

Original Research Article

***Gentiana macrophylla* Pall (Gentianaceae) extract exerts protective effects against osteoporosis in mice**

Teng Gong¹⁻³, Xue-Tao Su^{1*}, Qun Xia¹, Jing-Gui Wang¹

¹Department of Orthopedic Surgery, The Affiliated Hospital of Logistics College of Chinese People's Armed Police Force, No. 220 Chenglin Road, Dongli District, Tianjin City, 300162, ²Department of Spinal Surgery, Tianjin Hospital, Clinical Medical College of Tianjin Medical University, No. 406 Jiefang Nan road, Hexi District, Tianjin City, 300211, ³Post-doctoral Station, Tianjin Medical University, No. 22 Qixiang Tai Road, Tianjin City, 300070, China

*For correspondence: **Email:** skymberlysoul@yahoo.com; **Tel/Fax:** 0086-022-60578952

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Abstract

Purpose: To evaluate the anti-osteoporotic effect of *G. macrophylla* extract in a mice model.

Methods: Antioxidant activity was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) for radical scavenging, nitro-blue tetrazolium (NBT) reducing, and ferric reducing antioxidant power (FRAP) assays using standard biochemical procedures. The expression of some bone-related genes was measured by quantitative real-time polymerase chain reaction (qTR-PCR). Phytochemical analysis was carried out by liquid chromatography-mass spectrometry (LC/MS).

Results: The extract exhibited strong antioxidant activity as evident from DPPH, NBT and FRAP data, with half-maximal inhibitory concentration (IC₅₀) values of 275, 262 and 180 µg/ml, respectively. The antioxidant activity was concentration-dependent. The results showed that the extract restored bone characteristics by suppressing bone resorption through induction of expressions of several bone-associated genes. The results from phytochemical analyses showed the presence of loganic acid, swertiamarin, sweroside, gentiopicroside, gentesin and isogentesin.

Conclusion: These results indicate that the extract may be beneficial in the treatment of osteoporosis.

Keywords: *Gentiana macrophylla*, Osteoporosis, Bone mass, Loganic acid, Swertiamarin, Sweroside, Gentiopicroside, Gentesin

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INTRODUCTION

Osteoporosis is one of the bone degenerative disorders associated with high bone fragility and lower bone mass often leading to bone fractures [1]. It has been reported that the incidence of osteoporosis increases with increase in age, which implies that the disease is more common during old age [2]. Currently, the main option for

prevention of osteoporosis is hormone replacement therapy (HRT). However, with advancements in science and technology, HRT has now been reported to be associated with risk of development of breast and ovarian cancers in post-menopausal women [3–5]. Therefore, there is a need to develop alternative, effective, efficient and more viable treatment options for osteoporosis. In this context, it is believed that

Chinese herbal medicine may prove beneficial in treatment and management of osteoporosis.

Gentiana macrophylla belongs to family *Gentianaceae* and it is considered an important medicinal herb. It is popularly known as “Qin Jiao” and it is abundant mostly in China and Siberia. The roots of *G. macrophylla* taste bitter due to the presence of some phyto-constituents. Studies have shown that *G. macrophylla* extracts possess laxative properties, and its ethanol extract exhibits broad range antimicrobial activity [6]. Often, *G. macrophylla*, is used in combination with other *Gentiana* species for maintenance of healthy sinus, and for treating rheumatoid arthritis and osteoporosis [7]. The plant is also used in Chinese herbal medicine for the management of diabetes, osteoporosis and rheumatism [8]. However, till date, the use of *G. macrophylla* in the prevention of osteoporosis has not been validated scientifically. This study was therefore designed to evaluate the potential of *G. macrophylla* for prevention of osteoporosis. Moreover, phytochemical analysis of the extract was carried out to putatively identify the phytochemical constituents potentially responsible for its protective effect.

