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Original Article

Anti-inflammatory activities of gentiopicroside against iNOS and COX-2 targets

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ABSTRACT

Objective: To isolate high-purity gentiopicroside from the Chinese herbal *Gentiana officinalis* and investigate its anti-inflammatory activity against iNOS and COX-2 targets.
Methods: The purity and structures of gentiopicroside were determined by HPLC, IR, NMR, and MS. The anti-inflammatory effects of gentiopicroside were investigated by *in vivo, in vitro,* and molecular experiments.
Results: *In vitro* experiment results showed that gentiopicroside inhibited nitric oxide (NO), prostaglandin E2 (PGE_2), and interleukin-6 (IL-6) production in mouse macrophages RAW 264.7 stimulated by lipopolysaccharide. *In vivo* experiment found that xylene-induced mouse ear swelling was inhibited by gentiopicroside with an inhibition rate of 34.17%. Molecular docking of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) with gentiopicroside showed that hydrogen bonds (H-bonds) were formed between the sugar fragments in gentiopicroside structure with Tyr355, Ser353, Leu352, Ser530, Arg120, and His90 of COX-2, and Glu377, Asp382, Tyr373, Tyr347, Gln263, Asn370, and Gly371 of iNOS. Thus, gentiopicroside had a lower docking score and displayed satisfactory anti-inflammatory activities.
Conclusion: These results suggested that the mechanism of anti-inflammatory activity of gentiopicroside was associated with the downregulation of inflammatory cytokines, such as NO, PGE_2, and IL-6, and the suppression of iNOS and COX-2. Therefore, gentiopicroside is a potential and selective iNOS and COX-2 inhibitor.

1. Introduction

Inflammation is a common clinical disease worldwide (Jo et al., 2017; Khatri, Indalkar, Patil, Goyal, & Chaturbhuj, 2017; Li et al., 2017). Excessive secretion of inflammatory cytokines, such as nitric oxide (NO), prostaglandin E2 (PGE_2), and interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), is one of the leading causes of inflammation (Li et al., 2017; Niu et al., 2016). Among pro-inflammatory mediators, NO and PGE_2 are some of the most important chemokines for inflammation (Li et al., 2017; An et al., 2017). Overproduction of NO and PGE_2 is associated with the overexpression iNOS and COX-2 (Lee et al., 2017). INOS and COX-2 are two main targets in current inflammation research and treatment; The production of inflammatory cytokines NO and PGE_2 would be controlled by inhibiting their activity (Khatri C K et al., 2017; Kiem, Huyen, & Hang, 2017; Lee et al., 2017). Thus, selective COX-2 and iNOS inhibitors should be given particular attention for the treatment of inflammation.

The natural product gentiopicroside belongs to iridoid glycosides, which is widely distributed in Gentianaceae plants (Xiong, Gao, Zhang, Wang, & Han, 2017; Ma, Xiao, & Wang, 2006). It is the main active constituent of many Chinese materia medica, such as *Gentiana macrophylla* Pall (Wang et al., 2015). A large number of pharmacological studies found that gentiopicroside shows many activities including antiproliferative activity, wound healing activity, and choleretic, anti-hepatotoxic, adaptogenic, and analgesic effects (Wu et al., 2017; Zhao et al., 2015). The

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anti-inflammatory activity of gentiopicroside has been reported, such as in the treatment of osteoarthritis (Zhao et al., 2015) and inflammatory bowel disease (Niu et al., 2016). Moreover, it is safe and nontoxic, and causes few side-effects (Niu et al., 2016). However, the mechanism of the anti-inflammatory activity of gentiopicroside and its relationship with inflammation targets COX-2 and iNOS are lacking.

This study aimed to determine the anti-inflammatory activity and mechanism of gentiopicroside by *in vivo*, *in vitro*, and molecular docking experiments.

2. Materials and methods

2.1. Materials and reagents

Gentiana officinalis H. Smith was obtained from GanNan (Gansu, China) and identified by Dr. Ling Jin from Gansu University of Chinese Medicine. Gentiopicroside was isolated in our laboratory. The mouse macrophage RAW 264.7 was purchased from Procell Life Science and Technology Co., Ltd. (Wuhan, China). MTT, LPS and other biological reagents were purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Griess assays (using determined NO), PGE_2, and IL-6 ELISA Kits were purchased from Neobioscience Co., Ltd. (Shenzhen, China), ZORBAX SB-C_{18} column (250 mm × 4.6 mm, 5 μm, Agilent, USA).

