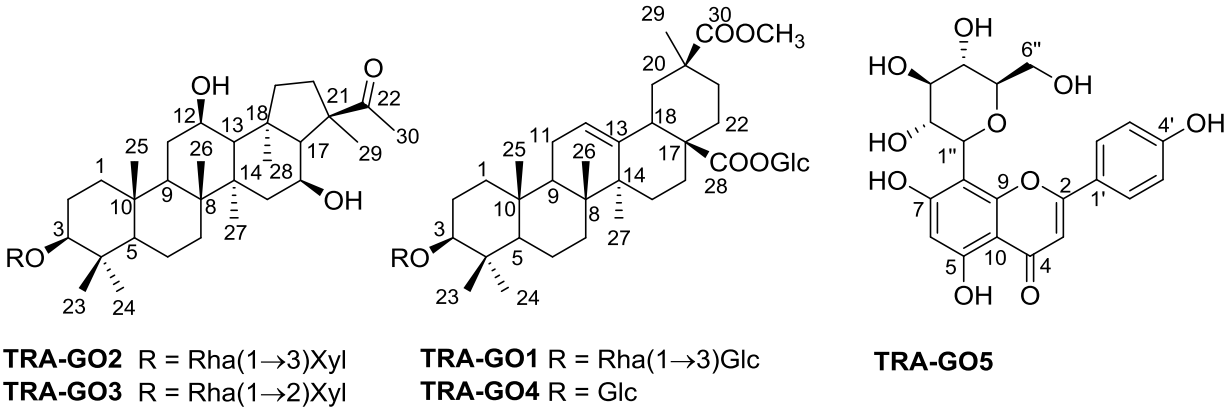

Figure 3. Chemical structures of compounds **TRA-GO1** – **TRA-GO5**.

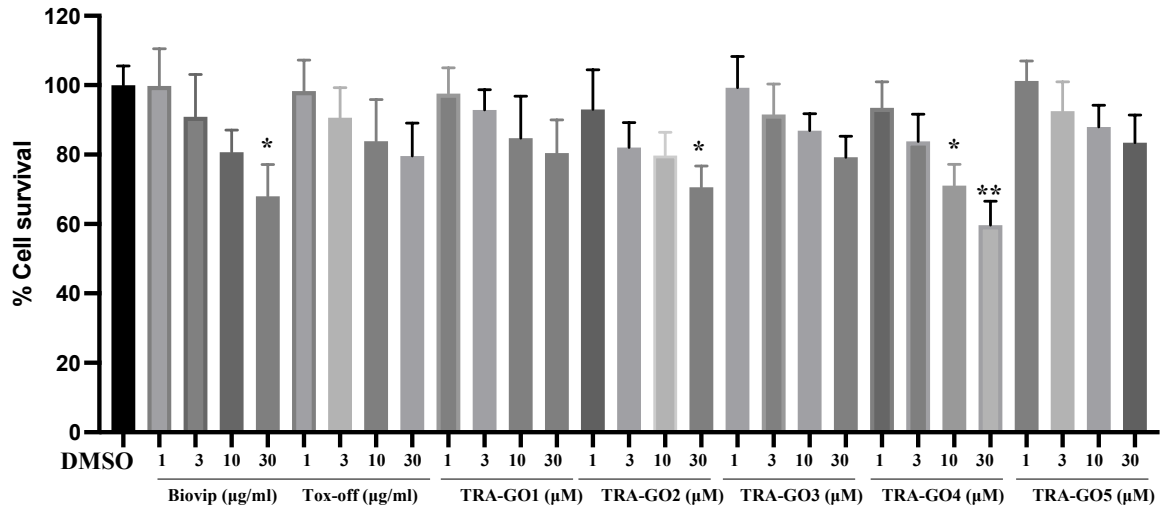

Figure 4. The effects of **TRA-GO1** – **TRA-GO5**, in dose-dependent on RAW 264.7 cells viability for 24 hours by using MTT assay. *$p < 0.05$; **$p < 0.01$; compared to DMSO control group.

To further determine the inhibitory effect of five compounds (**TRA-GO1** – **TRA-GO5**) on COX-2 mRNA level by qPCR, the cells were pre-treated with LPS 5 ng/mL and then stimulated with isolated compound at 10 µM for 24 h. The positive control experiment was treated with Dexamethasone (100 nM). Figure 5A presented the suppression of COX-2 mRNA expression by all 5 compounds compared to LPS. To confirm this data, the dose-dependent manner of **TRA-GO1** – **TRA-GO5** (1–10 µM) was treated with or without LPS for 24 hours by using qPCR and ELISA. As shown in Figure 5B-E, **TRA-GO1** – **TRA-GO3** and **TRA-GO5** (1–10 µM) significantly down-regulated COX-2 mRNA expression and PGE2 production in a dose-dependent manner. These data strongly suggest the inhibitory effect of isolated compounds from *G. oppositifolius* in RAW 264.7 cells.

