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In Vivo Antihyperuricemic Activities of 3,4,5-Tri-*O*-caffeoylquinic acid, 4,4',6'-Trihydroxy-2'-Methoxychalcone, and Caffeic Acid from the Aerial Parts of *Gnaphalium Affine*

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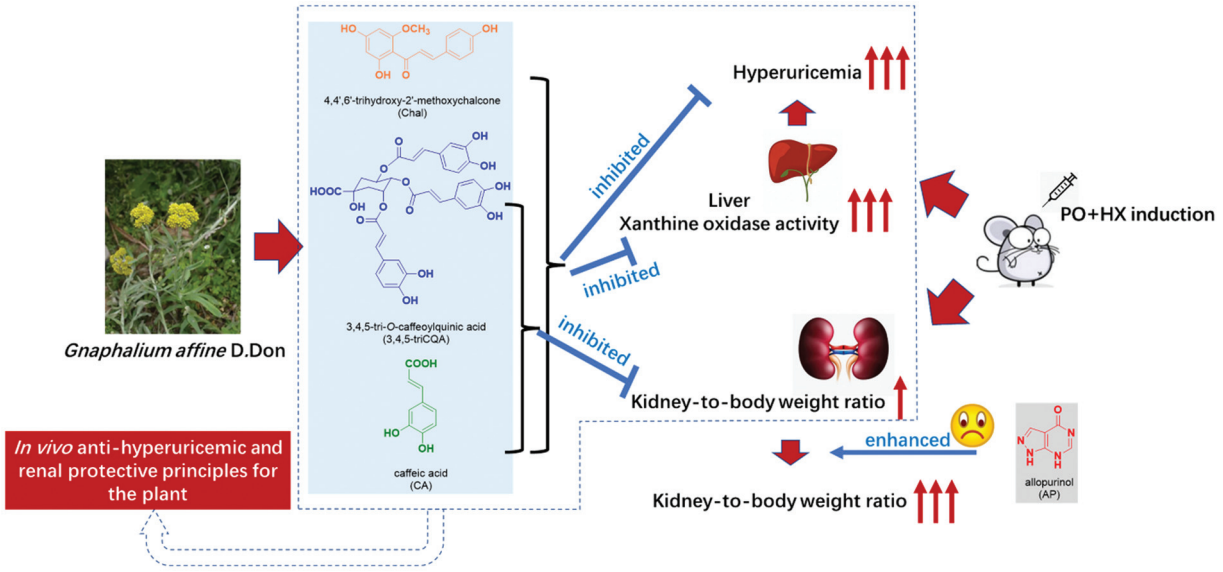

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Abstract

The extract of *Gnaphalium affine* has been reported to have antihyperuricemic and renal protective effects *in vivo*. The plant could alleviate acute hyperuricemia by inhibiting the activity of xanthine oxidase (XOD). 3,4,5-Tri-*O*-caffeoylquinic acid (3,4,5-triCQA), 4,4',6'-trihydroxy-2'-methoxychalcone (Chal), and caffeic acid (CA) were identified as the main ingredients of the plant attributed to the potential to retard XOD activity. However, whether the compounds were the effective ingredient of the plant exerting antihyperuricemic activity remained largely unknown. In this study, an experimental mouse model of hyperuricemia was induced by potassium oxonate and hypoxanthine, and orally administered with 3,4,5-triCQA (10 and 20 mg/kg/d), Chal (20 and 40 mg/kg/d), and CA (40 and 80 mg/kg/d) for 6 consecutive days, respectively. Then, serum urate levels and liver XOD activities were assessed. The liver- or kidney-to body weight ratio was calculated. Allopurinol (AP, 50 mg/kg/d) and benzbromarone (BBR, 10 mg/kg/d) were used as controls. Our data showed that there were 52.7 to 81.0% inhibitions in XOD activities in mice treated with 3,4,5-TriCQA (10 and 20 mg/kg/d), Chal (20 and 40 mg/kg/d), and CA (80 mg/kg/d), and 38.8 to 72.5% reduction in uric acid levels in mice treated with 3,4,5-TriCQA (20 mg/kg/d), Chal (20 and 40 mg/kg/d), and CA (40 and 80 mg/kg/d). A larger kidney-to-body weight ratio was observed in hyperuricemic mice and further enhanced by AP treatment. However, the increasing trend was significantly reversed by additional treatment of 3,4,5-triCQA (10 and 20 mg/kg/d) and CA (40 mg/kg/d). Given the above fundings, 3,4,5-triCQA, Chal, and CA may be the key component responsible for the *in vivo* activities of *G. affine* for urate-lowering therapy and even promising agents for the treatment of hyperuricemia.

