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Inhibitory Effects of *Apium graveolens* on Xanthine Oxidase Activity and Serum Uric Acid Levels in Hyperuricemic Mice

Karim Dolati[1], Hassan Rakhshandeh[1,2], Mohsen Golestani[1], Fatemeh Forouzanfar[3], Roya Sadeghnia[1], and Hamid R. Sadeghnia[1,2,3]

[1]Pharmacological Research Center of Medicinal Plants, [2]Department of Pharmacology, Faculty of Medicine, and
[3]Division of Neurocognitive Sciences, Psychiatry and Behavioral Sciences Research Center, Mashhad University of Medical Sciences, Mashhad 9177948564, Iran

ABSTRACT: Celery (*Apium graveolens*) is traditionally used to treat rheumatism and cardiovascular disorders. Hyperuricemia is considered as a predisposing factor for gout and is also suggested to be associated with coronary artery disease. In the present study, the effect of hydroalcoholic extracts from *A. graveolens* (AGE) against potassium oxonate (PO)-induced hyperuricemia was investigated in mice. AGE (250, 500, and 1,000 mg/kg) or allopurinol (5 mg/kg, as positive control) were orally administrated 1 h after PO injection (250 mg/kg, ip) for two weeks. After that, the serum uric acid level and hepatic xanthine dehydrogenase (XDH) and xanthine oxidase (XO) activities were measured. In addition, the antioxidant activity of AGE was determined by assessment of hepatic lipid peroxidation, *in vivo* and the ferric reducing/antioxidant power assay, *in vitro*. The extract exhibited good capacity to reduce ferric ion to ferrous ion with mean value of 63.8±8.5 μmol/g. The data also showed that oxonate treatment produced a significant increase in serum uric acid level (4.6 vs. 2.3 mg/ dL, $P<0.001$), liver XO/XDH activities ($P<0.01$ and $P<0.001$, respectively), and hepatic lipid peroxides levels (about two fold, $P<0.01$), compared to the healthy mice. AGE significantly decreased the serum uric acid level, hepatic XO/XDH activities, and lipid peroxidation, in a dose-dependent manner. Oral administration of 1,000 mg/kg AGE for two weeks reversed the elevated serum uric acid level (2.7 vs. 4.6 mg/dL, $P<0.001$) and significantly inhibited liver XO/XDH activities ($P<0.001$) and diminished hepatic lipid peroxidation (0.45 vs. 0.82 nmol/mg protein, $P<0.05$), compared with hyperuricemic mice. AGE (1,000 mg/kg) *per se* did not significantly modify these parameters. Our results demonstrated that AGE could reduce the serum uric acid level via inhibition of hepatic XDH/XO and indicated its potential utility as an effective hypouricemic bioactive agent or functional food.

Keywords: *Apium graveolens*, hyperuricemia, xanthine dehydrogenase (XDH), xanthine oxidase (XO), hypouricemic agent

INTRODUCTION

Excessive production of uric acid leads to deposition of monosodium uric acid crystals in soft tissues and joints which are associated with gout (1-3). These crystals cause the pathology of gout by activating the inflammatory responses, resulting in bone and cartilage erosions (4). Oxidative damage is also closely related to the pathogenesis of gout disease (2). Therefore, inhibition of uric acid overproduction, inflammation, and oxidative stress have importance in gout management (2). The most therapeutic strategies to treat hyperuricemia is the development of xanthine oxidase (XO) inhibitors which could effectively reduce the uric acid contents of plasma and urine and decrease tophaceous uric acid deposition (1). Allopurinol, a XO inhibitor, is widely used as a uric acid-lowering agent (5). However, the clinical use of allopurinol is limited because of severe adverse effects such as hepatotoxicity, nephrotoxicity or hypersensitivity reactions. Therefore, searching the new XO inhibitors, in particular from natural sources, is a promising approach (5-8).

Apium graveolens Linn. (Apiaceae), commonly known as celery, has a long history of usage in Ayurveda and Unani medicine. *A. graveolens* is widely cultivated in Central Europe, North Africa, North-Western Himalayas, and Western India. The whole plant or its seeds have been used as food or medicine (9,10). Celery has been effective in the prevention of cardiovascular disease (11) and lowering blood pressure (12). It also has antifungal (13), anti-inflammatory (14,15), antioxidant (16), anti-gastric ulcer

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Correspondence to Hamid R. Sadeghnia, Tel: +98-511-882-8566, E-mail: sadeghniahr@mums.ac.ir

(17), and anticoagulant activities (11). An antirheumatic formulation of the seeds has shown to induce significant arthritic pain relief (9,10).