EXPERIMENTAL

Collection of plant material and preparation of extract

The roots of *G. macrophylla* were harvested from their natural environment in Dongli District, Tianjin, China. The plant material was identified by Teng Gong at Post-doctoral station, Tianjin Medical University and a voucher specimen was deposited in Tianjin Medical University herbarium (voucher no. TMU/GM/09854/2016). The roots were dried at room temperature, ground to a fine powder (10 kg) and then extracted at least 3 times with 90 % methanol. The extract was then concentrated under vacuum to yield the final extract weighing 2.5 kg. The extract was stored at 4 °C prior to further usage.

Evaluation of antioxidant activities

The antioxidant activities of the methanol extract of *G. macrophylla* were assessed through DPPH radical scavenging, NBT reduction, and FRAP assays as described previously [9].

Animal groups

Three month-old female virgin mice were obtained from Tianjin Medical University. The animals were kept in well-ventilated rooms with controlled setting of light/dark cycle, temperature

of 24 ± 2 °C and humidity of 40-60 %. The study was approved by the Animal Ethics Committee of Tianjin Medical University under approval number C24/TJM/0987/2016. International guidelines were followed in carrying out the animal studies [10]. The mice were subjected to either laparotomy or closure (n = 20), or laparotomy followed by bilateral oophorectomy (n = 30). They were divided into five groups. Mice that underwent laparotomy and closure formed Group I (received normal saline, n = 10) or Group II (which received 300 mg/kg of the extract, n = 10), while those that underwent laparotomy and bilateral oophorectomy formed Group III (given normal saline). Group IV mice (n = 10) were given 150 mg/kg of the extract, while Group V mice (n = 10) received 300 mg/kg of the extract. To ensure that the mice received the proper dose of the extract, the extract was administered via a gastric tube. After 12 weeks of extract administration, urine was collected and then the mice were sacrificed by deep anesthesia with isoflurane. Blood was also withdrawn and serum was isolated from the blood as described previously [11]. The tibias and uteri were excised and stored at -80 °C for further investigation.

Serum and urine analysis

The serum and urine analysis of the mice from all groups were carried out in line with standard protocols. Alkaline phosphatase (ALP), serum calcium (Ca), phosphorus (P) and urine Ca, P and creatinine levels were determined as reported previously [12].

RNA isolation and RT-PCR

Total RNA was isolated from the tibia by TRIzol as per the manufacturer's instructions, and cDNA was synthesized by cDNA synthesis (Invitrogen) using 3µg of the isolated RNAs in 20 µL reaction, with random primers. The Rt-PCR reaction conditions were: one cycle at 95 °C for 4 min, 27 cycles at 95 °C for 40 sec, 55 °C for 30 sec, and 72°C for 40 sec. The primers used in the study were as described previously [13,14].

Phytochemical analysis

Phytochemical analysis was carried out by dissolving the extracts in methanol and filtering through nylon HPLC filters (0.22 µm). Liquid chromatography–mass spectrometry (LC/MS) apparatus Nexera UHPLC (130 MPa) connected to Shimadzu MS-8030 was employed for this study. Enable RP-C18 column (250 mm × 4.6 mm, 5 µM) was used and the injection volume was 2.5 µL at a flow rate of 0.3 ml/min. Mobile

phase A was water and acetonitrile in a volume ratio of 1 : 1, while mobile phase B was acetic acid (0.1 %). The initially column temperature was 75 °C. The mass chromatograms were obtained in both positive and negative ionization modes. The scan range was between 100 and 1000 m/z.

Statistical analysis

Data are presented as mean \pm SD. Statistical analysis was done with Student's t-test and differences were considered significant at * $p < 0.01$, ** $p < 0.001$ and *** $p < 0.0001$.

RESULTS

Antioxidant activity of *G. macrophylla* extract

The results of this study revealed that in all the three assays *G. macrophylla* exhibited significant antioxidant activity. This antioxidant activity was also found to exhibit a dose-dependent trend (Figure 1 A to 1 C). At 500 $\mu\text{g/ml}$, the inhibition of the extract was 75 % in case of DPPH assay, while for NBT assay, the inhibition was 72 %. In the FRASP assay, the absorbance increased from 0.36 at 100 $\mu\text{g/ml}$, to 0.94 at 500 $\mu\text{g/ml}$, indicating the antioxidant potential of the extract. The IC_{50} values of the extract for DPPH, NBT and FRAP assays were 275, 262, and 180 $\mu\text{g/ml}$, respectively.