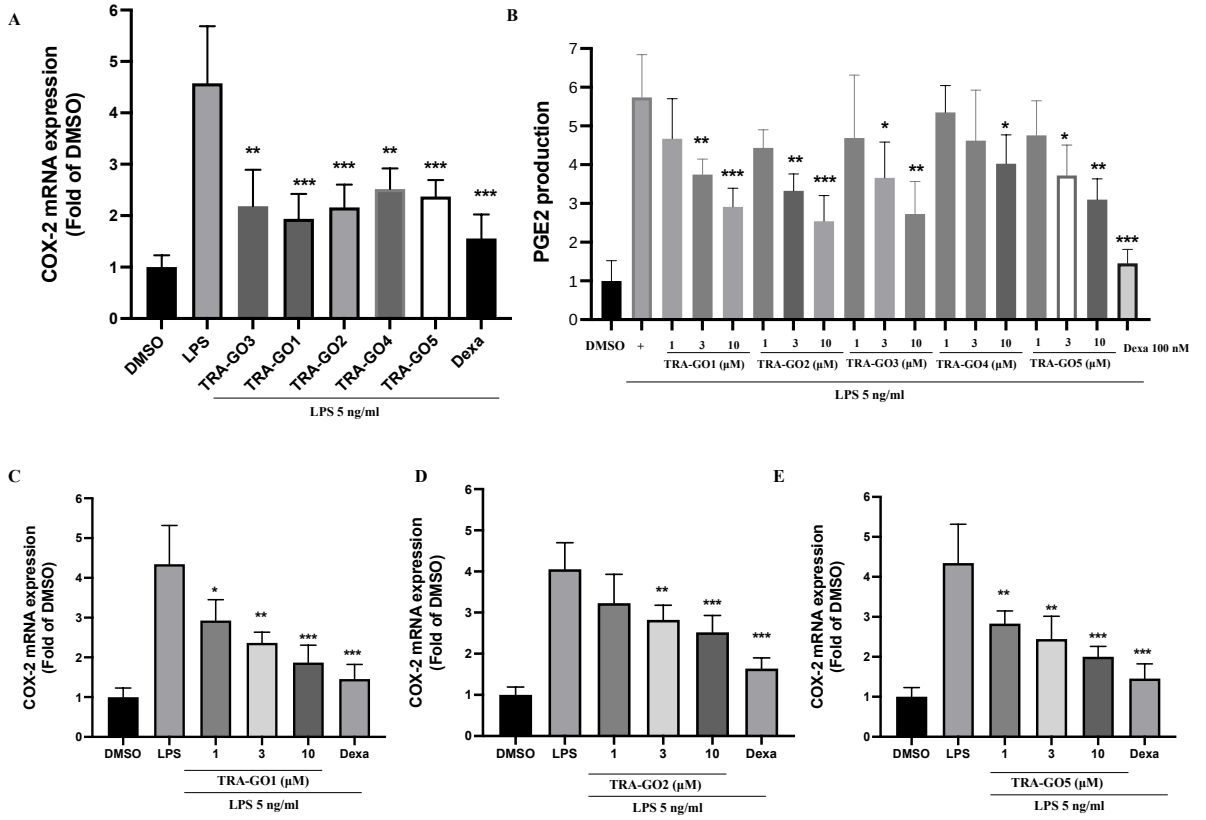

Figure 5. Effect of **TRA-GO1** – **TRA-GO5** (10 µM) on COX-2 expression and PGE2 production. (**A**) The effect of **TRA-GO1** – **TRA-GO5** (10 µM) on LPS-induced COX-2 was analyzed by qPCR. RAW 264.7 cells were treated with 5 ng/mL LPS for 24 h with or without **TRA-GO1** – **TRA-GO5** (10 µM) and then harvested and lysated for COX-2 mRNA level by qPCR. Relative changes in the COX-2 mRNA expression (*significant as compared to LPS, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, n=3). Dexamethasone 100 nM was used as a control. (**B**) Effect of **TRA-GO1** – **TRA-GO5** (1–10 µM) in a dose-dependent manner on PGE2 production. RAW 264.7 cells were incubated with 5 ng/mL LPS for 24 h with or without **TRA-GO1** – **TRA-GO5** and amounts of PGE2 in the medium were determined using PGE2-specific ELISA assays. (*significant as compared to LPS, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, n=3). (**C-E**) COX-2 mRNA expression by **TRA-GO1**, **TRA-GO2** and **TRA-GO5** compounds in dose dependent manner. RAW 264.7 cells were treated with 5 ng/mL LPS for 24 h with or without **TRA-GO1**, **TRA-GO2** and **TRA-GO5** (1-10 µM) and then harvested and lysated for COX-2 mRNA level by qPCR. Relative changes in the COX-2 mRNA expression (*significant as compared to LPS, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, n=3). Dexamethasone 100 nM was used as a control.