In the current study, the effect of hydroalcoholic extract of *A. graveolens* (AGE) on hyperuricemia induced by potassium oxonate (PO) was investigated in mice. Moreover, the activities of XO and xanthine dehydrogenase (XDH) in hepatic tissue were also measured.

MATERIALS AND METHODS

Materials

PO, 2,4,6-tri(2-pyridyl)-*s*-triazine, ferrous sulfate, ferric chloride, hydrogen peroxide (H_2O_2), xanthine, nicotinamide adenine dinucleotide (NAD), allopurinol, hydrochloric acid (HCl), and bicinchoninic acid protein assay kit were purchased from Sigma (St. Louis, MO, USA).

Animals

Adult male mice (20～30 g) were provided by the Institutional Animal House and kept under 12/12-h light/dark cycle at 21±2°C with access to water and food *ad libitum*. The animal experiments were approved by the Institutional Animal Ethics Committee and were in accordance with the Guidelines for the Care and Use of Laboratory Animals of National Institutes of Health (ethics number: IR900613).

Preparation of hydroalcoholic extract of *A. graveolens* (AGE)

The fresh herb of celery (*A. graveolens*) of a commercial cultivar was harvested and authenticated by the University's Herbarium Unit (voucher specimen No. AG-3278-21). The whole plant was washed, dried, and grounded to a fine powder with a blender. Then, the powder (100 g) was soaked in 1,000 mL of 70% ethanol, for 48 h at 40°C under gentle shaking. The resultant was filtered, and the solvent was removed using rotary evaporator. The residue (yield 11% w/w) was kept at −20°C until use (18,19).

Antioxidant capacity of *A. graveolens* extract *in vitro*

The total antioxidant (reducing) power of AGE was determined using the ferric reducing/antioxidant power (FRAP) assay, as described previously. Briefly, 50 μL of the extract was added to 2 mL of freshly prepared and prewarmed (37°C) FRAP reagent (300 mM acetate buffer, 10 mM tripyridyltriazine in 40 mM HCl, and 20 mM ferric chloride in the ratio of 10:1:1) in a test tube and incubated at 37°C for 10 min. The absorbance of the blue colored (ferrous tripyridyltriazine) complex was measured against the reagent blank at 593 nm. The standard curve was constructed using ferrous sulfate solution (100～1,000 μM), and the data were expressed as μmol ferric ions reduced to ferrous form per g dry extract mass (FRAP value). The measurements were performed in triplicate (20,21).

Experimental design

To explore the anti-gout activity of AGE, hyperuricemia was induced in mice using PO, as an uricase inhibitor (22). The mice were randomly divided into seven groups each comprising eight animals and fasted 2 h prior to drug administration. The control mice received saline solution as a vehicle. In hyperuricemic mice, PO (250 mg/kg) was injected intraperitoneally. In treatment groups, AGE (250, 500, and 1,000 mg/kg) or allopurinol (5 mg/kg, as positive control) were orally administrated 1 h before PO (250 mg/kg) injection and then once daily for 14 days. Another group of mice was orally administrated by AGE (1,000 mg/kg), alone for 2 weeks.

Oral gavage was performed using a 1 mL insulin syringe equipped with a ball tipped plastic (flexible) disposable feeding needle to deliver the solution to the back of the throat. A habituation to oral gavage was performed using water. Fresh solutions of AGE at concentrations of 25, 50, and 100 mg/mL in saline solution were prepared and administered at 10 mL/kg (19).

Blood and liver sampling

Mice were euthanized under deep anesthesia 1 h after the final drug administration, and blood samples were drawn from the heart. The blood was allowed to clot for approximately 1 h at room temperature and then centrifuged at 3,000 *g* for 15 min to obtain the serum. The serum was then stored at −20°C until analyzed. Serum uric acid was determined using alkaline phosphotungstate assay (22).