Effect of *G. macrophylla* extract on body weight, uterus index, and some serum and urine parameters

The results indicated that there was significant weight gain in group III mice, when compared to the group I mice (Table 1), while there was no significant difference between the weight of control (group I) mice and control mice receiving the 300 mg/kg extract (group II). However, in groups IV and V mice which received 150 and 300 mg/kg of the extract, significant reduction in weight was seen, when compared to the model animals (group III). Interestingly, the uterus index was significantly lower in group III group, when compared to group I (Table 1). However no significant changes were observed in group I mice. Administration of extract at doses of 150 mg/kg (group IV) and 300 mg/kg (group V) led to a significant increase in uterus index. In addition, ovariectomy of the mice (group III) augmented urinary calcium excretion and serum ALP level. This was in tandem with reduction in serum Ca and P levels, when compared to the sham group (group I). However, administration of 150 and 300 mg/kg of the extract reversed the ovariectomy-induced changes in serum Ca and P levels, and urinary Ca in mice ($p < 0.05$). The *G.*

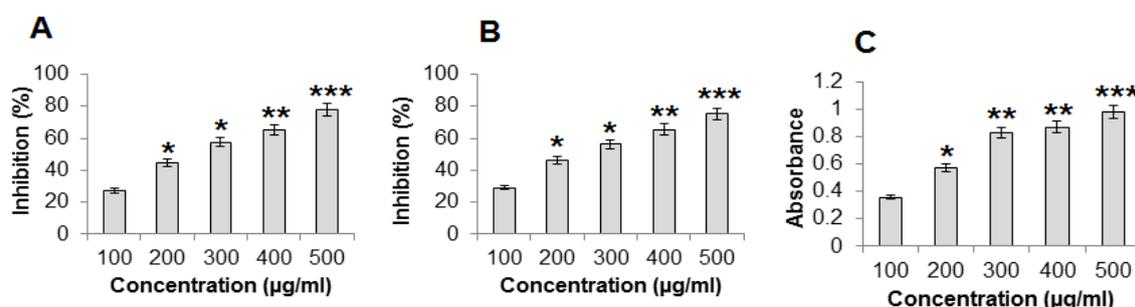


Figure 1: Antioxidant activity of methanol extract of *G. macrophylla* by (A) DPPH assay; (B) NBT assay, and (C) FRAP assay. The assays were carried out in triplicates and the values were considered significant at * $p < 0.01$, ** $p < 0.001$ and *** $p < 0.0001$

Table 1: Effect of *G. macrophylla* extract on uterus index, serum ALP, and serum and urinary levels of Ca and P. The experiments were repeated thrice in triplicates and the values were presented as mean \pm SD

Group	Weight (g)	Uterus index (mg/g)	Serum ALP (U/L)	Serum Ca (mg/dl)	Serum P (mg/dl)	Urine Ca/Cr (mg/mg)	Urine P/Cr (mg/mg)
I	1.7 \pm 0.05	3.1 \pm 0.04	70.4 \pm 3.21	10.3 \pm 0.51	9.1 \pm 0.75	0.27 \pm 0.02	4.53 \pm 0.22
II	1.6 \pm 0.03	3.2 \pm 0.02	68.4 \pm 2.78	10.6 \pm 0.76	9.0 \pm 0.66	0.25 \pm 0.01	4.55 \pm 0.32
III	2.5 \pm 0.07	1.4 \pm 0.05	90.1 \pm 4.10	7.6 \pm 0.55	7.4 \pm 0.34	0.38 \pm 0.01	5.71 \pm 0.44
IV	2.1 \pm 0.04	2.2 \pm 0.03	78.3 \pm 3.64	8.4 \pm 0.95	8.2 \pm 0.42	0.32 \pm 0.02	4.87 \pm 0.54
V	1.8 \pm 0.03	2.4 \pm 0.06	74.5 \pm 2.76	9.1 \pm 0.80	8.7 \pm 0.52	0.29 \pm 0.01	4.62 \pm 0.43

macrophylla extract administration at both doses significantly reduced serum ALP level in the OVX mice (Table 1). Furthermore, administration of the extract significantly ($p < 0.05$) decreased the urinary Ca and increased serum Ca level.