3.2 The extractions and purified compounds inhibited lps-induced nf-κb activation and tnf-α production in raw 264.7 cells.

NF-κB is one of the most common transcription factors that is activated during the inflammatory response to LPS. NF-κB activation induces the transcription of numerous genes related to pro-inflammatory enzymes COX-2 [31, 32] and inflammatory response. To examine whether **Biovip**, **Tox-off**, and isolated compounds affect NF-κB promoter activity, the luciferase of NF-κB was carried out. The cells were pre-treated with LPS 5 ng/mL and then stimulated with extractions or isolated compounds in a dose-dependent manner for 18 h. Figure 6 presents **Biovip**, **Tox-off**, and isolated compounds (except **TRA-GO4)** that significantly suppressed LPS-induced NF-κB promoter activity in a dose-dependent manner.

Tumor Necrosis Factor (TNF) is the well-known potent physiological inducer of the nuclear transcription factor NF-κB. To address whether **Biovip**, **Tox-off**, and isolated compounds affect TNF-α expression, an ELISA assay was performed.

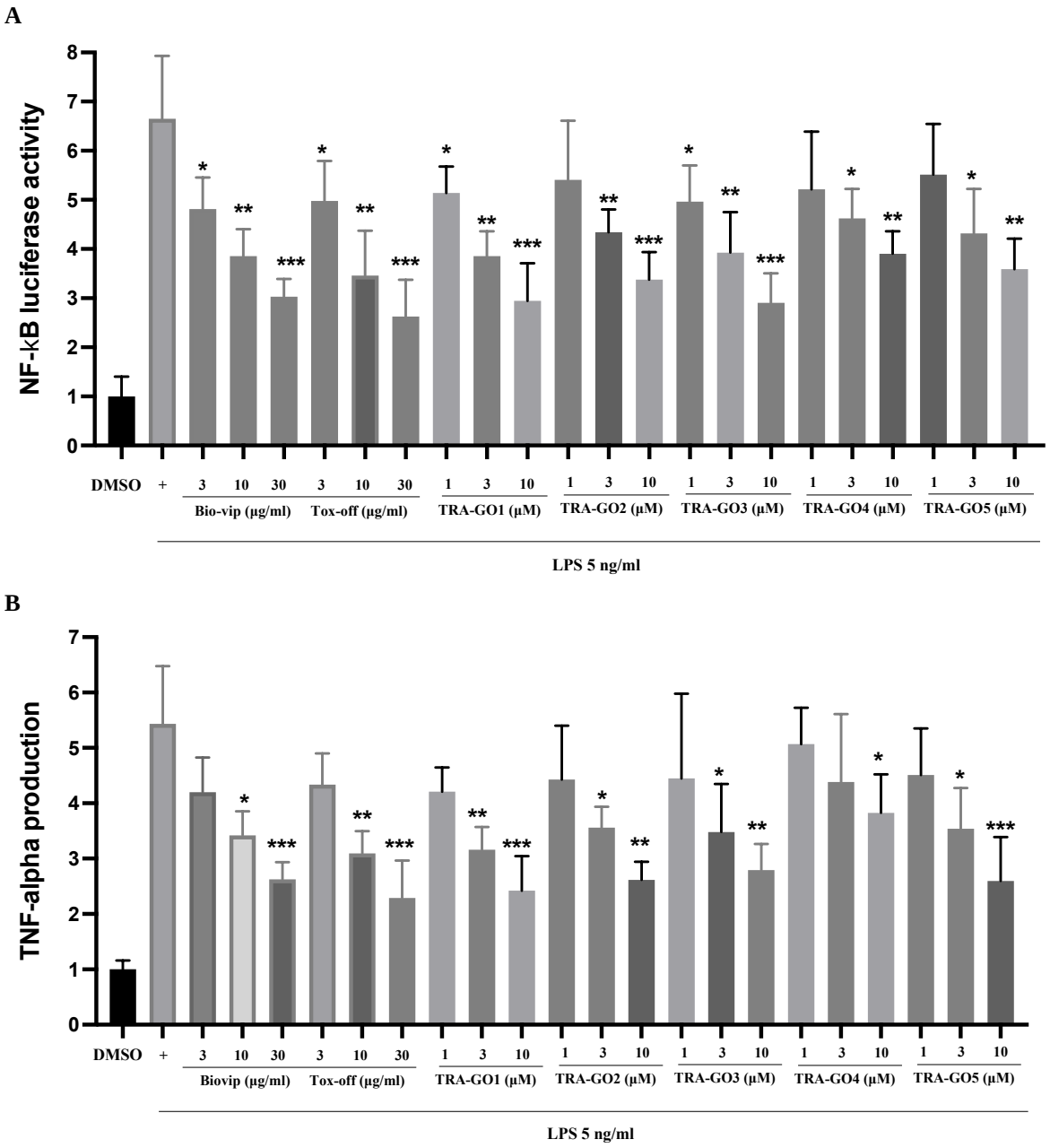

Figure 6. (A) NF-κB activity in RAW 264.7 macrophages in a dose-dependent manner. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, n=3, signifcant as compared to LPS group. (**B**) Effect of **Biovip**, **Tox-off** (3–30 µg/mL), and **TRA-GO1** – **TRA-GO5** (1–10 µM) in a dose-dependent manner on TNF-α production. RAW 264.7 cells were incubated with 5 ng/mL LPS for 24 h with or without **TRA-GO1** – **TRA-GO5** and amounts of TNF-α in the medium were determined using TNF-α -specific ELISA assays. (*significant as compared to LPS, *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$, n=3).

These results suggest that extractions and isolated compounds from *G. oppositifolius* inhibit LPS-induced inflammatory response: COX-2 protein, PGE2 production, and NF-κB expression and TNF-α in RAW 264.7 cells.

IV. DISCUSSION

G. oppositifolius is widely distributed and has been used as a traditional medicinal herb in Vietnam and other Asian countries. Several studies showed *G. oppositifolius* has potential antioxidants [3], hepatoprotectives [4], and immunomodulating effects [7]. *G. oppositifolius* is used in China for herpes zoster and herpangina and contains triterpenoid saponins. In this current study, we demonstrated the potential involvement of the NF-κB/ TNF-α pathway in the anti-inflammation of metabolites from *G. oppositifolius*. Among the active components, **TRA-GO1** was found to be the most anti-inflammatory activity in the inhibition of PGE2 and COX-2 production in LPS-stimulated RAW 264.7 macrophage cells.