Keywords

- *Gnaphalium affine*
- 3,4,5-tri-*O*-caffeoyl-quinic acid
- 4,4',6'-trihydroxy-2'-methoxychalcone
- caffeic acid
- antihyperuricemia

Introduction

Hyperuricemia (HUA; increased concentration of serum urate) is the most important risk factor for the development of gout and is associated with hypertension, insulin resistance, diabetes, renal diseases, and cardiovascular diseases.[1,2] It is very common in adults, especially in men, with increasing prevalence worldwide, ranging from 2.6 to 36% and estimated to be 13.3% in China.[2]

The aerial parts of *Gnaphalium affine* D. Don (Compositae) are a folk herbal medicine used for the treatment of rheumatic arthritis and gout in China.[3] It has been shown that *G. affine* extract has a urate-lowering effect on potassium oxonate (PO)-induced acute HUA by inhibition of xanthine oxidase (XOD) activity and urate transporter 1 (URAT1) expression.[4] Our previous study has also demonstrated the antihyperuricemic and renal protective activities of the plant in rats with chronic hyperuricemic nephropathy.[5]

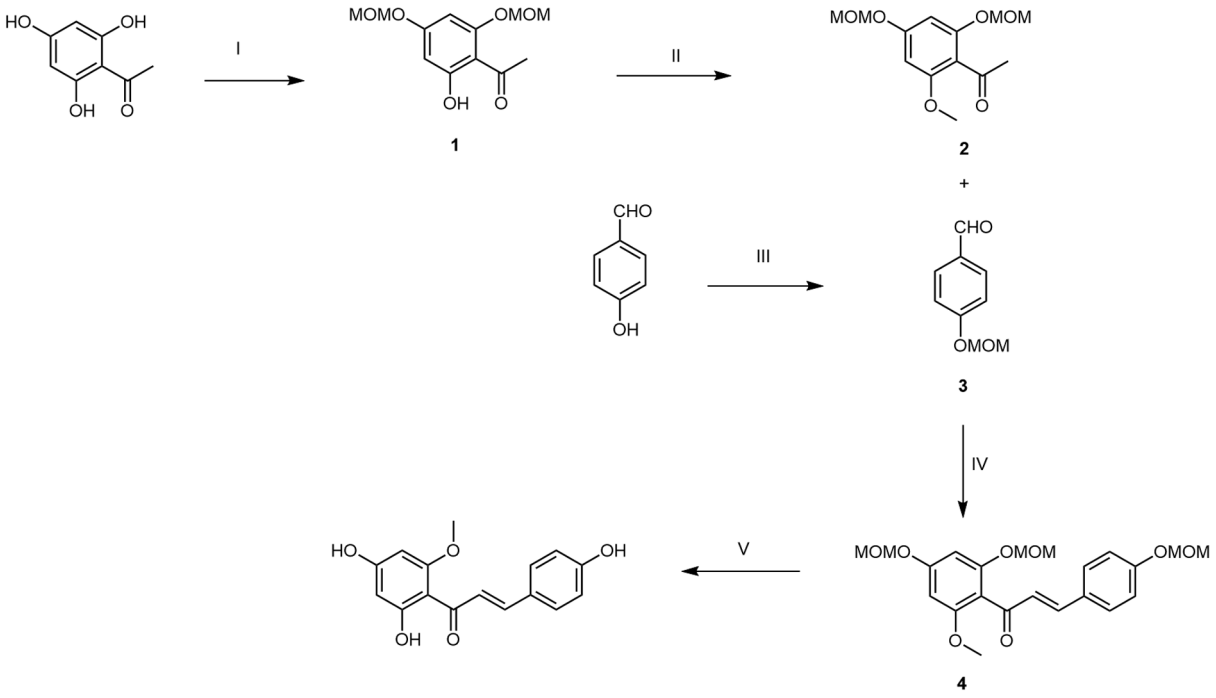
Evidence suggested that the extract of the herb is composed of caffeoylquinic acids, chalcones, caffeic acid (CA), flavonoids, and luteolin,[2,6] and except CA, all of them have potent XOD inhibitory activities with the IC_{50} values ranging from 2.4 to 27.9 μmol/L (positive drug-allopurinol, 1.6 μmol/L).[6] Interestingly, among those compounds, luteolin shows significant efficacy in treating HUA and gouty arthritis. Its derivative luteolin-4'-*O*-glucoside displays a similar effect, yet it does not have potent enzyme inhibition ($IC_{50} = 74.3$ μmol/L) *in vitro*.[6,7] However, whether those compounds have the potential antihyperuricemic activity remained largely unknown.

