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ORIGINAL ARTICLE

Inhibition of Xanthine Oxidase Activity by Gnaphalium Affine Extract

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Objective To evaluate the inhibitory effect of Gnaphalium affine extracts on xanthine oxidase (XO) activity *in vitro* and to analyze the mechanism of this effect.

Methods In this *in vitro* study, Kinetic measurements were performed in 4 different inhibitor concentrations and 5 different xanthine concentrations (60, 100, 200, 300, 400 $\mu\text{mol/L}$). Dixon and Lineweaver-Burk plot analysis were used to determine K_i values and the inhibition mode for the compounds isolated from Gnaphalium affine extract.

Results Four potent xanthine oxidase inhibitors were found in 95% ethanolic (v/v) Gnaphalium affine extract. Among them, the flavone Eupatilin exhibited the strongest inhibitory effect on XO with a inhibition constant (K_i) of 0.37 $\mu\text{mol/L}$, lower than the K_i of allopurinol (4.56 mol/L), a known synthetic XO inhibitor. Apigenin (K_i of 0.56 $\mu\text{mol/L}$, a proportion of 0.0053‰ in Gnaphalium affine), luteolin (K_i of 2.63 $\mu\text{mol/L}$, 0.0032‰ in Gnaphalium affine) and 5-hydroxy-6,7,3',4'-tetramethoxyflavone (K_i of 3.15 $\mu\text{mol/L}$, 0.0043‰ in Gnaphalium affine) also contributed to the inhibitory effect of Gnaphalium affine extract on XO activity.

Conclusions These results suggest that the use of Gnaphalium affine in the treatment of gout could be attributed to its inhibitory effect on XO. This study provides a rational basis for the traditional use of Gnaphalium affine against gout.

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GOUT is a growing health problem worldwide, especially in developed countries. Epidemiologic data indicate that the incidence of primary gout has increased by 2-fold over the past 20 years.¹ In recent years, xanthine oxidase (XO) has emerged as an important target not only for gout but also for cardiovascular and metabolic disorders involving hyperuricemia. XO is a key enzyme that catalyses the oxidation of xanthine and hypoxanthine into uric acid, and plays a vital role in the development of hyperuricemia and gout.² Allopurinol is a clinically used XO inhibitor in the treatment of gout, which blocks the terminal step in uric acid biosynthesis, therefore lowering the plasma uric acid concentration. However, due to its adverse effects, such as hepatitis, nephropathy, and allergic reactions, new alternatives with higher therapeutic potency and less adverse effects to replace allopurinol are desired. Moreover, superoxide anion radicals generated by XO are involved in various pathological states such as hepatitis, inflammation, aging, carcinogenesis, and ischemia-reperfusion.² Thus, the search for novel XO inhibitors would be beneficial not only to the treatment of gout but also to the improvement of various other diseases.

In China, natural herbs have been used for a long time in the treatment of gout and hyperuricemia-related disorders. Gnaphalium affine is the main drug in the empirical prescription "Baiai Tongfengling", which has proved therapeutic effect in clinical cases. Gnaphalium affine has the effects of drying dampness, resolving phlegm, and purging turbidity. The Gnaphalium affine, also named Cudweed or Qing Ming vegetable in China, is extensively harvested as a wild vegetable around the Qing Ming festival (in early April) and processed into drinks, canned products, and frozen vegetables, etc. Gnaphalium affine is believed to be of high nutritional value since it contains the 8 essential amino acids for human body in an appropriate proportion, a large content of minerals, trace elements, and vitamins. Besides the nutritional value, Gnaphalium affine was reported to exhibit many pharmacological activities.³

Based on a previous study of this research team, which demonstrated that water-boiled Gnaphalium affine extraction can lower blood uric acid in rats significantly,⁴ we conducted this *in vitro* investigation to identify the active substances, thus clarify potential pharmaceutical basis for the traditional use of Gnaphalium affine in treating gout and other diseases involving hyperuricemia.

MATERIALS AND METHODS

Plant material

The Gnaphalium affine used in this study was collected in

May 2009 from near Jing'an County in Jiangxi Province and identified by Professor Qian-feng Gong from School of Pharmaceutical sciences, Jiangxi University of Traditional Chinese Medicine. A voucher specimen was stored at 4°C in the Department of Traditional Chinese Pharmacy of Jiangxi Provincial Children's Hospital.

Air-dried Gnaphalium affine (7.5 kg) were extracted with 60 L 95% ethanol⁵ at room temperature (three times, once every week), and concentrated in vacuum to yield 910 g of crude extraction, prepared by drug manufacturing department of Jiangxi Provincial Children's Hospital.