Effect of *G. macrophylla* extract on the expression of bone-specific genes

The results of this study revealed that the expression levels of *Cbfa1* was down-regulated whereas the expression of *Ctsk* was up-regulated in tibia of mouse in response to ovariectomy. However administration of 150 and 300 mg/kg extract doses (groups IV and V) led to significant ($p < 0.01$) enhancements in the

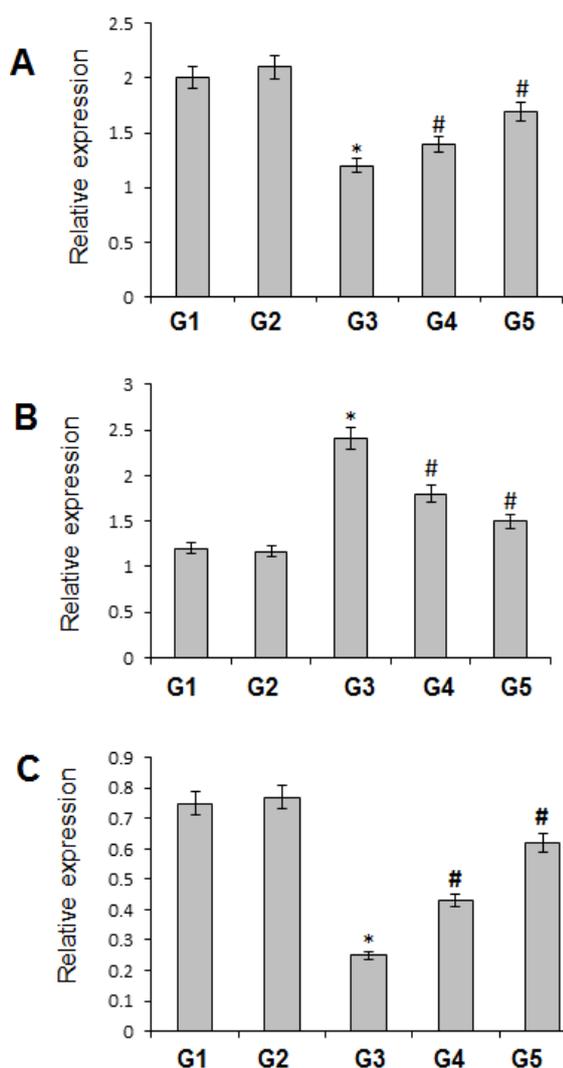


Figure 2: Effect of *G. macrophylla* extract on the expression of: (A) *Ctsk*, (B) *Cbfa1*, and (C) OPG/RANKL as determined using quantitative RT-PCR. Data are presented as mean \pm SD. The values were considered at * $p < 0.01$ G3 vs G1 and # $p < 0.01$ G3Vs G4. G1 to G5 represent groups 1 to 5

Following literature survey and m/z ratio, six compounds were putatively identified. These

transcript of *Cbfa1*, and suppression of *Ctsk* mRNA expression in mouse tibia in response to ovariectomy (Figures 2A and 2B). The results indicate that administration of extracts at doses of 150 and 300 mg/kg reversed OVX-prompted changes in the expression of OPG/RANKL in tibia (Figure 2B).

Phytochemical analysis of *G. macrophylla* extract

To gain understanding about the phyto-constituents of *G. macrophylla* that may be potentially responsible for its protective effects, LC/MS analysis was carried out (Figure 3).

included loganic acid, swertiamarin, sweroside, gentiopicroside, gentsin and isogentesin (Figure 4).

