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Hypouricemic Effect of Ethanol Extract of *Aster glehni* Leaves in Potassium Oxonate-Induced Hyperuricemic Rats

Ji-Eun Park,[1] **Zia Yeom**,[2] **Keun-Tae Park**,[2,3] **Eun Hye Han**,[4] **Heui Jong Yu**,[5] **Hyo Seok Kang**,[5] **Young-Hee Lim**[1,2,6]

[1]Department of Public Health Science (Brain Korea 21 PLUS Program), Graduate School, Korea University, Seoul 02841, Korea
[2]Department of Integrated Biomedical and Life Sciences, Graduate School, Korea University, Seoul, 02841, Korea
[3]Research and Development Center, Milae Resources ML Co. Ltd., Seoul 05542, Korea
[4]Department of R&D, Koreaeundan Co., Seongnam 06105, Korea
[5]Bioland Co. Ltd., Ansan 15407, Korea
[6]Department of Laboratory Medicine, Korea University Guro Hospital, Seoul 08308, Korea

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Correspondence to
Young-Hee Lim
Department of Public Health Science (Brain Korea 21 PLUS Program), Graduate School, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Korea.
E-mail: yhlim@korea.ac.kr

ORCID iDs
Ji-Eun Park
https://orcid.org/0000-0002-0741-9563
Zia Yeom
https://orcid.org/0000-0002-2981-2008
Keun-Tae Park
https://orcid.org/0000-0003-4490-0388
Eun Hye Han
https://orcid.org/0000-0001-7834-5218
Heui Jong Yu
https://orcid.org/0000-0002-4228-4983
Hyo Seok Kang
https://orcid.org/0000-0003-2276-8005
Young-Hee Lim
https://orcid.org/0000-0003-3634-6698

ABSTRACT

The prevalence of gout is increasing worldwide, and control of serum uric acid level has been regarded as one of the therapeutic methods for gout. Inhibition of xanthine oxidase (XO) activity which can oxidize hypoxanthine to uric acid has been commonly proposed to decrease serum uric acid level. The aim of this study was to demonstrate the hypouricemic effect of ethanol extract of *Aster glehni* leaves (EAG) by in vitro and in vivo study in potassium oxonate (PO)-induced hyperuricemic rats. EAG possessed 132.5 ± 6.8 mg QE/g of total flavonoid and showed antioxidant activity. EAG showed in vitro and in vivo inhibitory activity against XO and significantly decreased serum uric acid level in PO-induced hyperuricemic rats without liver toxicity. These results show that EAG significantly attenuates hyperuricemia by inhibiting XO activity, which resulted in the decrease of serum uric acid level. Therefore, EAG might possess a potential therapeutic ability for improving gout.

Keywords: *Aster glehni*; Hyperuricemia; Xanthine oxidase; Serum uric acid

INTRODUCTION

Hyperuricemia is characterized by a high level of uric acid in the blood. The crystallization of uric acid is considered as a key risk factor for the development of gout [1]. Epidemiological studies from different countries have shown that the prevalence of gout is increasing worldwide [2,3]. Increased level of uric acid not only causes gout but also leads to the development of various diseases such as cardiovascular disorders, obesity, diabetes, and renal disease [4]. It is widely accepted that control of serum uric acid level might play an important role in the prevention and treatment of those diseases. For this reason, development of drugs or agents capable of decreasing the level of uric acid is obviously needed to control those diseases.

Funding

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Conflict of Interest

The authors declare that they have no competing interests.

Xanthine oxidase (XO) mainly present in liver and intestine can oxidize hypoxanthine to xanthine and xanthine to uric acid. Inhibition of XO is commonly proposed to treat hyperuricemia by decreasing uric acid level. Allopurinol is widely used as a therapeutic agent for hyperuricemia because of its ability to inhibit XO activity. However, it has some side effects such as allergy, hypersensitivity reactions, and nephropathy [5]. A number of attempts have been made to find effective compounds that inhibit XO without side effects from traditional medicines and plants. Potential therapeutic agents for treating gout have been reported from soy sauce [6], *Phyllanthus niruri* methanol extract and lignans isolated from *P. niruri* [7], phytochemicals from *Tradescantia albiflora Kunth* extracts including bracteanolide A and butenolide [8], and astilbin [9].

