

Anti-inflammatory effect of the compounds from the flowers of *Trollius chinensis*

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Abstract: In order to investigate the anti-inflammatory activity of flavonoids, phenolic acids, and alkaloids from the flowers of *Trollius chinensis*, some representative compounds, namely, orientin, 2"-O- β -L-galactopyranosylorientin, vitexin, quercetin, isoquercetin, luteolin, veratric acid, proglobeflowery acid, trollioside, and trolline were selected to study their inhibitory effects against LPS-induced NO, IL-6, and TNF- α release in RAW264.7 cells. At the higher concentration, both phenolic acids and flavonoids inhibited the production of NO, whereas only phenolic acids showed this effect at the lower concentration. Although trolline had stronger cytotoxicity, it exhibited a potential effect of decreasing NO production induced by LPS in the non-toxic concentration range. In addition, all tested compounds decreased the production of IL-6 and TNF- α by almost 50% at both the higher and lower concentrations. It is concluded that the anti-inflammatory activity of the phenolic acids is stronger than that of the flavonoids.

Keywords: *Trollius chinensis*, compounds, NO, IL-6, TNF- α , anti-inflammatory activity.

INTRODUCTION

The flowers of *Trollius chinensis* are widely used in China to treat sore throat, swollen gums, acute lymphangitis and other diseases owing to their heat-clearing and toxic-resolving efficacy (Beijing Pharmacy Factory, 1973; Chinese Pharmacopoeia Commission, 1978). It has been proved that these flowers possess anti-inflammatory, antibacterial, and antiviral bioactivities (Jiangsu New College of Medicine, 1977; Wang *et al.*, 2004) and mainly contain three groups of compounds, viz, flavonoids, phenolic acids and alkaloids (Wang *et al.*, 2004; Cai *et al.*, 2006; Li *et al.*, 2009; Wang *et al.*, 2009). Flavonoids including vitexin, orientin, 2"-O- β -L-galactopyranosylorientin, and so on are the most abundant compounds in these flowers. Phenolic acids mainly consist of the derivatives of benzoic acid, such as veratric acid, globeflowery acid, proglobeflowery acid and trollioside (Wang *et al.*, 2004). Up to now, only one isoquinoline-type alkaloid named trolline has been isolated from these flowers.

NO is one of the important inflammatory mediators (Fang *et al.*, 2011). Its excessive generation is detrimental to the host cells and results in a series of inflammatory diseases (Lu *et al.*, 2011). Meanwhile, IL-6 and TNF- α are vital cytokines correlated with the generation of NO and thus deteriorate the inflammation (Zhou *et al.*, 2009). Therefore, it is considered as a treatment strategy for inflammatory diseases to reduce the production of NO, IL-6 and TNF- α (Rose-John *et al.*, 2007; Zhao *et al.*, 2008). In order to compare the anti-inflammatory activity

of the flavonoids, phenolic acids and alkaloids from the flowers of *T. chinensis*, the present article studied the inhibitory effects of the representative compounds of the three groups against LPS-induced production of NO, IL-6 and TNF- α in RAW264.7 cells through Griess reaction and enzyme-linked immune-sorbent assay (ELISA), respectively.

MATERIALS AND METHODS

Cell line

The murine RAW264.7 macrophages cells (Batch No. 3111C0001CCC000146) were purchased from the Cell Resource Center, Peking Union Medical College.

Chemical compounds

Orientin, vitexin, isoquercetin, luteolin, veratric acid, proglobeflowery acid, trollioside and trolline were isolated from the flowers of *T. chinensis* by our research group (Wang *et al.*, 2004) and their purities were determined to be over 98% by high performance liquid chromatography (HPLC); 2"-O- β -L-galactopyranosylorientin and quercetin at 98% minimum purities were purchased from Chengdu Puruifa Technology Development Company (Chengdu, Sichuan, China); aspirin was synthesized in our lab and its purity was determined to be over 98% by HPLC.