2.2. Animals

KM mice [SPF, (20 ± 2) g] were provided by the Laboratory Animal Center of Gansu University of Chinese Medicine (SCXK2011-0001, Lanzhou, China). The mice were housed under controlled conditions [relative humidity of (60 ± 5)% at temperature (25 ± 2) °C] at a 12/12 h day/night cycle. Standard laboratory food and water were provided. All animal experiments were approved by the Institutional Animal Care and Use Committee of Gansu University of Chinese Medicine. The mice were subjected to fasting for at least 12 h before the experiments and had free access to water (Xiong et al., 2017).

2.3. Preparation of gentiopicroside

Gentiopicroside was isolated as a white needle crystal from *G. officinalis* according to previous experiments (Zhao et al., 2015), Methods as the following: The *G. officinalis* plant (10 kg) was powdered and extracted with boiling water three times, the extract solution was subjected to chroma-tography on D101 macroporous resin column and the 30% alcohol elution was concentrated under low pressure, next, a column-chromatography on silicagel was performed with EtOAc:MeOH:H_2O (20:2:1) as the mobile phase. The purity of gentiopicroside was determined by high-performance liquid chromatography (HPLC). The HPLC analysis was performed using a ZORBAX SB-C_{18} column (250 mm × 4.6 mm, 5 μm), the temperature of the column was set at 25°C, the mobile phase was a mixture of methanol and water (25:75) at a flow rate of 1.0 mL/min, the detection wavelength was set at 274 nm. ^{13}C NMR (DEPT), ^{1}H NMR, MS, and IR spectra were used to elucidate the structure.

2.4. Ear-swelling model preparation

The mice were divided randomly into three groups, consisting of ten mice per group. Mice in each group were administered according to the medium dose in Table 1 for 3 d. The last day each animal received 0.1 mL of xylene on the anterior and posterior surfaces of the right ear lobe. The left ear was considered as control. After 15 min, the animals were killed by cervical dislocation and both ears were sampled. Circular sections were taken, using a cork borer with a diameter of 9 mm, and weighed. The degree of ear swelling was calculated based on the weight of left ear without applying xylene (Feng, Cheng, Chen, Yang, & Huang, 2010).

2.5. Anti-inflammatory activity

The anti-inflammatory activities of gentiopicroside were investigated by *in vivo* and *in vitro* experiments. In the *in vitro* study, the anti-inflammatory activities of gentiopicroside against inflammatory cytokines including NO, PEG_2, and IL-6 were evaluated in mice macrophages RAW264.7 stimulated by LPS. NO level was determined using Griess assay; PGE_2 and IL-6 levels were determined using ELISA Kits (Bist et al., 2017; Dirsch, Stuppner, & Vollmar, 1998; Guo et al., 2017; Zhang et al., 2015). The *in vivo* anti-inflammatory activities of gentiopicroside were evaluated using xylene-induced ear-swelling mice model.

2.6. Molecular docking of COX-2 and iNOS with gentiopicroside

Molecular docking experiments have realized through the calculation of Induced Fit Docking method in Schrodinger Software.

3. Results

3.1. Isolation and structure elucidation of gentiopicroside

Fig. 1A demonstrated typical chromatograms of gentiopicroside with purity > 99%. The structure of gentiopicroside (Fig. 1B) was identified on the basis of its spectral data. Data showed that the molecular formula was $C_{16}H_{20}O_9$. HR-ESI-MS at *m/z* 357.1049 $[M+H]^+$ (calcd357.1048), IR (KBr) ν (cm^{-1}) 3385.19, 2922.85, 1702.45, 1609.92, 1075.12. ^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm, *J* in Hz): 5.68 (1H, d, H-1), 7.47 (1H, s, H-3), 5.64 (1H, s, H-6), 5.05 (2H, m, H-7a, H-7b), 5.78 (1H, ddd, *J* = 24.0, 12.0, 8.0 Hz, H-8), 3.29 (1H, m, H-9), 5.25 (2H, m, H-10a, H-10b), 4.67 (1H, d, *J* = 8.0 Hz, H-1′), 3.47 (1H, s, H-2′), 3.35 (1H, overlaps, H-3′), 3.19 (1H, m, H-4′), 3.37 (1H, overlaps, H-5′), 3.92 (1H, dd, *J* = 12.0, 4.0 Hz, H-6′a),

Table 1
Effects of gentiopicroside on xylene-induced ear-swelling in mice (mean ± SD, *n* = 10).