Aster glehni, grown in Ulleung Island, Korea, has been used as food and traditional medicine in Korea. It has been reported that *A. glehni* shows beneficial effects on hypercholesterolemia, diabetes mellitus, cardiovascular disease, sedative effects, and insomnia [10]. *A. glehni* has been approved as a safe food source by Ministry of Food and Drug Safety in Korea. In this study, we prepared an ethanol extract of *A. glehni* leaves (EAG) and tested its effects on hyperuricemia in vitro and in vivo. To assess the hypouricemic effect in vivo, we used potassium oxonate (PO)-induced hyperuricemia in a rat model, in which hyperuricemia is induced by oral administration of PO (250 mg/kg, *b.w.*).

MATERIALS AND METHODS

Chemicals and reagents

Xanthine, XO from microbial, allopurinol, aluminum chloride, Folin-Ciocalteau reagent, quercetin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and PO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Daejung Chemicals & Materials CO., Ltd. (Siheung, Korea). Sodium chloride was purchased from Duksan (Ansan, Korea).

Preparation of the EAG

Leaves of *A. glehni* were collected from Ulleung Island and dried naturally. The dried leaves were pulverized. The pulverized sample (2 kg) was extracted with 25 L of 70% ethanol in a fermenter (KFC-30L; Korea Fermentor, Incheon, Korea) at 80°C for 4 hours while stirring at 150 rpm. After collecting 1st extract, 20 L of 70% ethanol was added into the fermenter. The sample in the fermenter was extracted again at 80°C for 2 hours, thus 2nd extract was collected. Perlite was added into 1st and 2nd extracts to be the final concentration of 15%. The extracts were filtered by a filter press (pore size 20 mm). The filtrates were combined and concentrated to 20 Brix by a concentrator (R10028; Dooyoung Hi-Tech, Seoul, Korea). After adding the same amount of dextrin into the concentrate, it was sterilized by an autoclave (AC-03; Jeio Tech, Seoul, Korea) at 95°C for 1 hour. After sterilization, the concentrate was spray dried. The spray dried sample was filtered through a 60-mesh sieve and the test material was prepared. The EAG used in this study was deposited in department of R&D, Koreaeundan Co. (Seongnam, Korea). 3,5-Dicaffeoylquinic acid (3,5-DCQA), a marker compound, was analyzed by high performance liquid chromatography (HPLC) (Agilent 1100 series, Agilent G1365B multiple wavelength detector [MWD]; Agilent, Santa Clara, CA, USA) at 330 nm. The analysis was performed on Kromasil 100-5-C_{18} column (250 × 4.6 mm, 5 μm) with flow rate at 1.4 mL/min. The mobile phase was phosphoric acid and distilled water (0.5:99.5, v/v). Standard 3,5-DCQA was purchased from Sigma-Aldrich. EAG used in this study contained 0.04% of 3,5-DCQA (data not shown).

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Analysis of total phenolic content (TPC)

The TPC of EAG was determined using the method described previously [11] with some modifications. To a 96-well plate, 25 μL of test sample and 75 μL of 10% Folin-Ciocalteau reagent were added and left for 5 minutes at room temperature. After 5 minutes, 100 μL of 7.5% sodium carbonate was added and incubated for 60 minutes in the dark with shaking. Following incubation for 60 minutes, the absorbance was measured at 725 nm using a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA). The TPC was calculated as gallic acid equivalents (GAEs). All the determinations were carried out 3 times.

Analysis of total flavonoid content (TFC)

The TFC of EAG was determined using the aluminum chloride assay described previously [12] with some modifications. To a 96-well plate, 40 μL of the test sample and 60 μL of 5% $AlCl_3$ solution were added and incubated for 30 minutes at room temperature. After 30 minutes, the absorbance was measured at 437 nm using a microplate reader (VersaMax; Molecular Devices). The TPC was calculated as quercetin equivalents (QEs). All the determinations were carried out 3 times.

DPPH free radical scavenging assay

For quantitative measurement of free radical scavenging activity of EAG, DPPH radical assay was carried out according to the method described previously [13]. Briefly, a stock solution (50 mg/mL) of EAG and a working solution of DPPH (250 μM) were freshly prepared and diluted with methanol. Several concentrations of EAG (50 μL) were loaded on a 96-well plate. And then, 150 μL of working solution was added to each well. After incubation for 10 min, absorbance was measured using SpectraMax 340PC^{384} microplate spectrophotometer (Molecular Devices) at 517 nm. Trolox was used as a positive control, and the 50% inhibitory concentration (IC_{50}) was calculated.