The extract was suspended in H₂O and then partitioned with petroleum ether (PE) and ethyl acetate (EA) to produce a PE-soluble fraction and EA-soluble fraction, respectively. The EA fraction (179 g) was subjected to a silica gel column chromatography eluted with a PE/acetone gradient (60:1 to 1:1) to give seven subfractions (fr. EA 1-7). Fr. EA 3 was chromatographed over a silica gel column chromatography (PE/EA, 20:1 to 10:1) to yield compound 1 (200 mg). Fr. EA 4 was subjected to a silica gel column chromatography (PE/EA, 10:1 to 4:1) and Sephadex LH-20 to yield compounds 4 (20 mg) and 5 (32 mg). Fr. EA 6 was subjected to Sephadex LH-20 to isolate compounds 6 (8 mg), 7 (24 mg) and 8 (40 mg). Fr. EA 7 was chromatographed over a reverse phase-18 gel column chromatography (MeOH/H₂O, 20:1 to 10:1) and Sephadex LH-20 to yield compounds 2 (22 mg) and 3 (10 mg). The isolated compounds were identified as β -sitosterol (1), rutin (2),⁶ quercetin-3-O- β -D glucopyranoside (3),⁷ eupatilin (4),⁸ 5-hydroxy-6,7,3',4'- tetramethoxyflavone (5),⁸ quercetin (6),⁹ luteolin (7)¹⁰ and apigenin (8)¹⁰ by comparison of their spectroscopic data with those reported in literature except for compound 1, which was identified by comparison of thin layer chromatography and melting point with those of β -sitosterol sample.

Drugs and chemicals

Allopurinol, xanthine and XO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents used in this study were of analytical grades and purchased from Aladdin Biomart Company (Shanghai, China).

In vitro inhibition on XO activity

The activity of XO was measured spectrophotometrically under aerobic conditions using xanthine as the substrate by following the increase in absorbance at 292 nm using an absorption coefficient of 9 600 L/(mol·cm).¹¹ Allopurinol as well as Gnaphalium affine and its isolated compounds were pre-incubated with xanthine at 25°C for 15 minutes.^{2,12} The reaction was started by adding XO. Kinetic measurements

were performed in 4 different inhibitor concentrations and 5 different xanthine concentrations (60, 100, 200, 300, 400 $\mu\text{mol/L}$).

Gnaphalium affine were used at 5, 10, 20, 40 $\mu\text{g/ml}$, its isolated compounds at 0.25, 0.5, 1, 2 $\mu\text{g/ml}$, with allopurinol (0.25, 0.5, 1, 2 $\mu\text{g/ml}$) as the positive control. Different concentrations of Gnaphalium affine were dissolved in dimethyl sulfoxide (DMSO) to reach a final concentration of DMSO less than 1%. DMSO was used as the negative control.

The reaction mixture for the XO inhibition assay consisted of 300 μL of 200 mmol/L sodium pyrophosphate buffer (pH 7.5), 60-400 μL of 1 mmol/L xanthine, 100 μL of sample solution dissolved in distilled water or 1% DMSO, and 200 μL of XO (0.1 U). A solution in 1% DMSO was used for those samples insoluble in distilled water. The increased UV absorption at 292 nm indicated the formation of uric acid.¹³ All measurements were performed in triplicate.

Statistical analysis

The data collected during these measurements were analysed using Dixon plots, drawn with Excel, to determine K_i values for the tested samples. The K_i values can be directly

obtained from the intersection of the straight lines representing constant substrate concentrations. The initial enzymatic rates using different inhibitor concentrations and a fixed xanthine concentration always fell into straight lines. Lineweaver-Burk plots were drawn to reveal the inhibition mode.

RESULTS

The Gnaphalium affine extracts and its compounds had inhibitory effect on XO activity, among which 4 compounds exhibited little or weak inhibition in XO activity, therefore not included in the following analysis. The K_i values of Gnaphalium affine extracts, its compounds, and the standard drug allopurinol are displayed in Table 1.

Lineweaver-Burk plots of reactions in the presence and absence of Gnaphalium affine in a XO reaction mixture is shown in Figure 1. Allopurinol exhibited a competitive inhibition. The inhibitory effect of 40 $\mu\text{g/ml}$ Gnaphalium affine extract is stronger than that of 2 $\mu\text{g/ml}$ allopurinol (Fig.1). The mode of XO inhibition by Gnaphalium affine and the 4 compounds are mixed inhibition (Fig. 2).

