Synergic effect in the reduction of serum uric acid level between ethanol extract of *Aster glehni* and vitamin B₆

Eun Hye Han¹ · Mi Kyung Lim¹ · Sang Ho Lee¹ · Hyoung Ja Kim² · Dahyun Hwang^{3,4}

Received: 25 January 2018 / Revised: 25 April 2018 / Accepted: 28 April 2018 / Published online: 8 June 2018
© The Korean Society of Food Science and Technology and Springer Science+Business Media B.V., part of Springer Nature 2018

Abstract In this study, synergistic hypouricemic activities between ethanol extract of *Aster glehni* (AG) and vitamin B₆ were investigated in vitro and in vivo. Xanthine oxidase inhibitory activities in the different parts, leaf, stem, and flower, during spring and autumn were compared. In addition, to improve hypouricemic activity, two chemicals (AG extract and vitamins) were mixed and measured inhibitory activity of xanthine oxidase. As a result, autumn leaf AG extracts showed the most effective xanthine oxidase inhibitory activity and we named autumn leaf AG extracts as AG-D006. In synergistic study, AG-D006 with vitamin B₆ showed significantly increased inhibitory activity on xanthine oxidase. AG-D006 with vitamin B₆ also showed significantly reduced uric acid level in hyperuricemic rats in vivo. In conclusion, AG-D006 with vitamin B₆ might be used functional foods in reducing serum uric acid level in gout.

Keywords *Aster glehni* · Vitamin B₆ · Xanthine oxidase · Hyperuricemic · Synergism

Introduction

Gout, one of the most prevalent inflammatory arthritides (Roddy, 2008), has historically known as “the disease of kings” or “rich man’s disease” (Chanqizi et al., 2012). The prevalence of gout increased more and more and 90% of gout patients were male and they are mostly in the middle age (mean age was 55.2) (Lee and Sung, 2011; Seo et al., 2011). Several studies have reported that gout is associated with increased serum uric acid level and gout caused by accumulation of uric acid crystals (Chen et al., 2009; Kuo et al., 2015). Men are more frequently affected by gout than women because men have lower ability to remove uric acid than woman. Since women have specific hormones which enhance uric acid excretion before menopause, serum uric acid level can be controlled appropriately (Sumino et al., 1999).

Eating high-purine foods such as fish, meat, or alcohol can cause a gout (Roddy et al., 2007). When purine is metabolized by xanthine oxidase (XO) in human body, hypoxanthine and xanthine oxidized from purine are converted into uric acid (Kanellis et al., 2004). Elevated serum uric acid forms solid crystal which deposits in tissues around joints, causing pain and inflammation (Chen et al., 2009).

Until now, allopurinol, benzobromarone, and febuxostat have been used to treat gout. These medicines reduce production of uric acid by inhibiting XO. However, these medicines have caused various side effects, including rash, fever, hepatotoxicity, and even death (Hassan et al., 2011; Singer and Wallace, 1986). In spite of the side effects, patients are taking risks because there was no alternative.

As an alternative treatment for gout, plants-based extracts are considering as important sources of nutraceuticals because of its considerable benefits such as safety in

✉ Dahyun Hwang
hdh@hoseo.edu

¹ R&D Center, Koreaeundon, Seongnam, Gyeonggi 13207, Republic of Korea

² Molecular Recognition Research Center, Korea Institute of Science and Technology, Seoul 02792, Republic of Korea

³ Department of Biomedical Laboratory Science, College of Life and Health Sciences, Hoseo University, 20, Hoseo-ro 79 beon-gil, Baebang-eup, Asan-si, Chungcheongnam-do 31499, Republic of Korea

⁴ The Research Institute for Basic Sciences, Hoseo University, Asan, Chungnam 31499, Republic of Korea

therapeutic agent (Nasri et al., 2014). *Aster glehni* (AG), a native plant of Ulleung Island in Republic of Korea, has known for its antioxidant effect (Kim et al., 2010). A recent study reported that ethyl acetate fraction from AG inhibited hyperuricemic activity (Son et al., 2015). Then, XO inhibitory effect was confirmed by ethanol extract of AG. Ethanol extract of AG was also treated with vitamins, because vitamin C supplementation helps reducing serum uric acid level (Huang et al., 2005). Experiments were designed in vitro and in vivo and measured inhibitory activity of XO and reduced uric acid level in serum of animal, respectively.