XO inhibitory assay

The inhibitory effect of EAG on XO activity in vitro was determined using a spectrophotometric method [14,15] with some modifications. Phosphate buffer (50 mM, pH 7.4) was used to dilute all materials in this assay. Briefly, the test solution and freshly prepared XO (0.02 U/mL) were incubated at 37°C for 15 minutes. After 15 minutes, xanthine (120 μM) was added to initiate the enzymatic reaction and the mixture was incubated at 37°C for 40 minutes. The reaction was terminated by the addition of 1N HCl solution, and the absorbance of each mixture was measured at 295 nm using a DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, NC, USA). Allopurinol was used as a positive control. XO inhibitory activity was shown as the percentage inhibition of XO compared with the mixture with xanthine and XO only. The degree of XO inhibition was calculated by following formula:

$$\% \text{ Inhibition} = [\frac{Ac - As}{Ac}] \times 100$$

where Ac, absorbance of the control; As, absorbance of the sample.

Animals

Male Sprague-Dawley rats (SD rats, 6–8 weeks old, 180–220 g, n = 36) were obtained from Koatech (Pyeongtaek, Korea). All animals had free access to water and were provided a normal diet (Purina Irradiated Laboratory Chow 38057; Purina Korea, Seoul, Korea). The temperature and humidity were kept at 24°C ± 1°C and 55%, respectively and light was

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controlled for 12 hours light and 12 hours dark. All experimental procedures were approved by the Korea University Institutional Animal Care and Use Committee (approval No. KUIACUC-2016-99) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health [NIH] publication No. 85-23, 1996).

Experimental design

A PO-induced hyperuricemic rat model was used to evaluate attenuation effect of EAG [16,17]. Thirty-six rats were randomly divided into 6 groups. EAG and allopurinol were dissolved in a mixture of de-ionized water and DMSO (9:1). PO was used to induce hyperuricemia in rats. Group I was treated with the mixture of de-ionized water and DMSO alone, served as a normal control; group II was treated with PO (250 mg/kg *b.w.*) alone, served as a model group; group III was treated with allopurinol (50 mg/kg *b.w.*) and PO, served as a positive control. Group IV, V, and VI were treated with EAG at doses of 50, 100, and 200 mg/kg *b.w.*, respectively and PO. All groups were orally administered once a day for 7 days. On the 1st, 3rd, and 7th days of the experiment period, PO (250 mg/kg *b.w.*) was injected intraperitoneally one hour before the administration of EAG or allopurinol to the animals except the normal control group to increase serum uric acid level. On the 7th day of the experiment, 2 hours after the final administration, all groups were anesthetized and sacrificed to obtain blood and liver. Parts of the livers were preserved in 10% formaldehyde, and the rest parts were kept at −80°C until use. The blood was allowed to clot for approximately 1 hour at room temperature and then centrifuged at 3,000× *g* for 5 minutes at 4°C to collect the serum. Serum chemical analysis was performed using a FUJI DRI-CHEM 4000i instrument and reagents (Fujifilm Co., Tokyo, Japan).

Preparation of liver homogenate and assay of XO activity in rat liver

Rats were monitored daily for weight change and any signs of diseases. On the final day of the experiment, animals were sacrificed and the livers were excised and stored at −80°C until used. The liver was washed in cold KCl (1.15%, w/v) containing 0.1 mM ethylenediaminetetraacetic acid. Chopped liver was put into 3 volumes of 50 mM phosphate buffer (pH 7.4) and homogenized. The homogenate was then centrifuged at 3,000× *g* for 10 minutes at 4°C. The lipid layer was carefully removed and the resulting supernatant was further centrifuged at 10,000× *g* for 60 minutes at 4°C. The supernatant was used for the assay of XO activity. The assay of XO activity in rat liver was determined using the same method described above with the supernatant obtained from the liver. Serum uric acid level was evaluated by using uric acid standard curve. The liver XO activity was expressed as nmol uric acid/mg protein/min. Protein concentration was quantified by Bradford assay [18].