Table 1. Inhibitory effect of Gnaphalium affine extracts and allopurinol on xanthine oxidase activity *in vitro*

Samples	Proportion in Gnaphalium affine (‰)	Inhibition mode	K_i
Gnaphalium affine crude extract	121.3	Mixed	11.38 $\mu\text{g/ml}$
Eupatilin	0.0027	Mixed	0.37 $\mu\text{mol/L}$
Apigenin	0.0053	Mixed	0.56 $\mu\text{mol/L}$
Luteolin	0.0032	Mixed	2.63 $\mu\text{mol/L}$
5-Hydroxy-6,7,3',4'-tetramethoxyflavone	0.0043	Mixed	3.15 $\mu\text{mol/L}$
Allopurinol	0	Competitive	4.56 $\mu\text{mol/L}$
DMSO	/	/	/

DMSO: dimethyl sulfoxide, serving as the negative control.

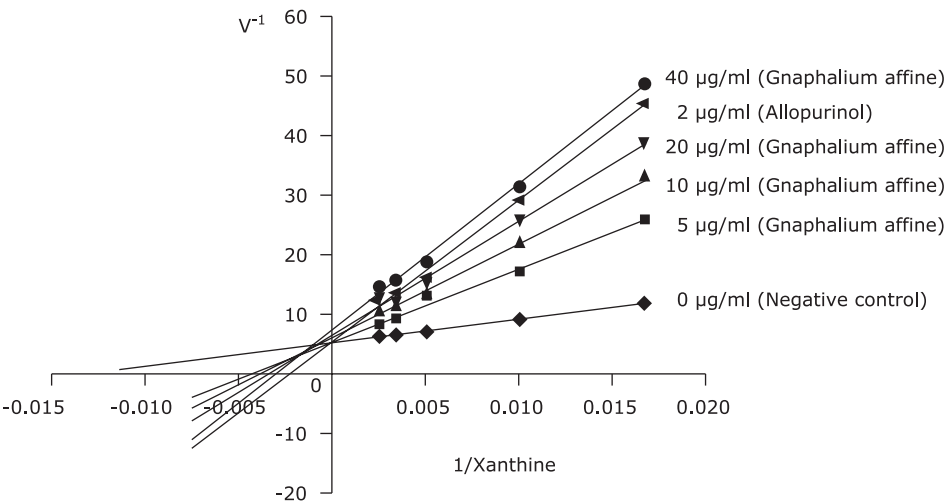


Figure 1. Lineweaver-Burk plot of the inhibition of xanthine oxidase by Gnaphalium affine, allopurinol, and negative control.

As shown in Figure 3A-F, the inhibition of the *Gnaphalium affine* extracts and its 4 compounds on XO activity was in a dose-dependent manner. From the intersection of these lines, the K_i values were determined as: 11.38 $\mu\text{g/ml}$ for *Gnaphalium affine* (Fig. 3A), 0.62 $\mu\text{g/ml}$ (4.56 $\mu\text{mol/L}$) for allopurinol (Fig. 3B), 0.13 $\mu\text{g/ml}$ (0.37 $\mu\text{mol/L}$) for eupatilin (Fig. 3C), 0.15 $\mu\text{g/ml}$ (0.56 $\mu\text{mol/L}$) for apigenin (Fig. 3D), 0.75 $\mu\text{g/ml}$ (2.63 $\mu\text{mol/L}$) for luteolin (Fig. 3E), and 1.13 $\mu\text{g/ml}$ (3.15 $\mu\text{mol/L}$) for 5-hydroxy-6,7,3',4'-tetramethoxyflavone (Fig. 3F).

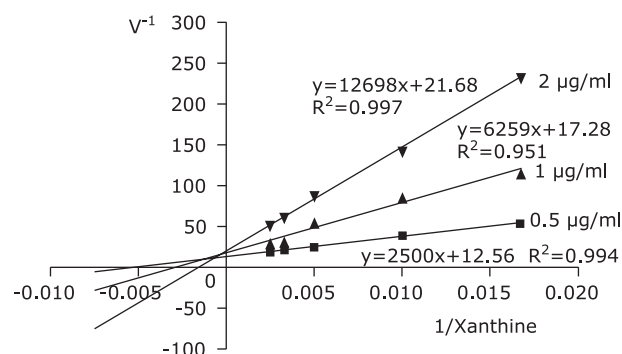


Figure 2. Lineweaver-Burk plot of the inhibition of xanthine oxidase by eupatilin.