Groups	Dose / ($mg \cdot kg^{-1}$)	Edema degree/mg	Inhibition / %
Control	–	12.81 ± 3.59	–
Celecoxib	66.67	6.91 ± 2.67**	46.05
Gentiopicroside	100	8.40 ± 3.14*	34.17

Note: $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group

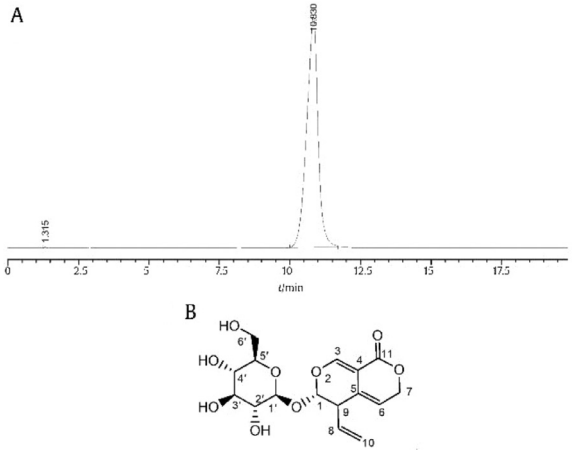

Fig. 1. HPLC chromatograms (A) and structure (B) of gentiopicroside.

3.68 (1H, dd, $J = 12.0$, 8.0 Hz, H-6′b).^{13}C NMR (100 MHz, $CDCl_3$, δ in ppm): 97.1 (C-1), 149.2 (C-3), 103.6 (C-4), 125.6 (C-5), 115.8 (C-6), 69.5 (C-7), 133.6 (C-8), 45.2 (C-9), 117.1 (C-10), 164.9 (C-11), 98.8 (C-1′), 73.2 (C-2′), 76.6 (C-3′), 70.1 (C-4′), 77.0 (C-5′), and 61.4 (C-6′).

3.2. In vitro anti-inflammatory activitiy

The mouse macrophages RAW264.7 were cultured at 37 °C in a humidified 5% CO_2 atmosphere. First, cell viability via MTT assay showed that gentiopicroside had no cytotoxicity within 100 μg/mL concentration. Accordingly, we set the concentration of compounds to 100, 50, and 25 μg/mL to examine the anti-inflammatory effects. Cells were pretreated with LPS (1 μg/mL) for 24 h except for the normal group, and then incubated with gentiopicroside (100, 50, and 25 μg/mL) for 24 h except the normal group and model group; Celecoxib was used as reference drug. NO, PGE_2, and IL-6 production from LPS-stimulated RAW 264.7 cells was assessed after treatment with gentiopicroside. Results showed that NO, PGE_2, and IL-6 production were strongly reduced (Fig. 2) after treatment (100, 50, and 25 μg/mL) compared with the model group (no drug intervention, adding equal volume of medium. $P < 0.05$).

3.3. In vivo anti-inflammatory activitiy

Ear edema induced by xylene in mice is an acute inflammatory model mediated by chemical substances. This model is mainly used to evaluate the effect of drugs on the increase of vascular permeability and inflammatory exudation in the early stage of inflammation, so as to reflect the effect of drugs on acute inflammation.

The target compounds were administered at 100 mg/kg, and Celecoxib (66.67 mg/kg) was used as reference drug. Results showed that gentiopicroside exhibited significant anti-inflammatory activities ($P < 0.05$) with an inhibition rate of 34.17% (Table 1).

3.4. Molecular docking of COX-2 and iNOS with gentiopicroside

Molecular docking experiment is a new method that using computer simulation of compound structure and related disease target to carry out molecular docking, calculate and analyze the biological activity of compound, and screen the pharmacodynamic material basis. This method could quickly and efficiently discover some new lead compounds with biological activity from the database.

The experiments results showed that inflammation target COX-2 (PDB code: 5IKR) and iNOS (PDB code: 3E7G) with gentiopicroside have strong interactions. Hydrogen bonds were formed between the sugar fragments in the gentiopicroside structure with Tyr355, Ser353, Leu352, Ser530, Arg120, and His90 of COX-2 (Fig. 3), and Glu377, Asp382, Tyr373, Tyr347, Gln263, Asn370, and Gly371 of iNOS (Fig. 4), thus, gentiopicroside had a lower docking score and binding energy (Table 2). This finding theoretically explains that gentiopicroside structure could combine with inflammation target COX-2 and iNOS effectively, and showed inhibition to the expression of inflammatory targets, it is consistent with the results of previous experiments (Abdelall et al., 2017; Qin et al., 2017). From this we came to a conclusion that gentiopicroside had the anti-inflammatory activity.