The livers were quickly removed and chopped on the ice. After freezing in liquid nitrogen, the samples were stored at −70°C until analysis. For determination of activities of XO and XDH, 0.5 g of livers was homogenized on ice in 2.5 mL phosphate buffer (50 mM, pH 7.4). The homogenates were centrifuged at 3,000 *g* for 10 min at 4°C, the supernatants were then carefully removed and underwent further centrifugation at 15,000 *g* for 60 min at 4°C. The supernatants were then used for further analyses (22,23).

Measurement of XO and XDH activities

The activities of XO and XDH were measured based on oxidation of xanthine to uric acid using NAD^+ (in the case of XDH) or H_2O_2 (for XO) as the electron acceptors. Briefly, 100 μL enzyme solution in 50 μM phosphate buffer (pH 7.4) was preincubated with 200 μM NAD^+ or H_2O_2 at 37°C for 15 min. Then, 50 μM xanthine was added to the mixture and further incubated for 30 min

at 37°C. After that, the reaction was stopped by adding 0.6 M HCl and the absorbance was monitored at 290 nm (22).

Thiobarbituric acid reactive species (TBARS) measurement

The extent of hepatic oxidative damage in different treatment groups was assessed using thiobarbituric acid (TBA) reagent that forms the pink colored complex with the end products of lipid peroxidation with peak absorbance at 532 nm. In brief, 1 mL of 10% homogenate sample (in phosphate buffered saline) was mixed with 2 mL of trichloroacetic acid (TCA)-TBA-HCl reagent (15% TCA, 0.67% TBA, and 0.25 N HCl) and heated for 45 min in a boiling water bath. After cooling, the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was collected, and the absorbance was read against blank, at 532 nm. The amount of malondialdehyde produced was calculated, using a molar absorption coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ and expressed as nmol/mg protein (21). The protein content of the samples was determined using bicinchoninic acid protein assay kit.

Statistical analysis

Data were presented as mean±standard error of mean (SEM). For data analysis, we used GraphPad Prism 6.01 software (GraphPad Software, La Jolla, CA, USA) and the values were compared using the one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. The $P<0.05$ was considered statistically significant.

RESULTS

***In vitro* total antioxidant capacity of AGE by the FRAP assay**

The extract exhibited good capacity in reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) with mean value of 63.8 ±8.5 μmol/g.

AGE significantly and dose-dependently decreased serum uric acid level in hyperuricemic mice

As shown in Fig. 1, serum uric acid level was significantly ($P<0.001$) increased in the PO animals compared to the control mice. Treatment with AGE markedly decreased the uric acid level from 4.6 mg/dL (PO group) to 2.7 mg/dL (500 mg/kg AGE group) ($P<0.001$), which is similar to the values of control mice (2.3 mg/dL). Also, in the positive control group, allopurinol caused a significant decrease in the serum uric acid level (0.8 mg/dL). As shown in Fig. 1, AGE by itself did not significantly change the serum uric acid level (2.0 mg/dL).

AGE significantly inhibited hepatic XO and XDH activities in hyperuricemic mice

Fig. 2 and 3 show the effects of AGE and allopurinol on the XO and XDH activities in mice liver. Following PO administration, the XO activity was significantly increased, reached to 134.2%, as compared to the control mice ($P<0.001$). The increased XO activity was probably responsible for the elevated serum uric acid level. In contrast, AGE significantly and dose-dependently decreased the elevated XO activity to 83.2%, 78.2% ($P<0.001$), and 49.2% ($P<0.001$) of control value (for 250, 500, and 1,000 mg/kg AGE, respectively). Treatment with allopurinol (as positive control) also significantly decreased XO activity to 33.2% of control value ($P<0.001$). On the other hand, AGE (1,000 mg/kg) *per se* did not significantly modify XO activity.