Materials and methods

Reagents

Allopurinol, xanthine, XO, dimethyl sulfoxide (DMSO), vitamin B₆, and potassium oxonate (PO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Vitamins B₁, B₃, B₉, B₁₂, and C were obtained from DSM Biochemicals (Het Overloon, Heerlen, Nederland). 3,5-Dicaffeoylquinic acid (3,5-DCQA) was purchased from MedChem Express. (Monmouth Jct., NJ, USA).

Ethanol extracts preparation

The parts (leaf, stem, flower) of AG collected in Ulleung Island during spring and autumn were naturally dried. The pulverized AG sample (2 kg) was extracted with 25 L of 70% ethanol in a fermenter (KFC-30L, Korea Fermentor, Republic of Korea) at 80 °C for 4 h stirring rate at 150 rpm. After collecting 1st extracts, 20 L of 70% ethanol was added into the fermenter. The samples in the fermenter were extracted again at 80 °C for 2 h, thus collecting 2nd extracts. 15% perlite equal to 15% of extracts was added into the 1st and 2nd extracts. The extracts were filtered by using a filter press (pore size 20 µm). The filtrates were concentrated to 20 °Bx by a concentrator (R10028, Dooyoung hi-tech, Republic of Korea). The concentrates adding the same amount of dextrin were sterilized by an autoclave (AC-03, Jeio Tech, Korea) at 95 °C for 1 h. After sterilization, the concentrates were spray dried and filtered through a 60-mesh sieve.

HPLC condition and content analysis of 3,5-DCQA in AG-D006

A standard reference 3,5-DCQA was prepared at a concentration of 50 µg/mL with 30% MeOH and filtered through 0.2 µm membrane filter. Content of 3,5-DCQA was determined by HPLC system (Waters ACQUITY Arc

system, Waters, Milford, MA, USA) equipped with Kromasil 100-5-C18 columns (250 × 4.6 mm, 5 µm, AKZO NOBEL, Bohus, Sweden) and Waters 2998 PDA detector (Waters). Column temperature was 25 °C and a mixed solvent of 0.5% (v/v) phosphoric acid in water (A) and 0.5% (v/v) phosphoric acid in acetonitrile (B) was used as mobile phase at a flow rate of 0.8 mL/min and eluent system was programmed as follows: 0 → 5 min (B 23%), 5 → 15 min (B 23% → 25%), 15 → 16 min (B 25% → 90%), 20 → 21 min (B 90% → 77%), 21 → 26 min (B 23%). Injection volume was 5 µL and detection of 3,5-DCQA was performed at a wavelength of 330 nm. To determine a quantity of 3,5-DCQA in the samples, 10 µg/mL of AG-D006 was prepared in 30% MeOH and sonicated for 10 min. Then, the solution was filtered by 0.2 µm membrane filter. After conducting analysis, the quantity of 3,5-DCQA was calculated by the formula below;

$$\text{Content}(\%) = \frac{A_t \times W_s \times D_t}{A_s \times W_t \times D_s} \times C_s$$

At peak area of test sample, A_s peak area of standard, W_s amount (g) of test sample, W_t amount (g) of standard, D_t dilution factor of test sample, D_s dilution factor of standard, C_s content (%) of standard.

XO inhibition assay in vitro

Inhibitory effect on XO was measured, following the method described by Son et al. (2015). Samples dissolved in DMSO or D.W were mixed with 0.1 M potassium phosphate buffer (pH 7.5) and 0.02 U/mL of XO and pre-incubated at 25 °C for 10 min. After finishing the pre-incubation, the reaction was initiated by 0.12 mM xanthine and the mixture was incubated at 25 °C for 30 min. The reaction was stopped by adding 1 N HCl. Absorbance was measured at 295 nm by spectrophotometer (Epoch2; Bio-Tek, Winooski, VT, USA). The degree of XO inhibition was calculated by the formula below;

$$\% \text{Inhibition} = \left[\frac{A_c - A_s}{A_c} \right] \times 100$$

A_c absorbance of control (sample in absence of XO inhibitors), A_s absorbance of sample (sample with potential XO inhibitors).

IC_{50} value was obtained by nonlinear regression analysis of a plot and data were expressed mean ± SD.