The so-far undescribed *in vivo* urate-lowering characterization of 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-triCQA) and 4,4',6'-trihydroxy-2'-methoxychalcone (Chal) (►**Fig. 1**) of the plant will be discussed in this study. Moreover, several pieces of literature have reported that caffeoylquinic acids can be hydrolyzed into CA (►**Fig. 1**) by the gut microbiota, and further re-absorption of CA into the bloodstream occurs in the colon.[8–11] Therefore, as both parent compound and metabolite after oral administration of *G. affine* extract, CA will also be tested for *in vivo* antihyperuricemic potential in a mouse experimental model in this study.

Material and Methods

Reagents and Chemicals

HPLC-grade acetonitrile, formic acid, and methanol were purchased from Adamas-β (Shanghai, China). XOD activity assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Uric acid (UA), PO, hypoxanthine (HX), benzbromarone (BBR, >98.0%), allopurinol (AP, >98.0%), and CA (>99.0%) were acquired from Sigma-Aldrich (St. Louis, United States), Macklin (Shanghai, China), Adamas-β (Shanghai, China), and J & K (Beijing, China), respectively. 3,4,5-triCQA (>95.0%) was isolated and prepared from *G. affine* according to a previous study.[6]



Scheme 1 Synthesis of Chal. Reagents and conditions: (i) acetone, K_2CO_3, MOMCl, 60°C, 2 hours, argon atmosphere; (ii) acetone, K_2CO_3, dimethyl sulfate, 60°C, 4 hours, argon atmosphere; (iii) acetone, K_2CO_3, MOMCl, 60°C, 2 hours, argon atmosphere; (iv) MeOH, KOH, 24 hours (ice); (v) MeOH, HCl, 65°C, 0.5 hours.

Animals

Male ICR mice (20 ± 2 g) were purchased from Sino-British-SIPPR/BK Laboratory Animal Ltd. (Shanghai, China). The animals were housed at a temperature of 20 ± 2°C, and humidity of 50 ± 5%, in a 12–12-hour dark–light cycle with *ad libitum* access to distilled water and sterilized food, for at least 7 days before the experiments.

Synthesis of Chal

The synthesis of Chal is described in ►**Scheme 1**. Following the procedure of Zhang et al[12] and Vogal et al,[13] a solution of 2,4,6-trihydroxybenzaldehyde (1 mmol) in acetone (10 mL, dry) was added to anhydrous K_2CO_3 (5 mmol) under an argon atmosphere. The resulting mixture was stirred for 30 minutes, then MOMCl (2.5 mmol) was added dropwise at 60°C under reflux. After being stirred for 2 hours, the resulting mixture was diluted with H_2O and extracted three times with EtOAc. The organic layer was combined and concentrated. The residue was purified by column chromatography (petroleum ether: EtOAc = 10:1) to obtain **1** (yield: 92%) as a yellow oil.

To a solution of **1** (1 mmol) in acetone (10 mL, dry) was added anhydrous K_2CO_3 (5 mmol) under an argon atmosphere and stirred for 30 minutes at r.t. Then dimethyl sulfate (1 mmol) was added dropwise and stirred at 60°C under reflux for 4 hours. The resulting mixture was diluted with H_2O and extracted three times with EtOAc. The organic layer was combined and concentrated. The residue was purified by column chromatography (petroleum ether:EtOAc = 15: 1) to obtain **2** (yield: 92%) as a light yellow oil.