Histological study

For histological study, each liver was sectioned, washed in ice-cold saline, fixed in the 10% formalin, and embedded in paraffin. Sections were cut at a thickness of 5 μm and were stained with haematoxylin and eosin (H & E) and observed using an optical microscope for histological changes.

Statistical analysis

The statistical analysis was performed using one-way analysis of the variance (ANOVA) followed by Fisher's least significant difference post hoc test using Statistical Package for the Social Science (SPSS) software package version 22.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± standard deviation from at least three independent experiments, and p value of < 0.05 was considered as significant.

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RESULTS

Quantification of total phenols and flavonoids in EAG

Phenolic, flavonoids, and carotenoids are abundant in plants extracts. As there was no information about the defined effective components of *A. glehni* for improving hyperuricemia, phenolic and flavonoid contents were measured. TPC and TFC were expressed in milligrams of gallic acid equivalents per gram of EAG (mg GAE/g) and milligrams of quercetin equivalents per gram of EAG (mg QE/g). TPC and TFC in EAG were 110.7 ± 7.7 mg GAE/g and 132.5 ± 6.8 mg QE/g, respectively (**Table 1**).

Free radical scavenging activity of EAG

The antioxidant activity of plant extracts has been decided by their free radical scavenging activities [19]. To investigate free radical scavenging effect of EAG, DPPH method was used. Trolox, a well-known radical scavenging compound, was used as a positive control. The IC_{50} values of Trolox and EAG were 31.2 ± 3.3 μg/mL and 150.6 ± 4.4 μg/mL, respectively (**Table 2**). Flavonoids show antioxidant activity due to their ability to decrease free radical formation [20]. Although free radical scavenging activity of EAG was lower than Trolox, EAG showed free radical scavenging ability, indicating that EAG possesses antioxidant activity.

Inhibitory activity of EAG on XO activity in vitro

To demonstrate the inhibitory effect of EAG on uric acid production, XO assay was performed. Allopurinol, which is known as a XO inhibitor, was used as a positive control. The IC_{50} value, the concentration that can decrease enzyme activity by 50%, was calculated. The IC_{50} values of EAG and allopurinol against XO were 97.2 ± 5.0 μg/mL and 45.4 ± 8.0 μg/mL, respectively (**Table 3**). Although the inhibitory activity of EAG against XO showed a half of allopurinol, EAG might be used as a XO inhibitor to decrease serum uric acid level.

Body weight and liver histology in PO-induced hyperuricemia rats

The body weights (g) on the last day of the experiment were measured and the results showed no significant difference between the experimental groups and did not show any

Table 1. Quantification of total phenolic and flavonoid contents in EAG

Total phenolic content, mg GAE/g	Total flavonoid content, mg QE/g
110.7 ± 7.7	132.5 ± 6.8

Data are the mean ± standard deviation of 3 separate experiments.
EAG, ethanol extract of *Aster glehni* leaves; mg GAE/g, milligrams of gallic acid equivalents per gram of EAG; mg QE/g, milligrams of quercetin equivalents per gram of EAG.

Table 2. Free radical scavenging activities of EAG and Trolox by DPPH assay

Sample	IC_{50}, μg/mL
Trolox	31.2 ± 3.3
EAG	150.6 ± 4.4

Data represent mean ± standard deviation.
EAG, ethanol extract of *Aster glehni* leaves; DPPH, 2,2-diphenyl-1-picrylhydrazyl; IC_{50}, 50% inhibitory concentration.

Table 3. Inhibitory effect of EAG on XO activity in vitro

Sample	IC_{50}
Allopurinol, μM	45.4 ± 8.0
EAG, μg/mL	97.2 ± 5.0

Data represent mean ± standard deviation.
EAG, ethanol extract of *Aster glehni* leaves; XO, xanthine oxidase; IC_{50}, 50% inhibitory concentration.