RAW 264.7 cells are the murine macrophage cell line that plays an important response for anti-inflammatory agents. The macrophage cells are activated by LPS leading to the release of cytokines. Among them, TNF-α is a major regulator of inflammatory responses and is known to be involved in the pathogenesis of some inflammatory and autoimmune diseases [33, 34]. Both COX-1 and COX-2 are the isozymes that convert arachidonic acid to prostaglandin, however, COX-2 responds mainly to produce a huge number of PGEs in macrophage cells [35]. Numerous reports demonstrated the suppression of COX-2, PGE2 production as well as cytokines may contribute to the effects of several types of inflammatory treatments [36, 37].

NF-κB is the most well-known common transcription factor and NF-κB inhibitors were demonstrated as a potential therapy for cancer, osteoarthritis, infections, Paget's disease, and metastatic bone disease [38-41].

In this current study, we first show the extractions of **Biovip** and **Tox-off** and isolated compounds from *G. oppositifolius* suppressed LPS-induced NF-κB/TNF-α activation. These results may suggest **Biovip**, **Tox-off**, and isolated compounds as potential therapies for anti-inflammatory agents.

Due to time restrictions and funding, there are some limitations to this study. First, we could not conduct additional experiments regarding the expression of NF-κB p65, and IκB and translocate to the nucleus to regulate the expression of inflammatory mediators by **Biovip**, **Tox-off** and isolated compounds. Also, we did not perform other effects of other COX-2 promoters: AP-1, C/EBP, or CRE [42]. We have a further plan in the future to clarify the biological effects and cellular mechanism of **Biovip**, **Tox-off**, and isolated compounds from *G. oppositifolius* as well as *in vivo* tests.

Taken together, this study suggested that **TRA-GO1** can be used as a marker compound for quantitative analysis of GO and GO extracts. In addition, two standardized extracts (**Biovip** and **Tox-off**) might be promising candidates for further studies in animal models and pre-clinical trials in order to develop products originating from this valuable plant.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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