4. Discussion and conclusion

Inflammation is a common clinical diseases worldwide, it seriously threaten the health of human beings and has an increasing incidence. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the first-line drugs to treat inflammation, they mainly include COX-2 selective inhibitors, such as Celecoxib. However, NSAIDs increase the risk of cardiovascular and gastrointestinal diseases. Based on the current state of adverse reaction of NSAIDs, searching effective and safe COX-2 selective inhibitors from natural products is an effective way. The natural product gentiopicroside belongs to iridoid glycosides, which are widely distributed in Chinese herbal medicine *G. officinalis*.

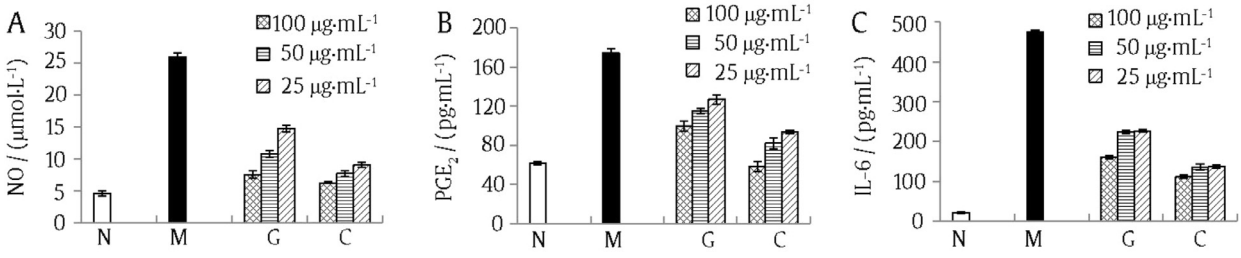

Fig. 2. Effect of gentiopicrosideon LPS-induced NO (A), PGE_2 (B), and IL-6 (C) production in RAW 264.7 macrophages. (mean ± SD, $n = 3$).
N: Normal group; M: Model group; G: Gentiopicroside group; C: Celecoxib group.

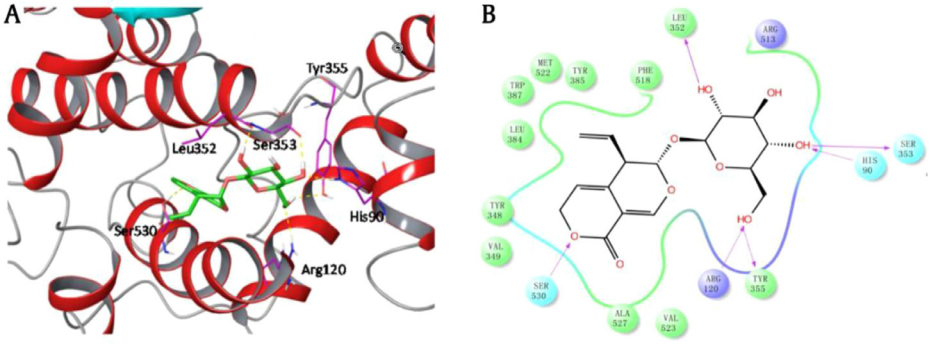

Fig. 3. 3D (A), and 2D (B) docking model of gentiopicroside with COX-2.

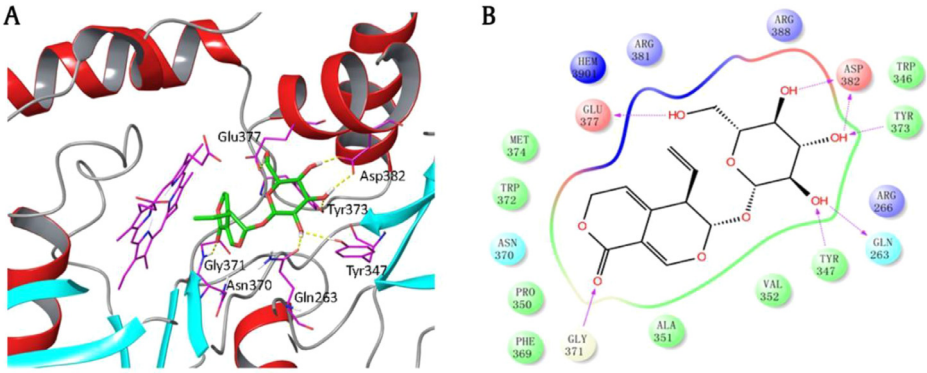

Fig. 4. 3D (A), and 2D (B) docking model of gentiopicroside with iNOS.