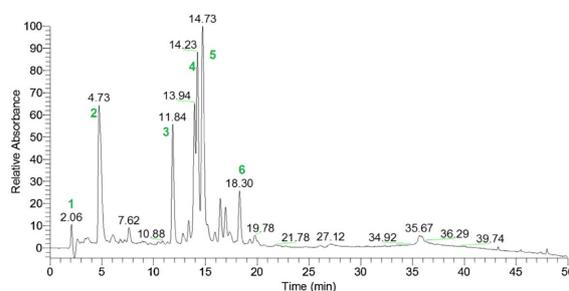


Figure 3: LC/MS chromatogram of the methanol extract of *G. macrophylla*. Peaks 1-6 were tentatively identified as shown in Figure 4

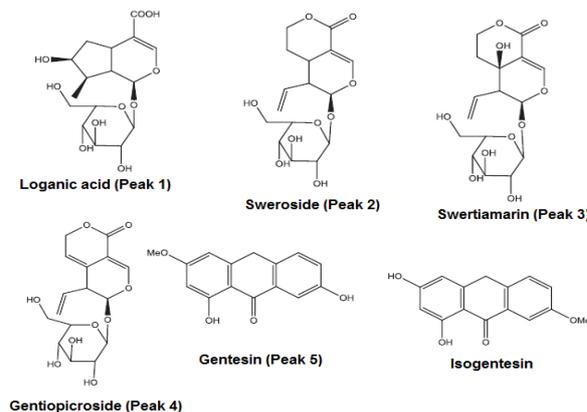


Figure 4: Structure of the compounds identified by LC/MS analysis. The peak numbers are as per chromatogram shown in Figure 3

DISCUSSION

Osteoporosis is a one of the lethal bone degenerative diseases which cause bone fragility [1]. Reactive oxygen species (ROS) have been implicated in the onset and development of different diseases and disorders including

osteoporosis. Currently, there are limited treatments available for osteoporosis, but these treatments are associated with lots of side effects. In this context, Chinese medicine is believed to be beneficial in the treatment of osteoporosis [14]. This study was therefore directed towards evaluation of the protective effects of *G. macrophylla* in experimental mice models of osteoporosis. Since ROS have been implicated in the etiology of osteoporosis, the antioxidant activity of the extract was evaluated through three different assays [15]. The results of the study indicated that *G. macrophylla* exhibited antioxidant activity in all the three assays viz DPPH, NBT and FRAP. These results are in agreement with previous reports wherein several *Gentiana* spp. were shown to exhibit antioxidant activities [16].

The effect of *G. macrophylla* was also evaluated *in vivo* in mice models and it was observed the *G. macrophylla* extract reversed the ovariectomy-induced alterations in blood and serum composition. Several plant extracts have been reported to cause such effects *in vivo* in osteoporosis mice models [17]. In addition, the extract also caused alterations in the expressions of several bone associated genes. It was observed that *G. macrophylla* extract enhanced the transcription of *Cbfa1* mRNA and cathepsin K mRNA, and increased the ratio of OPG/RANKL mRNA expression in the tibia. The results are well supported by a previous study wherein *Sambucus williamsii* exhibited similar effects [18]. The results of LC/MS analysis showed the presence of several compounds which may be potentially responsible for the protective effects of the extract. These results are consistent with previous findings on other *Gentiana* species [19].

CONCLUSION

Methanol extract of *G. macrophylla* was found to exhibit significant antioxidant activity. *In vivo* studies showed that this extract exerted protective effects in mice model of osteoporosis. Therefore, *G. macrophylla* extract may be beneficial in the treatment of osteoporosis.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Teng Gong did all the experiments under supervision of Xue-Tao Su. Qun Xia and Jing-Gui Wang collected data and offer help for analysis of the data as well.