Reagents and instruments

Lipopolysaccharide (LPS) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA); Dulbecco's Modified Eagle's Medium (DMEM) and trypsin were supplied by Gibco (Carlsbad, CA, USA); fetal bovine serum (FBS) was obtained from Zhejiang

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Tianhang Biotechnology Company (Hangzhou, Zhejiang, China); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Amresco (Solon, OH, USA); penicillin-streptomycin and 96-well plates were purchased from Corning (San Diego, CA, USA); the Griess reagent composed of N-(1-naphthyl) ethylenediamine dihydrochloride and sulfonamides was produced by Tianjin Fuchen Chemical Reagent Company (Tianjin, China); the cytokine enzyme linked immunosorbent assay kits for IL-6 and TNF- α were purchased from MultiSciences (Lianke) Biotechnology Company (Hangzhou, Zhejiang, China); MCO-18AIC(UV)CO₂ constant temperature incubator and Epoch micro plate reader were the products of Sanyo (Tokyo, Japan) and BioTek (Winooski, VT, USA), respectively.

Cell culture

The murine RAW264.7 macrophages cells were cultured with DMEM medium containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin in 25 cm² flasks inside an incubator with 5% CO₂ at 37°C and constant moisture.

Cell viability

The cytotoxicity of the compounds was assayed with MTT method as described in a literature (Zhou *et al.*, 1993). RAW264.7 cells were added into 96-well plates at the density of 1.5×10^4 cells/well. After being incubated for 12h, they were cultured in quadruplicate with 0, 25, 50, 100, 200 and 400 $\mu\text{mol}\cdot\text{L}^{-1}$ orientin, vitexin, quercetin, isoquercetin, 2''-O- β -L-galactopyranosyl-orientin, veratric acid, proglobeflowery acid, trollioside and aspirin (positive control), and 3.13, 6.25, 12.5, 25, 50, 100, 200 and 400 $\mu\text{mol}\cdot\text{L}^{-1}$ luteolin and trolline for 20 h. The cells were reacted with MTT (100 μL /well) at the concentration of 0.5mg·mL⁻¹ for 4 h; then, the supernatant was discarded and 150 μL of DMSO was added to dissolve the cells. After shaken for 10 min, their absorbance values were measured at 490 nm by a micro plate reader.

NO assay

As described in the previous reports, the NO assay was conducted (Green *et al.*, 1982; Li *et al.*, 2012). RAW264.7 cells (1×10^5 /well) were dispensed into 96-well plates for 12h; then, they were cultured in quadruplicate with 50, 100 and 200 $\mu\text{mol}\cdot\text{L}^{-1}$ orientin, 2''-O- β -L-galactopyranosyl-orientin, vitexin, quercetin, isoquercetin, veratric acid, proglobeflowery acid, trollioside, and aspirin (positive control), and 3.13, 6.25 and 12.5 $\mu\text{mol}\cdot\text{L}^{-1}$ luteolin and trolline in the presence of 1 $\mu\text{g}\cdot\text{mL}^{-1}$ LPS. After incubated for 20 h, the same volumes of supernatant and Griess reagent were mixed with each other to react for 10min at room temperature. Then, their absorbance values were read by the micro plate reader at 540nm.

IL-6 and TNF- α assay

The IL-6 and TNF- α assay was performed as reported in a literature (Yang *et al.*, 2013). RAW264.7 cells were

cultured into 48-well plates at the density of 1×10^6 /mL for 24h, then they were treated in quadruplicate with or without compounds (50, 100 and 200 $\mu\text{mol}\cdot\text{L}^{-1}$ orientin, 2''-O- β -L-galactopyranosyl-orientin, vitexin, quercetin, isoquercetin, veratric acid, proglobeflowery acid, trollioside, and aspirin (positive control), and 3.13, 6.25, and 12.5 $\mu\text{mol}\cdot\text{L}^{-1}$ luteolin and trolline) in the presence of LPS (10 $\mu\text{g}\cdot\text{mL}^{-1}$). After 24h, the supernatant was collected into a sterile Eppendorf tube and stored at -80°C until all of the samples were diluted 5-folds for ELISA analysis. The experiment of ELISA was conducted with the commercial ELISA kits following the instructions from the manufacturer.