To a solution of 4-hydroxybenzaldehyde (1 mmol) in acetone (10 mL, dry) was added anhydrous K_2CO_3 (5 mmol) under an argon atmosphere. The resulting mixture was stirred for 30 minutes, then MOMCl (1.2 mmol) was added dropwise at 60°C under reflux. After being stirred for 2 hours, the resulting mixture was diluted with H_2O and extracted three times with EtOAc. The organic layer was combined and concentrated to obtain **3** (yield: 84%) as a yellow oil.

To a solution of **2** (1 mmol) and **3** (1 mmol) in MeOH (10 mL) was added an aqueous solution of KOH (2 mol/L, 5 mL). The mixture was stirred at 0°C for 24 hours, and then diluted with 200 mL of ice water. The resulting solution was acidified with HCl (3 mol/L) to pH 3.0 and extracted with EtOAc three times, and the organic layer was combined and evaporated. The residue was purified by column chromatography to obtain **4** (yield: 78%) as a yellow powder.

To a solution of **4** (380 mg) in MeOH (3 mL) was added HCl (3 mol/L, 1 mL). The mixture was stirred for 30 minutes at 65° C, then diluted with ice water, and extracted with EtOAc three times. The organic layer was concentrated to give a residue, which was purified by column chromatography to obtain the target product (200 mg, yield: 70%) as an orange-yellow powder. mp: 251°C. HR-ESIMS (*m*/*z*): 285.0869 $[M-H]^-$; ^{1}H NMR (500 MHz, CD_3OD), δ 7.78 (d, J = 15.6 Hz, 1H), 7.67 (d, J = 15.6 Hz, 1H), 7.50 (d, J = 8.6 Hz, 2H), 6.83 (d, J = 8.6 Hz, 2H), 6.00 (d, J = 2.1 Hz, 1H), 5.92 (d, J = 2.1 Hz, 1H), 3.92 (s, 3H). ^{13}C-NMR (125 MHz, CD_3OD), δ 194.0, 168.6, 166.4, 164.6, 161.1, 143.8, 131.3 (× 2), 128.3, 125.5, 116.9 (× 2), 106.6, 97.0, 92.4, 56.4.

In Vivo Study

A PO and HX-induced hyperuricemic mouse model was established according to a reported study with a slight modification.[14] Briefly, 144 mice were randomly assigned to 6 groups ($n = 8$) for 3 separate experiments with the treatment described as follows: (1) normal control (NC), HUA, AP (50 mg/kg/d), BBR (10 mg/kg/d), and 3,4,5-triCQA (10 and 20 mg/kg/d); (2) same as (1), but were treated with Chal (20 and 40 mg/kg/d) instead of 3,4,5-triCQA; (3) same as (1), but were treated with CA (40 and 80 mg/kg/d) instead of 3,4,5-triCQA.

All drugs were suspended in 0.5% sodium carboxymethylcellulose (CMC-Na) and administered orally (po) to mice once daily for 6 consecutive days. The acute HUA in mice was induced by intraperitoneal (ip) administration of 100 mg/kg PO (0.9% physiological saline) and oral gavage of 500 mg/kg HX (0.5% CMC-Na) 0.5 hour after the last drug administration. The mice of the NC group were treated with the same volume of 0.9% physiological saline solution (ip) and 0.5% CMC-Na (po). One hour after modeling, the blood samples were collected and centrifuged at 5,000 rpm for 10 minutes at 4° C. Serum was separated and mixed with the same volume of MeOH. After centrifugation at 12,000 rpm for 10 minutes at 4°C, the upper layer was collected for UA determination by an HPLC method.[5]

After blood collection, the animals were sacrificed. The liver and kidney samples were removed, rinsed, and weighed rapidly. Liver tissue was cut into pieces, mixed with 0.9% physiological saline, and homogenized. After centrifugation at 5,000 rpm at 4°C for 10 minutes, the supernatants were collected for XOD activity assay according to the manufacturer's protocols.

Statistical Analysis

All data were expressed as mean ± standard deviation. The data between groups were further subjected to one-way ANOVA (analysis of variance) followed by Dunnett's multiple comparison test. $p < 0.05$ was considered statistically significant.