REFERENCES

- Zhang JK, Yang L, Meng GL, Fan J, Chen JZ, He QZ, Chen S, Fan JZ, Luo ZJ, Liu J. Protective effect of tetrahydroxystilbene glucoside against hydrogen peroxide-induced dysfunction and oxidative stress in osteoblastic MC3T3-E1 cells. *Euro J Pharmacol* 2012; 689: 31-37.
- Johnell O, Kanis JA. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *OsteoporosInt* 2006; 17: 1726-1733.
- Roux C. Osteoporosis and hormone replacement therapy. *Presse Med.* 2005; 34: 379-384.
- Ryan PJ, Harrison R, Blake GM, Fogelman I. Compliance with hormone replacement therapy (HRT) after screening for post-menopausal osteoporosis. *BJOG: Int J Gynaecol Obstet* 1992; 99: 325-328.
- Nelson HD, Humphrey LL, Nygren P, Teutsch SM, Allan JD. Postmenopausal hormone replacement therapy: scientific review. *Jama* 2002; 288: 872-881.
- Jia N, Li Y, Wu Y, Xi M, Hur G, Zhang X, Cui J, Sun W, Wen A. Comparison of the anti-inflammatory and analgesic effects of *Gentiana macrophylla* Pall and *Gentiana straminea* Maxim., and identification of their active constituents. *J Ethanopharmacol* 2012; 144: 638-645.
- Yu F, Yu F, Li R, Wang R. Inhibitory effects of the *Gentiana macrophylla* (Gentianaceae) extract on rheumatoid arthritis of rats. *J Ethanopharmacol* 2004; 95(1): 77-81.
- Jia N, Chu W, Li Y, Ding L, Duan J, Cui J, Cao S, Zhao C, Wu Y, Wen A. Iridoid glycosides from the flowers of *Gentiana macrophylla* Pall. ameliorate collagen-induced arthritis in rats. *J Ethanopharmacol* 2016; 189: 1-9.
- Baba SA, Malik AH, Wani ZA, Mohiuddin T, Shah Z, Abbas N, Ashraf N. Phytochemical analysis and antioxidant activity of different tissue types of *Crocus sativus* and oxidative stress alleviating potential of saffron extract in plants, bacteria, and yeast. *S Afr J Bot* 2015; 99: 80-87.
- McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL. Guidelines for reporting experiments

- involving animals: the ARRIVE guidelines. *British J Pharmacol*, 2010; 160: 1573-6.
11. Teahan O, Gamble S, Holmes E, Waxman J, Nicholson JK, Bevan C, Keun HC. Impact of analytical bias in metabolomic studies of human blood serum and plasma. *Analytical Chem* 2006; 78: 4307-18.
 12. Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, Nicholson JK. Metabolic profiling, metabolomic and metabolomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc* 2007; 2: 2692-703.
 13. Trouvin AP, Goeb V. Receptor activator of nuclear factor- κ B ligand and osteoprotegerin: maintaining the balance to prevent bone loss. *Clin Interv Aging* 2010; 5: 345-354.
 14. Zhang H, Xing WW, Li YS, Zhu Z, Wu JZ, Zhang QY, Zhang W, Qin LP. Effects of a traditional Chinese herbal preparation on osteoblasts and osteoclasts. *Maturitas* 2008; 61: 334-339.
 15. Baba SA, Malik SA. Evaluation of antioxidant and antibacterial activity of methanol extracts of *Gentiana kurroo royle*. *Saudi J BiolSci* 2014; 21: 493-498.
 16. Pereira JV, Modesto-Filho J, de FAgra M, Barbosa-Filho JM. Plant and plant-derived compounds employed in prevention of the osteoporosis. *Acta Farm Bonaer* 2002; 21: 223-34.
 17. Zhang Y, Li Q, Li X, Wan HY, Wong MS. *Erythrina variegata* extract exerts osteoprotective effects by suppression of the process of bone resorption. *Br J Nutr* 2010; 104: 965-971.
 18. Zhang Y, Li Q, Wan HY, Xiao HH, Lai WP, Yao XS, Wong MS. Study of the mechanisms by which *Sambucus williamsii* HANCE extract exert protective effects against ovariectomy-induced osteoporosis in vivo. *Osteoporosis Int* 2011; 22: 703-709.
 19. Wani BA, Ramamoorthy D, Rather MA, Arumugam N, Qazi AK, Majeed R, Hamid A, Ganie SA, Ganai BA, Anand R, et al., . Induction of apoptosis in human pancreatic MiaPaCa-2 cells through the loss of mitochondrial membrane potential ($\Delta\Psi_m$) by *Gentiana kurroo* root extract and LC-ESI-MS analysis of its principal constituents. *Phytomed* 2013; 20: 723-33.