Fig. 3 presents the liver XDH activity of hyperuricemic mice treated with AGE or allopurinol. Compared with the control group, PO-treated animals had increased levels ($P<0.001$) of XDH activity, indicating uric acid overproduction. In the same way, AGE significantly decreased XDH activity. For the high-, medium-, and low-dose AGE

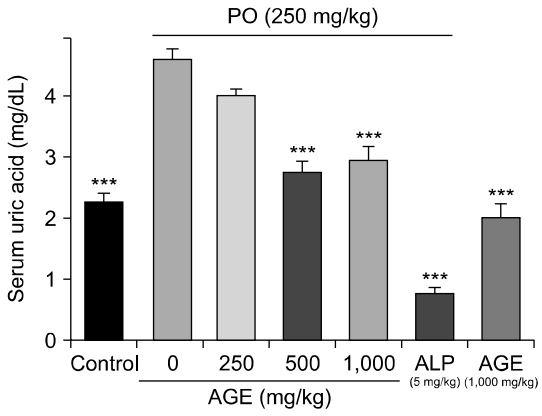

Fig. 1. Effects of *A. graveolens* hydroalcoholic extract (AGE) and allopurinol (ALP) on the serum uric acid level of potassium oxonate (PO)-induced hyperuricemic mice. Values were expressed as the mean±SEM (n=8). ***$P<0.001$ compared with PO group.

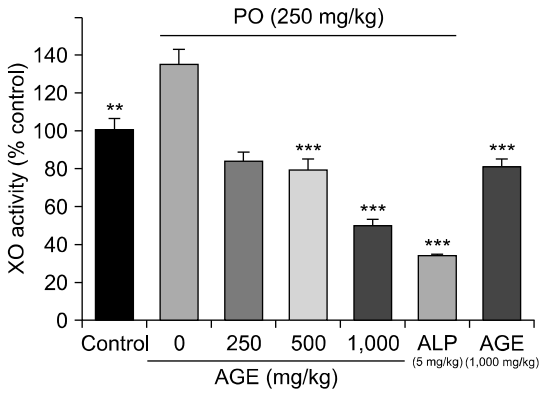

Fig. 2. Effects of *A. graveolens* hydroalcoholic extract (AGE) and allopurinol (ALP) on the inhibition rate of xanthine oxidase (XO). Values were expressed as the mean±SEM (n=8). **$P<0.01$ and ***$P<0.001$ compared with PO group.

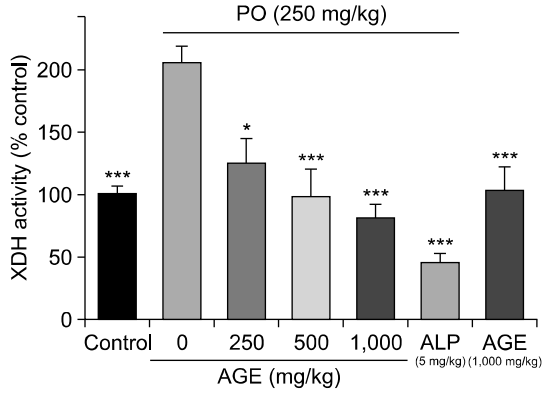

Fig. 3. Effects of *A. graveolens* hydroalcoholic extract (AGE) and allopurinol (ALP) on the inhibition rate of xanthine dehydrogenase (XDH). Values were expressed as the mean±SEM (n=8). $^{*}P<0.05$ and $^{***}P<0.001$ compared with PO group.

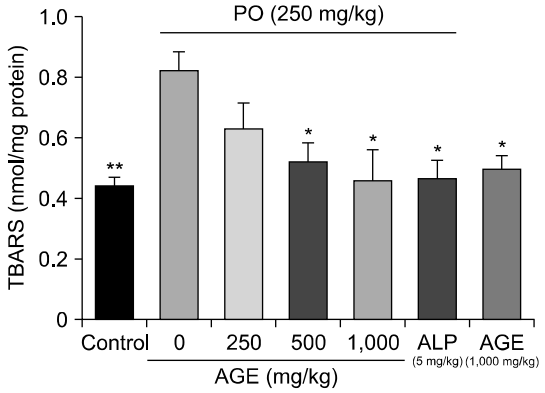

Fig. 4. Effects of *A. graveolens* hydroalcoholic extract (AGE) and allopurinol (ALP) on hepatic lipid peroxidation (as measured using thiobarbituric acid reactive species, TBARS). Values were expressed as the mean±SEM (n=8). $^{*}P<0.05$ and $^{**}P<0.01$ compared with PO group.

groups, XDH activity inhibited to about 80.1%, 97.5%, and 123.5% of control. Allopurinol also caused about a five-fold decrease in the enzyme activity, as compared to PO group ($P<0.001$, Fig. 3).