Results and Discussion

Because of its time-consuming and labor-intensive process of isolation, Chal was successfully obtained by total synthesis for further animal study. Aldol coupling with the MOM-protected benzaldehyde led to the protected chalcone, then deprotection with HCl in MeOH yielded the chalcone. The HR-ESIMS spectrum of Chal revealed an $[M - H]^-$ ion at m/z 285.0869, corresponding to the molecular formula $C_{16}H_{14}O_5$, which was indicative of 10 degrees of unsaturation. The ^{1}H-NMR spectrum of Chal showed an aromatic methoxyl group at $\delta = 3.92$ and eight aromatic protons. Two sharp doublets at $\delta = 7.78$ and 7.67 with $J = 15.6$ Hz are characteristic of the chalcone *trans* double bond. Three distinct sets of aromatic ring proton absorptions were shown as follows: an AA'BB' system for four protons, two at $\delta = 7.50$ and two at $\delta = 6.83$ ($J = 8.6$ Hz), indicating a *para*-substituted benzene ring; and two doublets at $\delta = 6.00$ and 5.92 ($J = 2.1$ Hz), characteristic of a 1,2,3,5-tetrasubstituted aromatic ring. In addition, the ^{13}C-NMR spectrum showed a signal for an α,β-unsaturated ketone at $\delta = 194.0$. The ^{1}H and ^{13}C resonances for Chal were in agreement with those reported by Aponte et al.[15] The spectroscopic data led to the identification of Chal as 4,4',6'-trihydroxy-2'-methoxychalcone.

In the animal study, all test articles and the positive control were preadministered to mice for 6 consecutive days before acute HUA induction. The experimental mice were dosed according to their body weight and body surface area. The human dose of BBR ranges from 25 to 100 mg/d, and that of AP ranges from 50 to 800 mg/d.[2] The mouse dose was converted into the corresponding human dose using the formula: mouse dose (mg/kg) = human dose (mg, reference body weight: 70 kg)/body weight of mouse (reference body weight: 0.02 kg)/body surface area ratio (387.9).[16] The mouse dose of BBR was calculated to be 3.2 to 12.9 mg/kg (10 mg/kg in this study), and that of AP was calculated to be 6.4 to 102.4 mg/kg (50 mg/kg in this study). *Gnaphalium affine* extract used in a previous animal study in rats showed a potent hypouricemic effect at doses of 450 (equiv. to 630 mg/kg in mice) and 900 mg/kg (equiv. to 1260 mg/kg in mice),[5] and it contained 3,4,5-triCQA (14.6 mg/g), Chal (29.8 mg/g), and CA (65.6 mg/g). The calculated doses of 3,4,5-triCQA, Chal, and CA in mice were approximately 10 (and 20), 20 (and 40), and 40 (and 80) mg/kg, respectively, and thus were chosen for the following experiment.

The HUA group had significantly higher serum UA levels than the NC group, indicating that acute HUA was effectively established. The positive control drug AP (an XOD inhibitor) was able to reduce serum urate levels of hyperuricemic mice to values lower than those found in normal animals. Another positive control drug BBR (a URAT1 inhibitor) also displayed significant reductions (59.8 to 77.8%) in UA levels in the three separate experiments. A 6-day pretreatment with 3,4,5-triCQA at the dose of 20 mg/kg/d, but not at 10 mg/kg/d, significantly reduced serum UA levels by 72.5% compared with the HUA group ($p < 0.01$). Chal showed significant antihyperuricemic activity at doses of 20 and 40 mg/kg/d with decreases in UA levels by 38.8 and 59.6%, respectively. CA was able to significantly reduce serum UA levels by 71.6 and 52.2% at doses of 40 and 80 mg/kg/d, respectively.

XOD is the enzyme that catalyzes the metabolism of HX and xanthine into UA. Recently, some *in vivo* data indicated that mono-caffeoylquinic acid (e.g., chlorogenic acid) and di-caffeoylquinic acid (ester) can inhibit the activities of XOD in the liver to decrease UA levels.[17,18] Our previous *in vitro* study of the structure–activity relationship also demonstrated that the introduction of more caffeoyl groups in the quinic acid skeleton may give more potency.[6] Inspiringly, the *in vivo* XOD inhibitory activity and the antihyperuricemic effect of tricaffeoylquinic acid were evaluated in the present study for the first time. Our data showed that 3,4,5-triCQA, at doses of 10 and 20 mg/kg/d, was able to inhibit liver XOD activities by 52.7 and 75.4% when compared with the HUA group. Evidence suggests that the administration of naturally occurring[19,20] or synthetic[21] chalcones could decrease UA levels by inhibiting liver XOD activities *in vivo*. Chal had a methoxy group at the 2'-position of the chalcone skeleton, which is