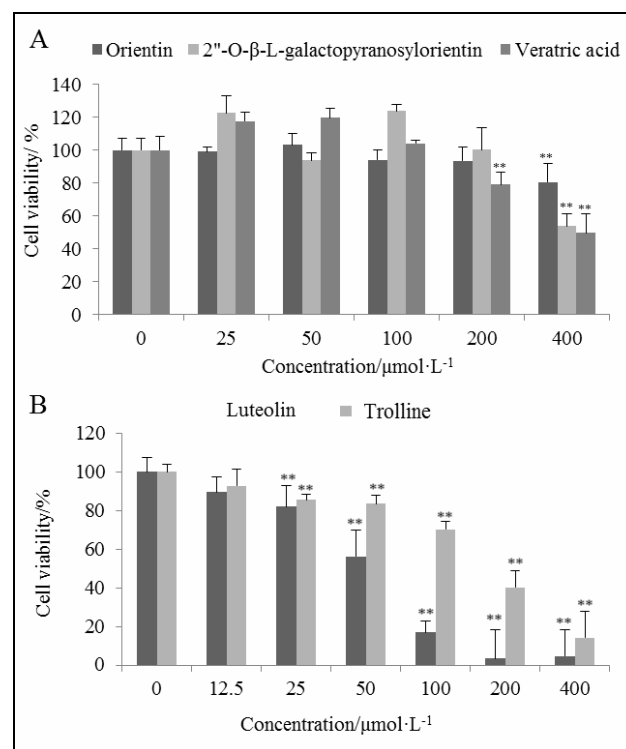


Fig. 1: Effects of the compounds from the flowers of *T. chinensis* on viability of RAW264.7 cells (** $P < 0.01$, compared with the control group)

STATISTICAL ANALYSIS

The results were presented as means $\pm$ SD, and statistical evaluations were analyzed by ANOVA or nonparametric test using SPSS 16.0. The level of significance was set at $P < 0.05$.

RESULTS

Effects of compounds on Cell viability

The results showed that none of vitexin, quercetin, isoquercetin, proglobeflowery acid, trollioside and aspirin exhibited cytotoxicity at 0-400 $\mu\text{mol}\cdot\text{L}^{-1}$. Neither orientin nor 2''-O- β -L-galactopyranosyl-orientin affected cell