AGE dose-dependently attenuated liver lipid peroxidation in hyperuricemic mice

As illustrated in Fig. 4, TBARS levels were significantly increased (about two fold) in hyperuricemic mice compared to control animals ($P<0.01$). On the other hand, hyperuricemic mice treated with relatively high doses of the extract showed significantly lower lipid peroxides levels. Hepatic TBARS levels significantly decreased to 0.63±0.08, 0.52±0.06 and 0.45±0.09 nmol/mg protein for hyperuricemic mice treated with 250, 500, and 1,000 mg/kg AGE, respectively (both $P<0.05$ compared with the PO group; Fig. 4).

DISCUSSION

Hyperuricemia is considered as a predisposing factor for gout and is also thought to be associated with a number of clinical disorders, including endothelial dysfunction and atherosclerosis, hypertension, coronary artery disease, diabetes, dyslipidemia, and chronic kidney disease. It has been reported that hyperuricemia may increase production of oxygen free radicals, promote lipid peroxidation, and also upregulate proinflammatory mediators (1,2,24-28). It can be caused by uric acid overproduction, decreased renal excretion of uric acid, or both (3). Hyperuricemia can be effectively controlled by inhibiting XO, which is generated from XDH during oxidative stress and inflammatory processes. The enzyme oxidizes hypoxanthine to xanthine and then to uric acid. Therefore, inhibition of uric acid overproduction, inflammation and oxidative stress has considerable values in the management of gout, cardiovascular and metabolic disorders (1,2).

As mentioned earlier, it was shown that the methanol and petroleum ether extracts of celery leaves and seeds could significantly decrease serum uric acid level in rats (7). However, the mechanism underlying the hypouricemic effects including XO and XDH activities were not explored. Therefore, we explored the effects of AGE on hepatic XO and XDH activities and serum uric acid level as well as hepatic lipid peroxidation. We found that the AGE dose-dependently and significantly inhibited hepatic XO and XDH activities, lowered serum uric acid level, and decreased liver lipid peroxidation in PO-induced hyperuricemic mice. Moreover, the extract exhibited good antioxidant capacity *in vitro*, as measured by FRAP assay, which is consistent with other results (29).

A. graveolens contains furocoumarins (apigravin, celerin, and umbelliferone), flavonoids (apigenin, apiin, kaempferol, and luteolin), phenolic compounds (caffeic acid, *p*-coumaric acid, and ferulic acid), and tannins (30,31). Celery essential oil has also been shown to have a series of phthalide derivatives (32). Anti-gout activity has been described for phthalide derivatives, phenolic compounds, and tannins (7,33). The hyperuricemic and XO inhibitory activity of apigenin were investigated in several studies. Lin et al. (34) reported that apigenin could interact with the XO active site and had the comparable inhibitory effect as allopurinol *in vitro*. Flemmig et al. (35) also showed that apigenin could moderately inhibit XO activity *in vitro*. Apigenin, at dose of 25 mg/kg, also significantly decreased serum uric acid and inhibited liver XO activity by 38.4% in hyperuricemic mice (36). In contrast, Huang et al. (37) indicated that apigenin at doses as high as 700 mg/kg failed to decrease serum uric acid level and liver XO activity of hyperuricemic mice. Luteolin, another major component of celery, also reversibly inhibited XO in a competitive manner, by interacting with the primary amino acid residues located within the active site pocket of XO (38). It was also reported that kaempferol,

another flavonoid found in celery, reversibly inhibited XO by occupying the catalytic center of the enzyme. In addition, luteolin exhibited a strong synergistic effect with kaempferol (39).