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Table 4. Average body weights and levels of serum biochemical parameters (GOT, GPT, and BUN) in PO-induced hyperuricemic rats treated with EAG

Treatment	Average body weight, g	Serum levels		
		GOT, U/L	GPT, U/L	BUN, mg/dL
Normal control	227.8 ± 3.6	154.5 ± 30.6	41.3 ± 5.3	14.1 ± 6.3
Normal control + PO	227.0 ± 2.9	146.3 ± 21.0	38.3 ± 5.3	11.2 ±4.9
Allopurinol (50 mg/kg *b.w.*) + PO	228.8 ± 6.2	103.5 ± 14.4	34.5 ± 1.0	12.9 ± 5.7
EAG (50 mg/kg *b.w.*) + PO	224.0 ± 4.7	140.7 ± 15.1	44.3 ± 6.1	13.0 ± 5.7
EAG (100 mg/kg *b.w.*) + PO	231.0 ± 4.1	88.5 ± 10.4	37.8 ± 4.7	12.0 ± 5.0
EAG (200 mg/kg *b.w.*) + PO	210.3 ± 21.6	87.5 ± 17.8	32.8 ± 7.4	11.8 ± 4.4

Data represent mean ± standard deviation.
GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; BUN, blood urea nitrogen; PO, potassium oxonate (250 mg/kg *b.w.*); EAG, ethanol extract of *Aster glehni* leaves; DPPH, 2,2-diphenyl-1-picrylhydrazyl; IC_{50}, 50% inhibitory concentration.

significant signs of diseases (**Table 4**). Natural compounds might produce toxic substances as secondary metabolites which could give damage to liver [21]. When liver is damaged, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) are released into the blood resulted in the increase of levels of GOT and GPT in blood [22]. To evaluate hepatotoxic effect of EAG, GOT, and GPT levels were measured and H & E staining was performed. Oral administration of EAG at doses of 50, 100, and 200 mg/kg *b.w.* for 7 days did not significantly affect levels of serum GOT and GPT (**Table 4**).

For histological examination of liver sections, H & E staining was performed. Microscopic observation by H & E staining showed no significant changes in liver sections of rats treated with PO or PO and EAG (**Figure 1**). The results suggest that there was no hepatotoxic effect of EAG within the concentrations used in this study.