Animal study using PO-induced hyperuricemia rats

SD rats weighing 240 ± 20 g were purchased from the OrientBio (Gyeonggi, Republic of Korea). The rats were housed in environmentally controlled cages (temperature:

Table 1 Animal group

Group	Treatment	
	Agents	Dose (mg/kg/day)
1	Normal group	
2	Negative control	
3	Allopurinol	50
4	AG-D006	75
5	AG-D006	225
6	AG-D006	375
7	Vitamin B ₆	25
8	Vitamin B ₆	75
9	Vitamin B ₆	125
10	AG-D006	75
	Vitamin B ₆	25
11	AG-D006	225
	Vitamin B ₆	75
12	AG-D006	375
	Vitamin B ₆	125

24 ± 1 °C, humidity: 55 ± 5%) with 12 h light–dark cycle. All animals were given water and diet which was provided ad libitum. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996). All experimental procedures were approved by the Korea University Institutional Animal Care and Use Committee (Approval No. 000KUIACUC-2016-000).

Hyperuricemia rat was induced by PO, an uricase inhibitor for this study (Taejarernwiriyaikul et al., 2015). The animals were randomly divided into twelve groups (n = 8) (Table 1). Normal control only received water and 0.9% saline. Other groups received 300 mg/kg of PO suspended in 0.9% saline and PO were injected intraperitoneally in last day. AG-D006 and vitamin B₆ were orally administered once daily from day 1 to day 7. Allopurinol was used positive control and suspended in water before administration. Allopurinol was orally administered once in last day. Last day, rats were sacrificed, and blood samples were collected and allowed to clot at room temperature for 1 h.

Fig. 1 HPLC chromatogram of 3,5-DCQA. The HPLC chromatogram of standard material (A) and AG-D006 (B). The analysis of HPLC was performed on Kromasil 100-5-C18 columns (250 × 4.6 mm, 5 μm) with flow rate at 0.8 mL/min. 5 μL of sample was injected. Mobile phases were phosphoric acid and distilled water (D.W) [0.5:99.5 (v/v)]

And then, samples were centrifuged at 10,000 g at 4 °C for 20 min to separate out the serum. Uric acid was measured by biochemical blood analyzer (Hitachi 7180, Hitachi, Japan).

Results and discussion

Measurement of 3,5-DCQA in AG-D006

We chose 3,5-DCQA as a marker compound in AG-D006, because 3,5-DCQA occupy the highest proportion in HPLC analysis. In HPLC data analysis, the peaks which can interrupt 3,5-DCQA peak were not detected in the blank solution. Standard material of 3,5-DCQA with a purity of 98.86% was clearly detected at the retention time of 11.621 min in HPLC chromatogram [Fig. 1(A)]. Based on retention time of standard, the peak at 11.613 min shown in Fig. 1(B) was identified as 3,5-DCQA, and content of 3,5-DCQA in AG-D006 was calculated. Consequently, the result of HPLC analysis revealed that 3,5-DCQA content in AG-D006 was calculated as 0.51% (w/w).

XO inhibitory effect of AG in different parts of plant and seasons

Many of previous studies have reported that plants-based extracts reduced XO, a key targeting enzyme of gout (Gholamhoseinian et al., 2017). Because XO catalyzes the formation of uric acid from xanthine, inhibition of XO prevents the uric acid formation (Maiuolo et al., 2016). According to previous study (Son et al., 2015), AG extracted ethyl acetate showed hypouricemic activity, however, the solvent of ethyl acetate did not use for human consumption. So in this study, we used ethanol extracts of AG to investigate of hypouricemic activity. At a first, we screened inhibition activity of XO in different collecting seasons and each parts of the plant. It is because each plant parts in different harvesting seasons have different chemical compositions and bioactivity (Shih et al., 2011). After separating AG into leaf, stem, and flower, each part was extracted by 70% ethanol. To compare XO inhibitory activity among parts of plant, we calculated IC_{50} . IC_{50} value was obtained by nonlinear regression analysis of sample concentrations/inhibition of XO (%) (data not shown). As a result, IC_{50} of leaf extracts was 645.86 $\mu\text{g/mL}$. XO was less inhibited by the stem and flower of AG. IC_{50} values were 809.2 $\mu\text{g/mL}$ in stem and flower did not show inhibition effect [Fig. 2(A)]. We used allopurinol as a positive control, and measured XO inhibition. IC_{50} value of allopurinol was 20.23 $\mu\text{g/mL}$ (data not shown).

To compare the seasonal effect on XO inhibitory activity, AG were harvested in spring and autumn, and

Fig. 2 XO inhibitory effect of AG extracts. Inhibition of XO (%) was calculated according to XO inhibition assay in methods. We compared inhibition of XO (%) in the different parts, leaf, stem, and flower (A), during spring and autumn (B). Each bar represents the mean $\pm$ SD. ^{abc}Values not sharing a common superscript vary significantly ($P < 0.05$) from each other (leaf, stem, and flower). The symbols indicate the level of significance ($*P < 0.05$) between AG-Spring and AG-Autumn

extracted by 70% ethanol. As a result, extract of AG harvested in spring did not show inhibited XO. On the other hand, the XO inhibitory effect of AG harvested in autumn was significantly increased compared to spring [Fig. 2(B)]. Since the autumn leaf of AG showed the most effective inhibitory activity on XO in comparison to other parts and season, the autumn leaf of AG was selected as a sample (AG-D006) and used other study.