structurally different from the reported active chalcones. Interestingly, the methoxy group did not impact the activity. Chal (20 and 40 mg/kg/d) significantly inhibited liver XOD activities with an inhibitory ratio being 60.9 and 81.0%, respectively. Dose-dependent antihyperuricemic effects were also observed with the treatment of 3,4,5-triCQA and Chal, which, a priori, was consistent with results reported by Zhang et al regarding the XOD inhibition of the two compounds (3,4,5-triCQA, $IC_{50} = 20.45$ μmol/L; Chal, $IC_{50} = 3.12$ μmol/L) *in vitro*.[6] Furthermore, CA was a very weak XOD inhibitor ($IC_{50} > 50$ μmol/L),[6,22,23] and only significantly inhibited liver XOD activity at a high dose (80 mg/kg/d). However, despite the weakness of its XOD inhibition, CA had a potent urate-lowering effect (►**Fig. 2**). The mechanism

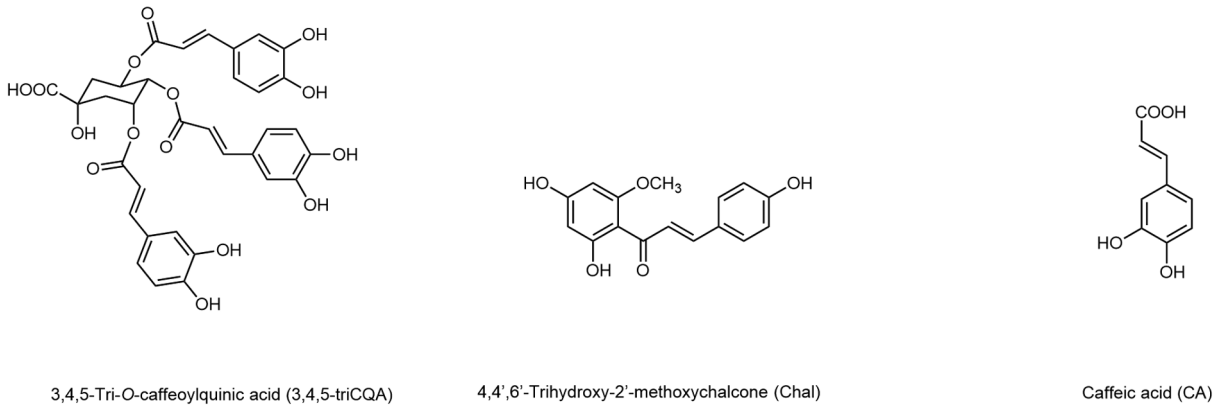


Fig. 1 Chemical structures of 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-triCQA), 4,4',6'-trihydroxy-2'-methoxychalcone (Chal), and caffeic acid (CA).