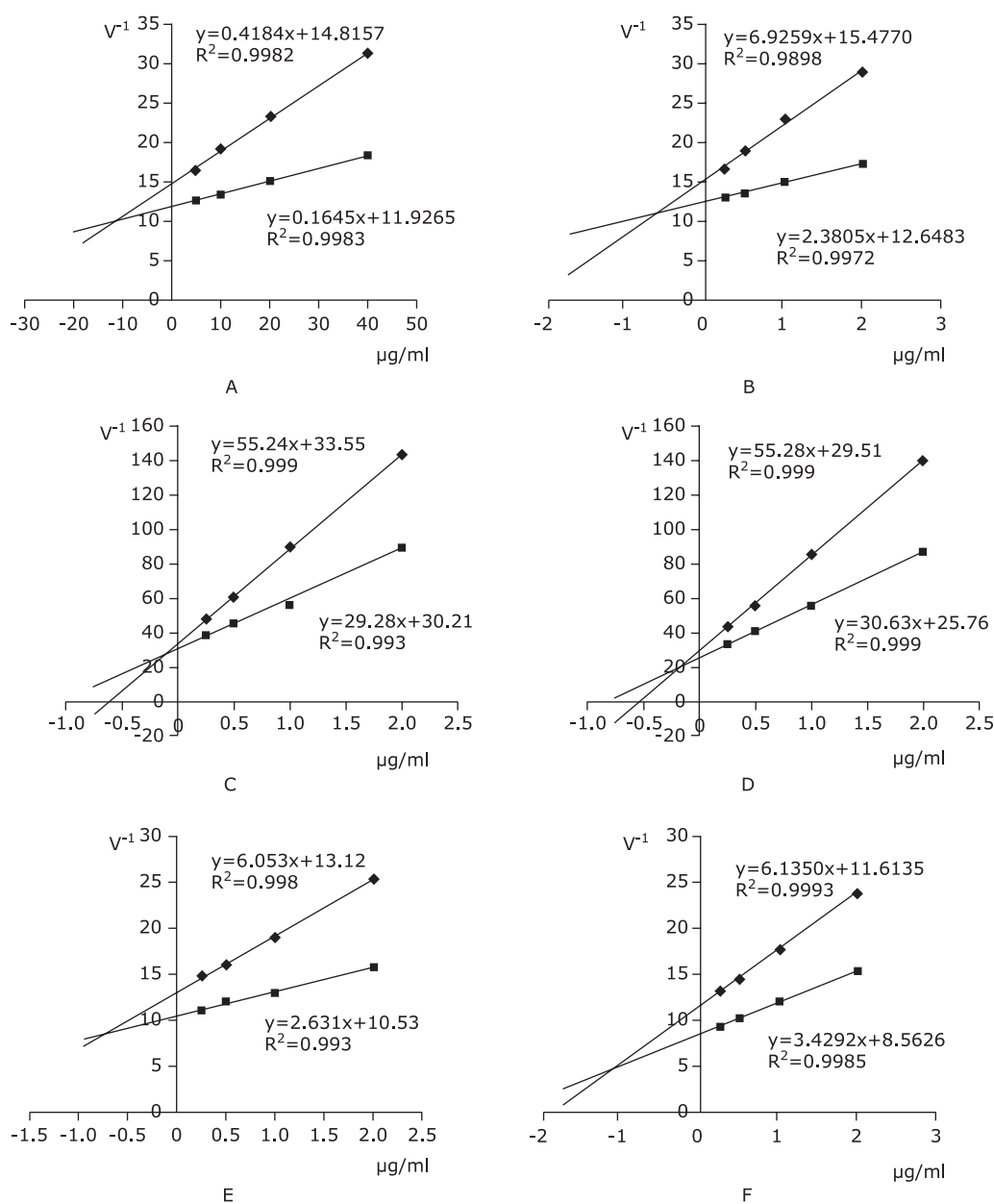


Figure 3. Dixon plot analysis of the inhibitory effect of *Gnaphalium affine* (A), allopurinol (B), eupatilin (C), apigenin (D), luteolin (E), and 5-hydroxy-6,7,3',4'-tetramethoxyflavone (F) on xanthine oxidase, with the intersection point of the lines indicating the K_i value of each compound. ♦ xanthine 100 $\mu\text{mol/L}$ ■ xanthine 200 $\mu\text{mol/L}$.

DISCUSSION

The increased risk of hyperuricemia has been linked with not only gout, but also the development of hypertension, hyperlipidaemia, cancer, diabetes, and obesity.¹⁴ Recent investigations strongly indicate an important pathophysiological role of XO in various forms of post-ischemic injuries, inflammatory diseases, and chronic heart failure.¹⁵

In general, allopurinol is the most common clinical inhibitor of XO. However, about 5% of patients are unable to tolerate its adverse effects, which include gastrointestinal irritation, bone marrow suppression, and hypersensitivity syndromes ranging from simple skin rash to life-threatening conditions in which the patients develop toxic epidermal necrolysis, fever, hepatitis, eosinophilia, and worsening renal function.¹⁶⁻¹⁸ Thus, new alternatives with higher therapeutic potency and less adverse effects are desired.