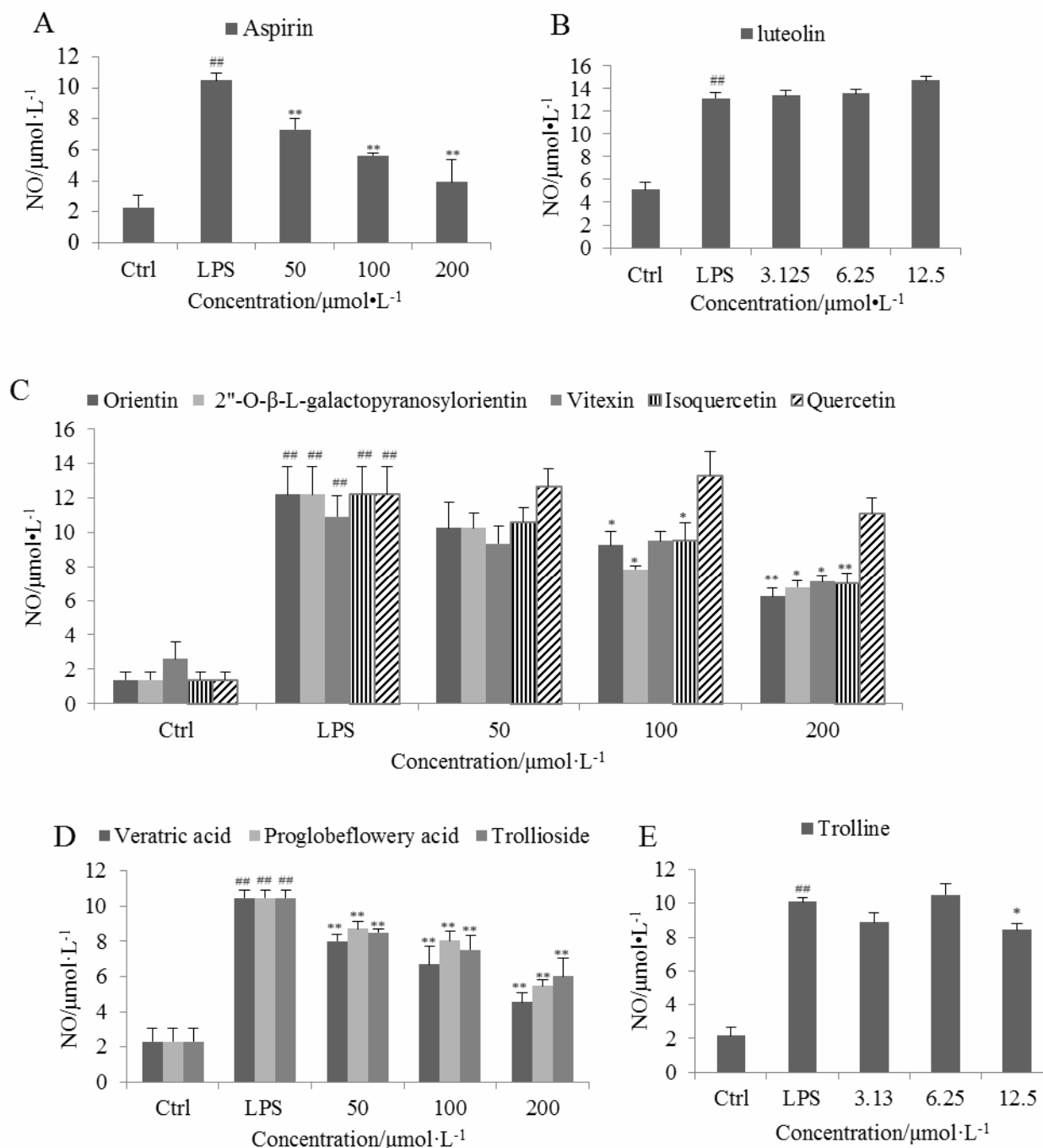


Fig. 2: Effects of the compounds from the flowers of *T. chinensis* against LPS-induced NO production in RAW264.7 cells (^{##} $P < 0.01$, compared with the control group; ^{*} $P < 0.05$, compared with the LPS group; ^{**} $P < 0.01$, compared with the LPS group)

viability at 0-200 $\mu\text{mol}\cdot\text{L}^{-1}$, but they reduced the cell viability at 400 $\mu\text{mol}\cdot\text{L}^{-1}$ with the survival rates of 80.4% and 53.7%, respectively (fig. 1A). Veratric acid at 0-100 $\mu\text{mol}\cdot\text{L}^{-1}$ did not show any cytotoxicity; however, it significantly reduced the cell viability to 79.2% and 43.2% at 200 and 400 $\mu\text{mol}\cdot\text{L}^{-1}$, respectively. As for luteolin and trolline, the cytotoxicity increased in a concentration-dependent manner. Although none of these two compounds impaired the cell viability at 12.5 $\mu\text{mol}\cdot\text{L}^{-1}$

¹, they decreased the cell survival rates to 82.0%, 56.3%, 19.2%, 3.5% and 4.6%, and 85.4%, 83.6%, 70.1%, 40.2% and 14.2%, respectively, at the concentrations of 25, 50, 100, 200 and 400 $\mu\text{mol}\cdot\text{L}^{-1}$ (fig. 1B).

Effects of compounds against NO production

The effects of compounds against NO production in RAW264.7 cells stimulated by LPS were tested through Griess reaction and the results are shown in fig. 2. All of