Synergistic effect of XO inhibition in AG-D006 with vitamin B₆ in vitro

To investigate of synergistic effect between AG-D006 and vitamins (vitamins B₁, B₃, B₆, B₉, B₁₂, and C) were tested. Even though human require in relatively small quantities of vitamins (some milligrams to micrograms per day), they are key regulators for maintaining body functions to be

Fig. 3 Synergistic effect of XO inhibition in AG-D006 with vitamin B₆. Inhibition of XO (%) was calculated according to XO inhibition assay in methods. We compared inhibition of XO (%) between AG-D006 and AG-D006 with vitamin B₆. Each bar represents the mean $\pm$ SD. The symbols indicate the level of significance (* P < 0.05) between AG-D006 and AG-D006 with vitamin B₆

healthy (Lukaski, 2004). For example, vitamin C supplementation lead to the lower serum uric acid levels (Choi et al., 2009). First, we screened single substance effect on XO inhibition. As a result, vitamins B₆, B₉, and C showed XO inhibitory activity (data not shown). To investigate of synergistic effect, we treated AG-D006 with vitamins B₆,

B₉, and C. Various concentrations AG-D006 were treated with various concentrations of vitamins B₆, B₉, and C. As a result, AG-D006 with vitamin B₆ showed synergistic effect on XO inhibition. Firstly, we screened concentration of vitamin B₆ for optimizing synergy effect and as a result, over 500 μ g/mL showed synergistic activity (data not shown). We fixed concentration of vitamin B₆ as 1000 μ g/mL (which is close to IC₅₀ value of itself) and treated with various concentrations of AG-D006. As a result, remarkable inhibitory effects on XO were observed (Fig. 3). AG-D006 with vitamin B₆ showed about 100% inhibition even though single substance of 200 μ g/mL of AG-D006 inhibited XO slightly. All concentrations (200–1400 μ g/mL) of AG-D006 with vitamin B₆ showed significantly increased XO inhibition activity compare to single treatment of AG-D006.

Synergistic effect of hypouricemic activity in AG-D006 with vitamin B₆ in vivo

To investigate hypouricemic activity in AG-D006 with vitamin B₆ in vivo, we used PO-induced hyperuricemia rats. We used PO-induced hyperuricemia rats to analyze reduced serum uric acid level by chemicals. The PO blocks the effect of hepatic uricase and successfully produces hyperuricemia in rats (Stavric and Nera, 1978). Allopurinol

Fig. 4 Synergistic effect of AG-D006 with vitamin B₆ on serum uric acid level. Uric acid level in serum was analyzed in last day. Each bar represents the mean $\pm$ SD. Normal control indicates mice treated with saline. The symbols indicate the level of significance (* P < 0.05 and ** P < 0.01 compared with normal control group; * P < 0.05 and ** P < 0.01 compared with the group treated with PO only)

is a potent inhibitor of XO, and is commonly used a positive control for rat model of hyperuricemia. To measure of synergistic effect between AG-D006 and vitamin B₆, we divided animal group into twelve and treated different concentrations of AG-D006 and vitamin B₆ (Table 1). As a result, PO is well-induced hyperuricemia in rats, because uric acid levels were significantly increased compared to normal control. Allopurinol-treated rats, a positive control group, showed 87.1% reduction of uric acid level compared to control. Among sample-treated groups, only 225 mg/kg AG-D006 with 125 mg/kg vitamin B₆ showed significantly reduced uric acid level about 15.9% (Fig. 4). Overall, AG-D006 mixed vitamin B₆ showed better hypouricemic activity than single substance. This result means that AG-D006 with vitamin B₆ showed synergistic effect in hypouricemic activity in vivo and this result is in agreement with XO inhibition activity in vitro.

Acknowledgements This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (115005-3).