Table 2
Binding energy and docking score of gentiopicroside with iNOS and COX-2.

Compound	COX-2 (5ikr)		iNOS (3e7g)	
gentiopicroside	docking score	−13.329	docking score	−11.386
	mmGBSA /(kcal·mol^{-1})	−96.050	mmGBSA/(kcal·mol^{-1})	−86.877

In this paper, high-purity gentiopicroside was isolated and purified, and confirmed by HPLC, IR, NMR, and MS analyses. The anti-inflammatory activity has been investigated by *in vivo, in vitro*, and molecular docking experiments. The *in vitro* study found that gentiopicroside could inhibit the production of NO, PGE_2, and IL-6 in LPS-stimulated mouse macrophage RAW 264.7. Overproduction of NO and PGE_2 in LPS-stimulated cells is associated with the overexpression of iNOS and COX-2 in cells (Lee et al., 2017), so gentiopicroside could inhibit expression of iNOS and COX-2. The *in vivo* experiment results indicated that gentiopicroside could exhibit anti-inflammatory activity to xylene-induced mice ear-swelling model effectively, so gentiopicroside has therapeutic effect on acute inflammation mediated by chemical substances. These findings suggested that the anti-inflammatory activity of gentiopicroside was associated with the downregulation of the inflammatory cytokines NO, PGE_2, and IL-6. Molecular docking experiments indicated that gentiopicroside could effectively combine with inflammation targets COX-2 and iNOS by H-bonds and to suppress COX-2 and iNOS expression, thereby facilitating the anti-inflammatory activity. Therefore, we conclude that gentiopicroside may be a potential and selective iNOS and COX-2 inhibitor. This study will be very helpful for the design and development of novel anti-inflammatory agents in drug field.

However, the anti-inflammatory activity of gentiopicroside is lower than the first-line drug Celecoxib, likely due to the presence of sugar fragments in its structure, which resulted in high hydrophilicity. This characteristic leads to reduction of oral bioavailability, acceleration of metabolism, shortening of half-life, and restriction of the effectiveness. Our group will conduct the further research to address this issue.

Conflicts of interest

The authors declare no competing financial interest.

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References

Abdelall, E. K. A., & Abdelhamid, A. O. (2017). Synthesis and biological evaluations of new nitric oxide-anti-inflammatory drug hybrids. *Bioorganic & Medicinal Chemistry Letters, 27*(18), 4358–4369.

An, J. Y., Lee, H. H., Shin, J. S., Yoo, H. S., Park, J. S., Son, S. H., & Kim, N. J. (2017). Identification and structure activity relationship of novel flavone derivatives that inhibit the production of nitric oxide and PGE 2 in LPS-induced RAW 264.7 cells. *Bioorganic & Medicinal Chemistry Letters, 27*(11), 2613–2616.

Bist, G., Pun, N. T., Magar, T. B. T., Shrestha, A., Oh, H. J., Khakurel, A., & Lee, E. S. (2017). Inhibition of LPS-stimulated ROS production by fluorinated and hydroxylated chalcones in RAW 264.7 macrophages with structure-activity relationship study. *Bioorganic & Medicinal Chemistry Letters, 27*(5), 1205–1209.

Dirsch, V., Stuppner, H., & Vollmar, A. (1998). The griess assay: Suitable for a bio-Guided fractionation of anti-inflammatory plant extracts? *Planta Medica, 64*(05), 423–426.

Guo, R., Guo, C., He, D., Zhao, D., & Shen, Y. (2017). Two new C19-diterpenoid alkaloids with anti-inflammatory activity from *Aconitum iochanicum*. *Chinese Journal of Chemistry, 35*(10), 1644–1647.

Khatri, C. K., Indalkar, K. S., Patil, C. R., Goyal, S. N., & Chaturbhuj, G. U. (2017). Novel 2-phenyl-4,5,6,7-tetrahydro[*b*]benzothiophene analogues as selective COX-2 inhibitors: Design, synthesis, anti-inflammatory evaluation, and molecular docking studies. *Bioorganic & Medicinal Chemistry Letters, 27*(8), 1721–1726.