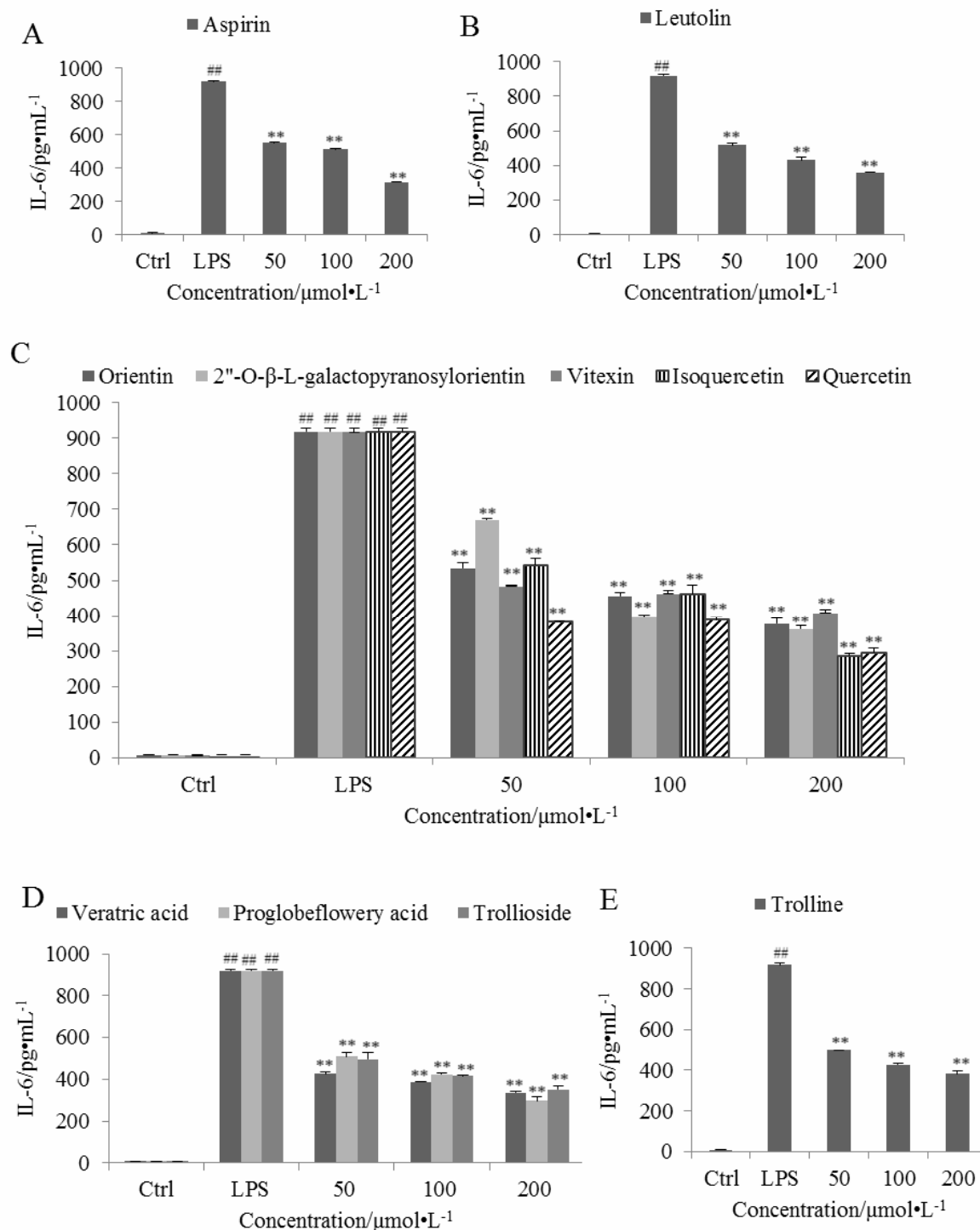


Fig. 3: Effects of the compounds from the flowers of *T. chinensis* against LPS-induced IL-6 production in RAW264.7 cells (^{##} $P < 0.01$, compared with the control group; ^{**} $P < 0.01$, compared with the LPS group)

the inflammatory models were successfully established since the NO concentrations were increased drastically in the presence of LPS. The inhibition of NO production was observed in a concentration-dependent manner in aspirin group. As the concentrations of aspirin were 50, 100 and $200\mu\text{mol}\cdot\text{L}^{-1}$, the NO inhibitory rates were 30.4%, 46.1%,

and 62.5%, respectively.

For flavonoids, the production of NO was inhibited by 16.0%, 14.4%, 16.1%, 12.9% and -3.9%; 24.3%, 12.8%, 35.8%, 21.8% and -8.8%; 48.9%, 34.8%, 44.6%, 42.6% and 9.0% by orientin, vitexin, 2''-O-β-L-galactopyranosylorientin, isoquercetin and quercetin at the concentrations