Gnaphalium affine is often used by Chinese people to cure musculoskeletal pains, carbuncle ulcers, etc. The *in vitro* inhibition of XO by Gnaphalium affine and its isolated compounds demonstrated one pharmaceutical effect of them. The ingredients of Gnaphalium affine are rather complicated, some of which exhibit strong inhibitory effect on the activity of XO. Aritomi and Kawasaki¹⁹ derived a new chalcone and two flavonoids from the methanol extract of fresh Gnaphalium affine flower, and defined them as 2',4,4'-trihydroxy-6'-methoxychalcone, luteolin and luteolin-4'-D-glucoside by infrared, ultraviolet, MRI, spectrum and chemical analysis. The antifeedant flavonoids, 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone, 5-hydroxy-3,6,7,8-tetramethoxyflavone, 5,6-dihydroxy-3, 7-dimethoxyflavone, and 4,4',6'-trihydroxy-2'-methoxychalcone, have been isolated from cudweed Gnaphalium affine D. Don using high performance liquid chromatography.²⁰ With chromatographic fractionation and spectroscopic methods, Aquino et al²¹ identified some cinnamic acid derivatives and 6 flavonoid constituents from an ethanol extract of the Gnaphalium uniflorum Lan, which showed a significant *in vitro* antioxidant effect and *in vivo* photoprotective activity. The flavonoids are rutin, quercetin-3-O- β -D-galactopyranoside-4'-O- β -D-glucopyranoside, quercetin-3-O- β -D-glucopyranoside, isorhamnetin-3-O- β -D-galactopyranoside, quercetin and kaempferol.

Flavonoids are a group of polyphenolic compounds reported to inhibit XO activity.²² Flemmig et al¹¹ reported that in comparison to the known synthetic XO inhibitor allopurinol with a K_i of 7.3 $\mu\text{mol/L}$, the flavone aglycone apigenin exhibited a strong inhibitory effect on XO with a K_i

of 0.52 $\mu\text{mol/L}$, luteolin²³ had a K_i of 2.9 $\mu\text{mol/L}$ and luteolin-7-O- β -D-glucoside had a K_i value of 15.0 $\mu\text{mol/L}$. Eupatilin has been reported to act as a novel antioxidant and may play an important role in the formulated ethanol extract DA-9601, which is now commercially available as a hepatoprotective, antioxidative, anti-inflammatory and antibacterial agent²⁴ in South Korea, and will be on sale in other Asian countries in the near future.²⁵

In comparable experiments published, XO inhibitors were pre-incubated with the enzyme before addition of xanthine.²⁶ In contrast, we applied a more physiological approach, i.e. pre-incubation of the inhibitor with the substrate. Therefore, the presented kinetic data cannot be directly compared to the results obtained under different experimental conditions or using a non-kinetic approach.² In the experimental conditions of this study, the 4 phenolics even demonstrated inhibitory effects exceeding that of allopurinol (Table 1).

In this *in vitro* investigation, we identified 4 potent XO inhibitors. Among them, the flavone eupatilin exhibited the most potent inhibitory effect on XO with a K_i value of 0.37 $\mu\text{mol/L}$. In comparison, the known synthetic XO inhibitor, allopurinol, showed a K_i of 4.56 $\mu\text{mol/L}$. Apigenin (K_i of 0.56 $\mu\text{mol/L}$), luteolin (K_i of 2.63 $\mu\text{mol/L}$) and 5-hydroxy-6,7,3',4'-tetramethoxyflavone (K_i of 3.15 $\mu\text{mol/L}$) also contributed considerably to the inhibitory effect of Gnaphalium affine on XO.

Eupatilin and 5-hydroxy-6,7,3',4'-tetramethoxyflavone have not been isolated from this plant before. Apigenin and luteolin were found in the Gnaphalium hypoleucum.²⁷ We also found 2 compounds, rutin and quercetin, which have been reported as weak XO inhibitors.²² They might also contribute to the inhibitory effect of Gnaphalium affine on XO.

In conclusion, this study suggests that Gnaphalium affine and its components have potent inhibitory effect on XO, which might be helpful in preventing or slowing the progress of gout. Further *in vivo* research is being carried out to identify a potential chemical entity for clinical use in the prevention and treatment of gout and related inflammatory disorders.

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