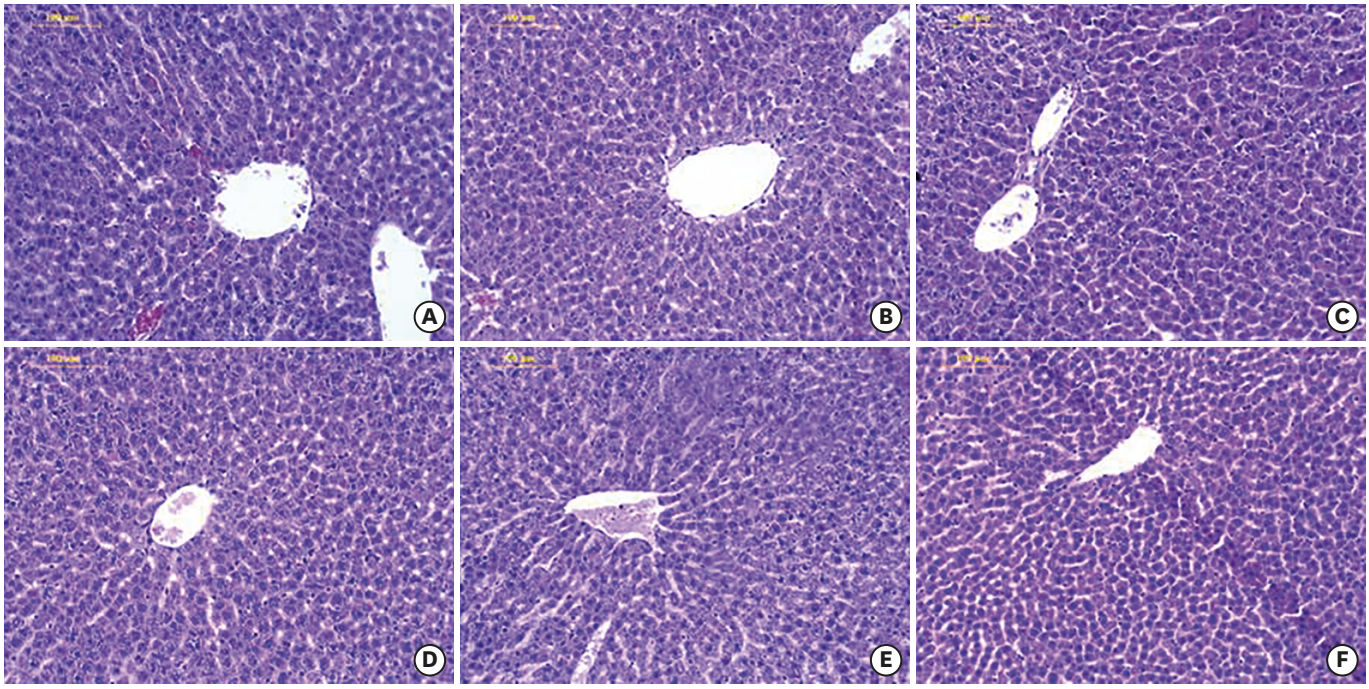

Figure 1. Effect of EAG on liver histology in PO-induced hyperuricemic rats. (A) Normal group, (B) hyperuricemia group induced by PO (250 mg/kg *b.w.*), (C) allopurinol (50 mg/kg *b.w.*) + PO, (D) EAG (50 mg/kg *b.w.*) + PO, (E) EAG (100 mg/kg *b.w.*) + PO, (F) EAG (200 mg/kg *b.w.*) + PO. All images were captured at 100× magnification.
EAG, ethanol extract of *Aster glehni* leaves; PO, potassium oxonate.

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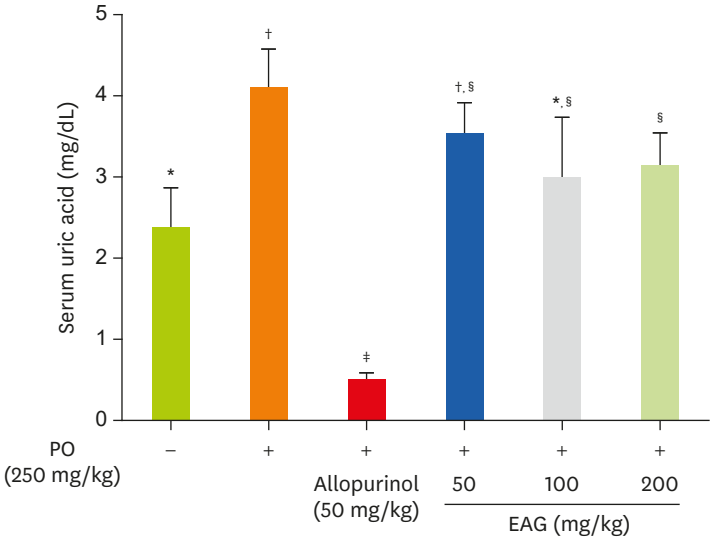

Figure 2. Effect of EAG on serum uric acid levels in PO-induced hyperuricemic rats. Data represent mean ± standard deviation of 3 independent experiments performed in triplicate.
EAG, ethanol extract of *Aster glehni* leaves; PO, potassium oxonate.
*,†,‡,§Values not sharing a common superscript vary significantly ($p < 0.05$) from each other.

Hypouricemic effect of EAG in hyperuricemic rats

In animal study, to investigate the hypouricemic effect of EAG, PO was used to increase serum uric acid level. In control rats, serum uric acid level was 2.38 ± 0.49 mg/dL (**Figure 2**). In hyperuricemic induced rats, serum uric acid level significantly increased (4.10 ± 0.48 mg/dL), which means that PO successfully induced hyperuricemia in rats. Serum uric acid levels in rats treated with allopurinol (50 mg/kg *b.w.*) and EAG at doses of 50, 100, and 200 mg/kg *b.w.* were 0.50 ± 0.08 mg/dL, 3.53 ± 0.39 mg/dL, 3.00 ± 0.74 mg/dL, and 3.15 ± 0.39 mg/dL, respectively. The results showed that serum uric acid levels in rats treated with allopurinol and EAG (100 and 200 mg/kg *b.w.*) significantly decreased compared with the model group. Although EAG (50 mg/kg *b.w.*)-treated group decreased serum uric acid level, it did not significantly decrease compared with the model group. These results show that EAG has potential hypouricemic effect.