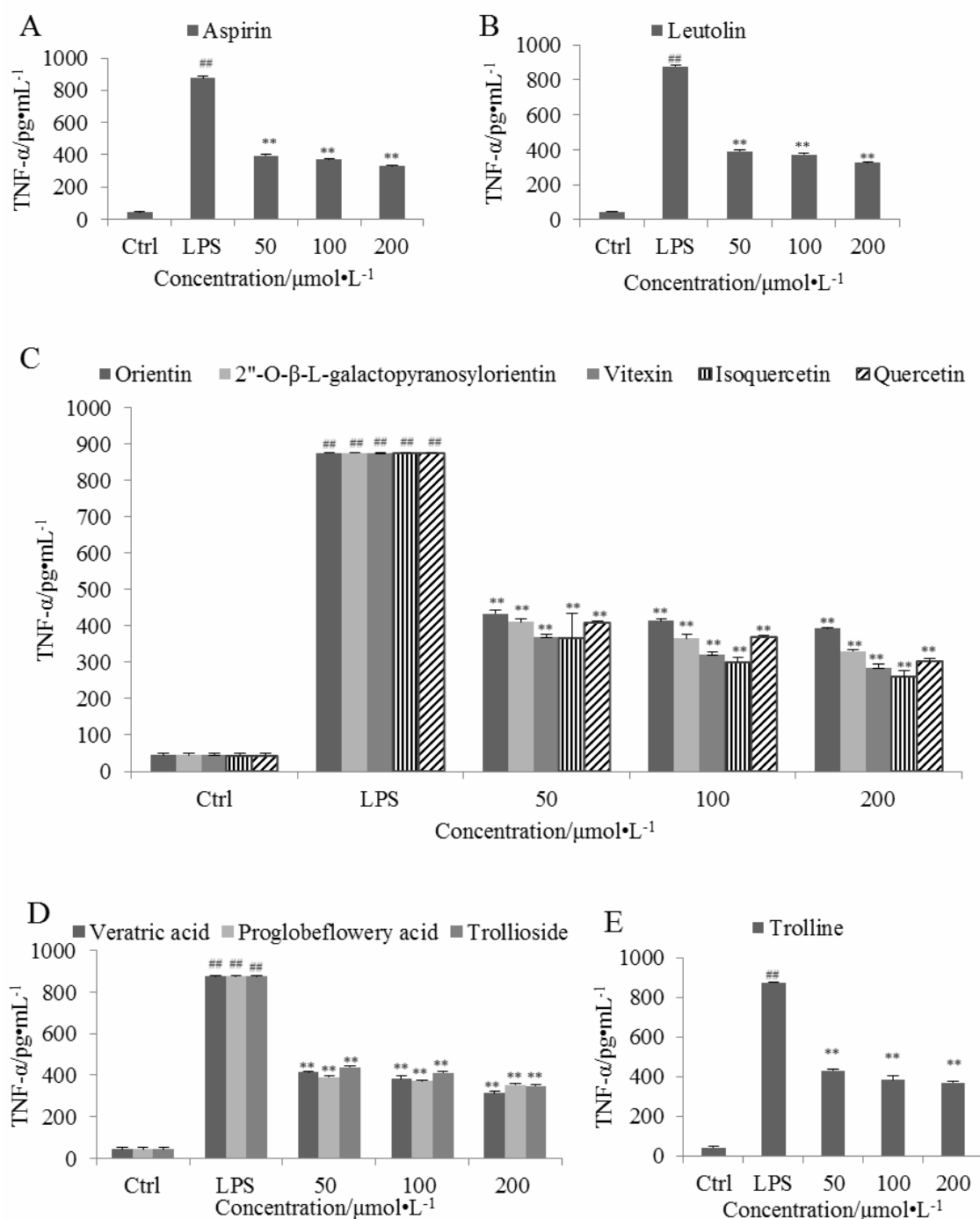


Fig. 4: Effects of the compounds from the flowers of *T. chinensis* against LPS-induced TNF- α production in RAW264.7 cells (^{##} $P < 0.01$, compared with the control group; ^{**} $P < 0.01$, compared with the LPS group)

of 50, 100 and 200 $\mu\text{mol}\cdot\text{L}^{-1}$, respectively. At 50 $\mu\text{mol}\cdot\text{L}^{-1}$, None of these five flavonoids inhibited the production of NO. However, orientin and 2''-O-β-L-galactopyranosylorientin at 100 and 200 $\mu\text{mol}\cdot\text{L}^{-1}$ and vitexin at 200 $\mu\text{mol}\cdot\text{L}^{-1}$ significantly inhibited the release of NO. In addition, quercetin and luteolin at none of the tested concentrations showed significant inhibitory effect against the NO production.