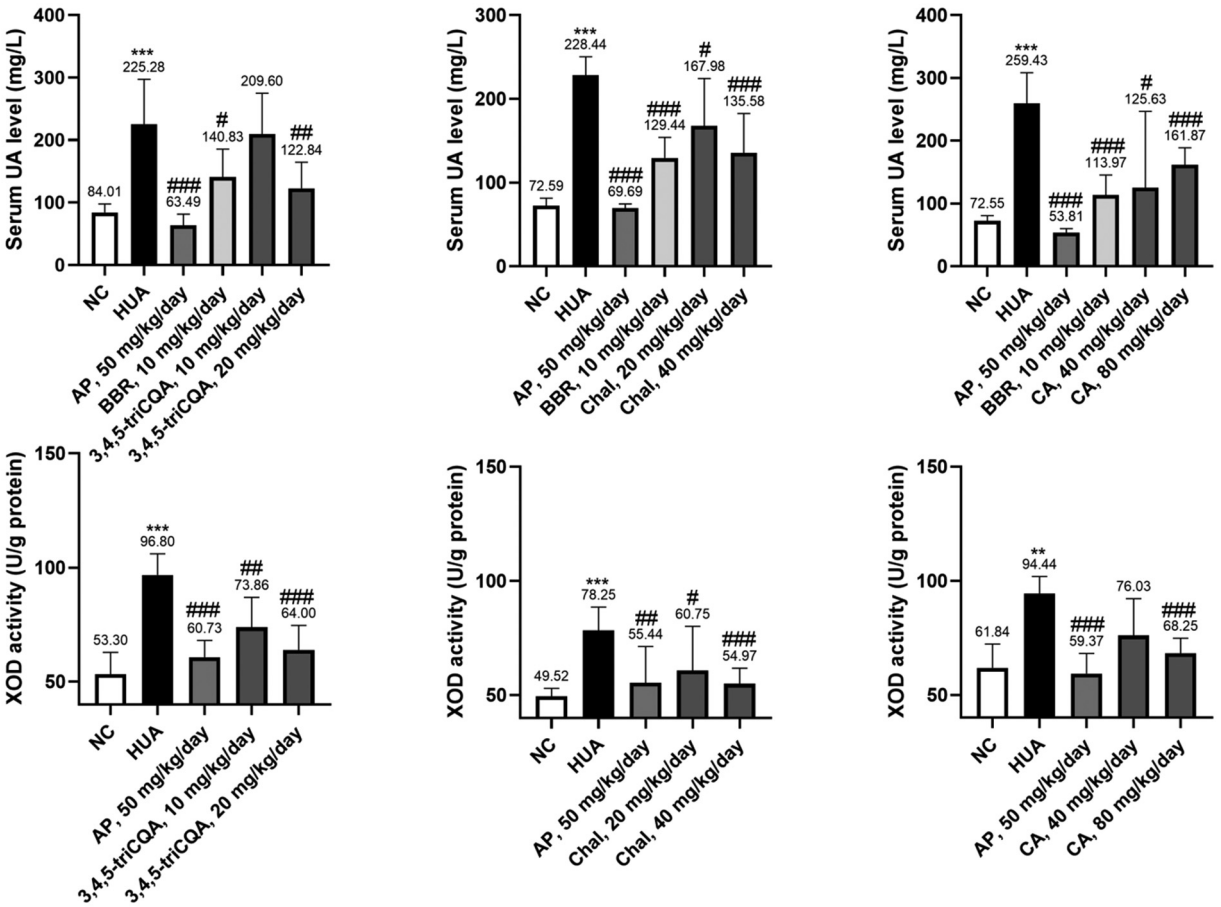


Fig. 2 Effects of 3,4,5-triCQA (10 and 20 mg/kg/d), Chal (20 and 40 mg/kg/d), and CA (40 and 80 mg/kg/d) on serum UA levels and liver XOD activities in HUA mice after 6-day oral administration. Values were expressed as mean ± standard deviation of eight animals. For statistical significance, one-way ANOVA was used followed by Dunnett's test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus NC group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus HUA group. 3,4,5-triCQA, 3,4,5-tri-*O*-caffeoylquinic acid; Chal, 4,4',6'-trihydroxy-2'-methoxychalcone; CA, caffeic acid; AP, allopurinol; BBR, benzbromarone; UA, uric acid; XOD, xanthine oxidase; NC, normal control; HUA, hyperuricemia.

may be that CA downregulates URAT1 and glucose transporter 9 (GLUT9) mediating UA re-absorption and upregulates organic anion transporter 1 (OAT1) and ATP-binding cassette transporter G2 (ABCG2) mediating UA efflux[23] in rats with HUA. In addition, in this study, AP (50 mg/kg/d), as a known XOD inhibitor, inhibited 79.4 to 107.6% of liver XOD activities.