References

- Chanqizi AS, Golestanpour A, Shamsi M, Tabatabaei S, Ramazani M. Rhazes' prescriptions in treatment of gout. *Iran. Red Crescent Med. J.* 14: 108–112 (2012)
- Chen JH, Chuang SY, Chen HJ, Yeh WT, Pan WH. Serum uric acid level as an independent risk factor for all-cause, cardiovascular, and ischemic stroke mortality: A chinese cohort study. *Arthritis Rheum.* 61: 225–232 (2009)
- Choi HK, Gao X, Curhan G. Vitamin C intake and the risk of gout in men: a prospective study. *Arch. Intern. Med.* 169: 502–507 (2009)
- Gholamhoseinian A, Sharifi-Far F, Rahimi-Naiini M. Screening of methanol extracts of sixty plants from Kerman for their potential xanthine oxidase inhibitory activity. *J. Herb med Pharmacol.* 6:126–129 (2017)
- Hassan S, Wetz R, Zouein E. Allopurinol causing drug rash with eosinophilia and systemic symptoms syndrome: A challenging diagnosis. *Int. J. Gen. Med.* 4: 789–792 (2011)
- Huang HY, Appel LJ, Choi MJ, Gelber AC, Charleston J, Norkus EP, Miller ER. The effects of vitamin c supplementation on serum concentrations of uric acid: Results of a randomized controlled trial. *Arthritis Rheumatol.* 52: 1843–1847 (2005)
- Kanellis J, Feig DI, Johnson RJ. Does asymptomatic hyperuricaemia contribute to the development of renal and cardiovascular disease? An old controversy renewed. *Nephrology* 9: 394–399 (2004)
- Kim HH, Park GH, Park KS, Lee JY, An BJ. Anti-oxidant and Anti-inflammation Activity of Fractions from *Aster glehni* Fr. Schm. *Kor. J. Microbiol. Biotechnol.* 38: 434–441 (2010)
- Kuo CF, Grainge MJ, Zhang W, Doherty M. Global epidemiology of gout: prevalence, incidence and risk factors. *Nat. Rev. Rheumatol.* 11: 649–662 (2015)
- Lee CH, Sung NY. The prevalence and features of korean gout patients using the national health insurance corporation database. *J. Rheum. Dis.* 18: 94–100 (2011)
- Lukaski HC. Vitamin and mineral status: effects on physical performance. *Nutrition* 20: 632–644 (2004)
- Maiuolo J, Oppedisano F, Gratteri S, Muscoli C, Mollace V. Regulation of uric acid metabolism and excretion. *Int. J. Cardiol.* 213: 8–14 (2016)
- Nasri H, Baradaran A, Shirzad H, Rafieian-Kopaei M. New concepts in nutraceuticals as alternative for pharmaceuticals. *Int. J. Prev. Med.* 5: 1489–1499 (2014)
- Roddy E. Hyperuricemia, gout, and lifestyle factor. *J. Rheumatol.* 35: 1689–1691 (2008).
- Roddy E, Zhang W, Doherty M. The changing epidemiology of gout. *Nat. Clin. Pract. Rheumatol.* 3: 443–449 (2007)
- Seo YI, Son KM, Jung YO, Kim IJ, Kim KL, Bae YD, Kim HA. Survey of current trends for diagnosis and treatment in korean gout patients. *J. Rheum. Dis.* 18: 187–192 (2011)
- Shih MC, Chang CM, Kang SM, Tsai ML. Effect of different parts (leaf, stem and stalk) and seasons (summer and winter) on the chemical compositions and antioxidant activity of *Moringa oleifera*. *Int. J. Mol. Sci.* 12: 6077–6088 (2011)
- Singer JZ, Wallace SL. The allopurinol hypersensitivity syndrome. Unnecessary morbidity and mortality. *Arthritis Rheum.* 29: 82–87 (1986)
- Son MJ, Jin C, Lee YS, Lee JY, Kim HJ. Characterization of Caffeoylglucoside Derivatives and Hypouricemic Activity of the Ethyl Acetate Fraction from *Aster glehni*. *Bull. Korean Chem. Soc.* 36: 503–512 (2015)
- Stavric B, Nera E. Use of the uricase-inhibited rat as an animal model in toxicology. *Clin. Toxicol.* 13: 47–74 (1978)
- Sumino H, Ichikawa S, Kanda T, Nakamura T, Sakamaki T. Reduction of serum uric acid by hormone replacement therapy in postmenopausal women with hyperuricaemia. *Lancet* 354: 650 (1999)
- Taejarernwiriyaikul O, Anzai N, Jutabha P, Kruanamkam W, Chuanluang S. Hypouricemia and nephroprotection of *Coix lacrym-jobi* L. seed extract. *Songklanakarin J. Sci. Technol.* 37: 441–447 (2015)