At 50, 100 and 200 $\mu\text{mol}\cdot\text{L}^{-1}$, veratric acid, proglobe-flowery acid and trollioside significantly reduced the NO production by 23.6%, 36.1% and 56.5%; 16.9%, 23.4% and 48.0%; 18.8%, 28.3% and 42.6%, respectively. All of these data indicated that the NO production was gradually attenuated by increasing the concentrations of these three phenolic acids.

At 3.13, 6.25 and 12.5 $\mu\text{mol}\cdot\text{L}^{-1}$, trolline decreased the NO production by 12.6%, -4.4% and 16.8%, respectively. This indicated that trolline had an effect of attenuating the release of NO at the highest concentration tested.

Effects of compounds against IL-6 and TNF- α production

As shown in figs. 3 and 4, the release of IL-6 and TNF- α was significantly increased in LPS group, which indicated that the inflammatory models were established successfully. The inhibitory rates were 40.2%, 44.4% and 66.0% for IL-6 and 54.9%, 57.7%, and 62.2% for TNF- α when the concentrations of aspirin were 50, 100 and 200 $\mu\text{mol}\cdot\text{L}^{-1}$, respectively.

A concentration-dependent inhibitory manner was observed in IL-6 and TNF- α production for orientin, 2"-O- β -L-galactopyranosylorientin, vitexin, isoquercetin and quercetin at the test concentrations of 50, 100 and 200 $\mu\text{mol}\cdot\text{L}^{-1}$, while luteolin exhibited the similar concentration-dependent inhibitory effect against production of IL-6 and TNF- α at the much lower concentrations of 3.13, 6.25 and 12.5 $\mu\text{mol}\cdot\text{L}^{-1}$.

The phenolic acids including veratric acid, proglobeflowery acid, and trollioside also showed the similar inhibitory effect against the production of IL-6 and TNF- α as above flavonoids at the concentrations of 50, 100 and 200 $\mu\text{mol}\cdot\text{L}^{-1}$.

Like luteolin, trolline significantly reduced the production of both IL-6 and TNF- α in a concentration-dependent manner at the much lower test concentrations of 3.13, 6.25 and 12.5 $\mu\text{mol}\cdot\text{L}^{-1}$.

DISCUSSION

As mentioned previously, NO is a pro-inflammatory cytokine. It also enhances the production of various inflammatory mediators such as IL-6 and TNF- α (Yoon *et al.*, 2009). Thus, the inhibition of NO production is regarded as the therapeutic target for inflammation and vital for evaluation of the anti-inflammatory effect. The production of NO is mainly mediated by nitric oxide synthase (NOS) which includes three isoforms, namely, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Among them, iNOS is the main mediator to promote pathological inflammation, while the other two are constitutively expressed and play a vital role in the normal physiological activities (Yoon *et al.*, 2009). Therefore, the effects of the test compounds against NO production may be related to their inhibition on NOS, especially iNOS. The results of this article indicated that the flavonoids, phenolic acids and alkaloids possessed anti-inflammatory activity because they all decreased the production of NO, IL-6 and TNF- α stimulated by LPS in RAW264.7 cells. At all test concentrations, not only

flavonoids but also phenolic acids and alkaloid reduced the release of IL-6 and TNF- α and they showed almost similar inhibitory activities. However, it was not the case for NO. Although both phenolic acids and flavonoids inhibited the production of NO at the higher concentration, only phenolic acids showed this inhibitory effect at the lower concentration. Therefore, the anti-inflammatory activity of phenolic acids is stronger than that of the flavonoids. Although trolline, the only alkaloid isolated from the flowers of *T. chinensis* so far, exhibited stronger cytotoxicity, it had a potential effect of decreasing NO production in the non-toxic concentration range.

It is worth mentioning that, among the phenolic acids tested, veratric acid has the strongest anti-inflammatory activity and is abundant in these flowers (Yang *et al.*, 2009). Moreover, our previous study had also evidenced that veratric acid is absorbed easily and the retention duration *in vivo* is very long (An *et al.*, 2014).

CONCLUSION

Hence, veratric acid may be considered as the main anti-inflammatory phenolic acid in the flowers of *T. chinensis*.

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