Kiem, P. V., Huyen, L. T., & Hang, D. T. (2017). Sesquiterpene derivatives from marine sponge *Smenospongia cerebriformis* and their anti-inflammatory activity. *Bioorganic & Medicinal Chemistry Letters, 27*(7), 1525–1529.

Jo, A., Han, H., Seo, S., Shin, J. S., Lee, J. Y., Kim, H. J., & Lee, K. T. (2017). Inhibitory effect of moschamine isolated from *Carthamus tinctorius* on LPS-induced inflammatory mediators via AP-1 and STAT1/3 inactivation in RAW 264.7 macrophages. *Bioorganic & Medicinal Chemistry Letters, 27*(23), 5245–5251.

Lee, S., Kim, D. C., Park, J. S., Son, J. Y., Hak Sohn, J., Liu, L., & Oh, H. (2017). Penicillospirone from a marine isolate of *Penicillium sp*. (SF-5292) with anti-inflammatory activity. *Bioorganic & Medicinal Chemistry Letters, 27*(15), 3516–3520.

Li, J., Li, D., Xu, Y., Guo, Z., Liu, X., Yang, H., & Wang, L. (2017). Design, synthesis, biological evaluation, and molecular docking of chalcone derivatives as anti-inflammatory agents. *Bioorganic & Medicinal Chemistry Letters, 27*(3), 602–606.

Li, S., Sun, X., Li, Y., Liu, F., Ma, J., Tong, L., & Guo, Y. (2017). Natural NO inhibitors from the leaves of *Callicarpa kwangtungensis*: Structures, activities, and interactions with iNOS. *Bioorganic & Medicinal Chemistry Letters, 27*(3), 670–674.

Niu, Y. T., Zhao, Y. P., Jiao, Y. F., Zheng, J., Yang, W. L., Zhou, R., & Yu, J. Q. (2016). Protective effect of gentiopicroside against dextran sodium sulfate induced colitis in mice. *International Immunopharmacology, 39*, 16–22.

Qin, N. B., Li, S. G., Yang, X. Y., Gong, C., Zhang, X. Y., Wang, J., & Hua, H. M. (2017). Bioactive terpenoids from *Silybum marianum* and their suppression on NO release in LPS-induced BV-2 cells and interaction with iNOS. *Bioorganic & Medicinal Chemistry Letters, 27*(10), 2161–2165.

Wang, Z., Tang, S., Jin, Y., Zhang, Y., Hattori, M., Zhang, H., & Zhang, N. (2015). Two main metabolites of gentiopicroside detected in rat plasma by LC–TOF-MS following 2,4-dinitrophenylhydrazine derivatization. *Journal of Pharmaceutical and Biomedical Analysis, 107*(3), 1–6.

Wu, S., Yang, L., Sun, W., Si, L., Xiao, S., Wang, Q., & Zhang, Y. (2017). Design, synthesis and biological evaluation of gentiopicroside derivatives as potential antiviral inhibitors. *European Journal of Medicinal Chemistry, 130*, 308–319.

Xiong, K., Gao, T., Zhang, T., Wang, Z., & Han, H. (2017). Simultaneous determination of gentiopicroside and its two active metabolites in rat plasma by LC–MS/MS and its application in pharmacokinetic studies. *Journal of Chromatography B, 1065-1066*, 1–7.

Feng, X., Cheng, G., Chen, S., Yang, H., & Huang, W. (2010). Evaluation of the burn healing properties of oil extraction from housefly larva in mice. *Journal of Ethnopharmacology, 130*(3), 586–592.

Zhang, X., Sun, J., Xin, W., Li, Y., Ni, L., & Du, G. (2015). Anti-inflammation effect of methyl salicylate 2-*O*-β-D-lactoside on adjuvant induced-arthritis rats and lipopolysaccharide (LPS)-treated murine macrophages RAW264.7 cells. *International Immunopharmacology, 25*(1), 88–95.

Zhao, L., Ye, J., Wu, G. T., Peng, X. J., Xia, P. F., & Ren, Y. (2015). Gentiopicroside prevents interleukin-1 beta induced inflammation response in rat articular chondrocyte. *Journal of Ethnopharmacology, 172*, 100–107.