Liver XO activity in PO-induced hyperuricemic rat

To confirm the hypouricemic effect of EAG, XO activity was measured with liver homogenate. Liver XO activity in PO-induced hyperuricemic rats treated with allopurinol (15.5 ± 0.6 nmol uric acid/mg protein/min) significantly decreased compared with the XO activity in PO-induced hyperuricemic model rats (28.1 ± 3.4 nmol uric acid/mg protein/min) (**Table 5**). Liver XO activities in PO-induced hyperuricemic rats treated with EAG (50, 100, and 200 mg/kg

Table 5. Effect of EAG and allopurinol on liver XO activity in PO-induced hyperuricemic rats

Treatment	Liver XO, nmol uric acid/mg protein/min
Normal control	23.1 ± 2.3*
Normal control + PO	28.1 ± 3.4†
Allopurinol (50 mg/kg *b.w.*) + PO	15.5 ± 0.6‡
EAG (50 mg/kg *b.w.*) + PO	20.6 ± 3.6§
EAG (100 mg/kg *b.w.*) + PO	20.0 ± 4.5§
EAG (200 mg/kg *b.w.*) + PO	17.2 ± 2.7‖

Data represent mean ± standard deviation.
EAG, ethanol extract of *Aster glehni* leaves; XO, xanthine oxidase; PO, potassium oxonate (250 mg/kg *b.w.*).
*,†,‡,§,‖Values not sharing a common superscript vary significantly ($p < 0.05$) from each other.

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b.w.) were 20.6 ± 3.6, 20.0 ± 4.5, and 17.2 ± 2.7 nmol uric acid/mg protein/min, respectively, which show a significant decrease in liver XO activity compared with the model group. The results consisted with the inhibitory activity of EAG against XO in vitro.

DISCUSSION

Uric acid is formed as a byproduct in the process of protein digestion; especially it is derived from purine metabolism. Normally, uric acid is excreted by the kidney; however, if it is not fully cleared through urinary system because of renal dysfunction, excess uric acid is deposited in the joints and kidney, which results in the development of gout. Gout is a common disease worldwide and it is caused by excess deposition of urate crystal in the joint and kidney.

Recent studies have revealed the mechanism of hyperuricemia, which leads to the development of gout treatment. Interleukin-1 (IL-1) blockers have been developed to treat gout caused by the deposition of monosodium urate crystal [23]. New drugs targeted to renal urate transporters have been tried to lower serum uric acid level [24]. Notably XO inhibitors, for instance, allopurinol and febuxostat have been developed for the long-term treatment of gout [25]. Therefore, XO inhibitors might be good candidates for treatment of gout by decreasing uric acid level. The leaves of *A. glehni* have been used as food and traditional medicine in Korea. As it is approved as food sources in Korea, *A. glehni* is regarded as a relatively non-toxic material from which to develop functional food and medicine. In this study, we found that *A. glehni* leaves contain a high level of flavonoids and show a strong inhibitory activity against XO. The 3,5-DCQA methyl ester and 4,5-DCQA methyl ester from purified *A. glehni* leaves in ethyl acetate soluble fraction showed the most potent XO inhibitory activity [26]. The XO inhibitory activity of those compounds was comparable to that of allopurinol, a well-known positive control as a XO inhibitor. EAG used in this study contained a low amount of 3,5-DCQA (0.04%); therefore, it is unclear that 3,5-DCQA in EAG acts as an effective compound on its inhibitory activity against XO. In further study, effective compound(s) of EAG must be identified for applying EAG to improve hyperuricemia.

XO produces reactive oxygen species (ROS) by transferring electrons to oxygen from hypoxanthine and xanthine to generate H_2O_2, which means that antioxidants might be effective in preventing hyperuricemia by inhibiting XO activity. Oxidative stress in uremia is the consequence of higher ROS production and XO is one of the main factors to create excessive ROS [27]. EAG showed antioxidant activity and inhibited XO activity. These findings suggest that antioxidant activity might contribute to mechanism of action of EAG on hypouricemic effect in PO-induced hyperuricemic rats. Although antioxidants are very effective for treatment of diseases caused by excess ROS production in cells, a high-dose antioxidant showed negative effect in many clinical trials [28]. EAG showed moderate antioxidant activity compared with Trolox. Therefore, EAG could be a candidate of gout treatment, either alone or in combination with other gout treatments.

CONCLUSION

EAG inhibits XO activity in vitro and in vivo and decreases serum uric acid level in PO-induced hyperuricemic rats, indicating that EAG attenuates hyperuricemia by inhibiting XO

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activity. Histological analysis of liver tissues in PO-induced hyperuricemic rats indicates that EAG does not show any liver toxicity. Therefore, EAG could be used as a gout treatment.

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