Celery, or its constituents, has been shown to have antioxidant and anti-inflammatory effects (30,40), which may be involved in the anti-gout, cardiovascular, hepatic or renal protective effects of the medicinal herb (31). Several studies showed that celery contains high levels of phenolic and flavonoid compounds and exhibits significant antioxidant and free radical scavenging activities *in vitro*. An extremely significant positive correlation between the antioxidant activity and the contents of total flavonoids or total phenolics was observed (41,42). Popović et al. (43) suggested that the diethyl ether, chloroform, ethyl acetate, *n*-butanol, and water extracts of celery leaves and roots are good scavengers of hydroxyl (OH) and 1,1-diphenl-2-picrylhydrazyl (DPPH) free radicals and decrease liposomal peroxidation, which may be due to the presence of flavonoids. Moreover, *in vivo* experiments revealed antioxidant properties of the extracts by diminishing lipid peroxidation and promoting the antioxidant defense systems in the liver and blood of mice treated with carbon tetrachloride. In the same way, celery seeds showed antioxidant and cyclooxygenase inhibitory effects reflecting the anti-inflammatory action via the presence of phenolic compounds; sedanolide and senkyunolide-N (16). In another study, the methanolic extract of *A. graveolens* ameliorated liver oxidative stress in adjuvant-induced arthritic rats by reducing the generation of liver free radicals and increasing the liver antioxidant enzyme activity (44). Another study found that celery roots and leaves juices improved the biochemical parameters in rats' liver homogenate and blood hemolysate samples and revealed protective effects against doxorubicin toxicity (45). In a study by Al-Sa'aidi et al. (46), it was found that the *n*-butanol extract of celery seed modified the glycemic and insulin levels, ameliorated hepatic lipid peroxidation, and improved intracellular glutathione and antioxidant enzymes (mainly superoxide dismutase, catalase, glutathione transferase, and reductase) in streptozotocin-induced diabetic rats. Antioxidant and cyclooxygenase (COX-I and COX-II) inhibitory activities have also been described for several constituents in the celery plant (16). For example, apigenin, an antioxidant, inhibits the production of hydrogen peroxide and IgE, which are responsible for inflammation and allergic responses (47,48). Apigenin also had inhibitory effects on cyclooxygenase and lipoxygenase (49). Li et al. (50) showed that apiin, the flavonoid isolated from celery leaf, possess remarkable antioxidant activities *in vitro* and *in vivo*. Apiin effectively scavenged DPPH, OH, and superoxide (O_2^-) free radicals *in vitro*. *In vivo*, apiin also significantly decreased TBARS levels and improved the total antioxidant capacity, and enhanced activities of the enzymes superoxide dismutase, glutathione peroxidase and catalase in serum, brain, heart, liver and kidney of male mice.

A. graveolens extracts were also reported to have anti-inflammatory activity via suppression of carrageenan-induced paw edema in rats (51). Ramezani et al. (52) also showed that aqueous and hexane extracts from *A. graveolens* seeds have remarkable anti-inflammatory and anti-nociceptive activities in formalin and xylene-induced ear edema tests in mice. The croton-oil ear test in mice showed that the hydroalcoholic extract of celery leaves exerted anti-inflammatory activity with a potency seven-times lower than that of indomethacin. The anti-inflammatory activity was attributed to the inhibitory effect of the extract and its major constituent, apiin, on nitric oxide (NO) production and inducible NO synthase expression (14). In addition, two aglycones of a phthalide glycoside and a megastigmane glycoside, isolated from 70% ethanolic extract of *A. graveolens*, showed potent inhibitory activity against lipopolysaccharide-induced NO production in RAW 264.7 macrophages, *in vitro* (53). Apiuman, a pectic polysaccharide isolated from fresh celery stalks, also exerted anti-endotoxemic effect in LPS-intoxicated mice, and this effect was shown to be mediated by diminished neutrophils migration, decreased interleukin (IL)-1β and increased IL-10 production (54).

A. graveolens and the flavonoids content, mainly apiin and apigenin, also exhibit healing properties (30). Recently Kooti et al. (19) showed that hydroalcoholic extract of celery leaf could significantly increase the number of sperms, sertoli cells, and primary spermatocyte in the rat. In one study, L-3-*n*-butylphthalide from celery seeds, significantly reversed memory impairment in APP/PS1 transgenic mice model of Alzheimer's disease via brain-derived neurotrophic factor/tropomyosin receptor kinase B/phosphoinositide-3-kinase/Akt signaling pathway (55).

In conclusion, the present study demonstrated that hydroalcoholic extract of *A. graveolens* possesses hypouricemic activity in PO-induced hyperuricemia in mice by inhibiting XDH and XO activities. These findings suggest the use of *A. graveolens* as a dietary supplement or nutraceutical for the treatment of hyperuricemia, but the molecular mechanisms underlying these effects require further investigation.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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