Patients with HUA and gout need urate-lowering therapy for a long time or even their whole life.[2,24] Unfortunately, the long-term application of conventional drugs (XOD inhibitors and uricosuric agents) may result in higher risks of nephrotoxicity and hepatotoxicity.[24] It was confirmed by the abnormality in organ weights of positive drug–treated mice in this study. As shown in ►**Table 1**, a trend toward a larger kidney-to-body weight ratio was obtained by acute hyperuricemic induction and further enhanced by 6-day administration of AP 50 mg/kg/d ($p < 0.001$ or $p < 0.01$, compared with the NC group; $p < 0.001$, compared with HUA group). Meanwhile, 6-day administration of BBR 10 mg/kg/d induced a statistically significant increase in the liver-to-body weight ratio ($p < 0.001$ or $p < 0.05$, compared with the NC group; $p < 0.001$ or $p < 0.01$, compared with the HUA group). However, HUA-induced increases in kidney-to-body weight ratio were surprisingly inhibited after 6-day administration of 3,4,5-triCQA at doses of 10 and 20 mg/kg/d and CA at the dose of 40 mg/kg/d ($p < 0.01$ or $p < 0.05$, compared with HUA group). The results suggested that 3,4,5-triCQA and CA may be attributed to the renal protective potential of *G. affine*.[5] Meanwhile, additional studies investigating the effects and mechanisms of dual compounds on kidney function are required to confirm this. Furthermore, Chal (20 and 40 mg/kg/d) had no significant impact on the kidney-to-body weight ratio ($p > 0.05$ compared with NC or HUA group), and 3,4,5-triCQA and CA at all tested doses had no significant impact on the liver-to-body weight ratio ($p > 0.05$ compared with the NC or HUA group). It could be presumed that drugs extracted from this herb may be more beneficial with lesser adverse effects.[25]

Table 1 Effects of 3,4,5-triCQA, Chal, CA, AP, and BBR on kidney- or liver-to-body weight ratio in HUA mice after 6-day oral administration

Groups	Kidney-to-body weight ratio × 100%, %	Liver-to-body weight ratio × 100%, %
NC	1.42 ± 0.08	4.41 ± 0.31
HUA	$1.57 \pm 0.13^{*}$	4.52 ± 0.28
AP, 50 mg/kg/d	$2.11 \pm 0.36^{***,\#\#\#}$	4.41 ± 0.43
BBR, 10 mg/kg/d	$1.44 \pm 0.11^{\#}$	$5.55 \pm 0.26^{***,\#\#\#}$
3,4,5-triCQA, 10 mg/kg/d	$1.41 \pm 0.13^{\#}$	4.72 ± 0.33
3,4,5-triCQA, 20 mg/kg/d	$1.38 \pm 0.10^{\#\#}$	4.67 ± 0.55
Groups	**Kidney-to-body weight ratio × 100%, %**	**Liver-to-body weight ratio × 100%, %**
NC	1.57 ± 0.14	4.20 ± 0.23
HUA	1.65 ± 0.15	4.42 ± 0.46
AP, 50 mg/kg/d	$2.11 \pm 0.18^{***,\#\#\#}$	4.65 ± 0.34
BBR, 10 mg/kg/d	$1.43 \pm 0.10^{\#\#}$	$5.30 \pm 0.543^{*\#\#}$
Chal, 20 mg/kg/d	1.69 ± 0.26	4.07 ± 0.47
Chal, 40 mg/kg/d	1.57 ± 0.10	4.53 ± 0.15
Groups	**Kidney-to-body weight ratio × 100%, %**	**Liver-to-body weight ratio × 100%, %**
NC	1.46 ± 0.12	4.00 ± 0.16
HUA	1.63 ± 0.23	3.97 ± 0.16
AP, 50 mg/kg/d	$1.79 \pm 0.24^{**}$	4.13 ± 0.19
BBR, 10 mg/kg/d	1.57 ± 0.09	$4.57 \pm 0.42^{\#\#}$
CA, 40 mg/kg/d	$1.33 \pm 0.22^{\#}$	4.13 ± 0.19
CA, 80 mg/kg/d	$1.52 \pm 0.16^{\#\#}$	4.14 ± 0.14

Abbreviations: 3,4,5-triCQA, 3,4,5-tri-*O*-caffeoylquinic acid; AP, allopurinol; BBR, benzbromarone; Chal, 4,4',6'-trihydroxy-2'-methoxychalcone; CA, caffeic acid; NC, normal control; HUA, hyperuricemia.
Note: Values were expressed as mean ± standard deviation of eight animals. For statistical significance, one-way ANOVA was used followed by Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus NC group. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus HUA group.

A limitation of the present study was the time course of treatment: treatment is initiated before the disease pathology is initiated (prophylactic treatment). Drug accumulation is a prerequisite for the desired therapeutic effect. As low bioavailability, most herbal compounds need to be administered repeatedly to obtain the desired accumulation in the body. Thus, the efficacy of compounds during treatment would be assessed in a chronic animal model in further study.

Conclusion

In summary, to the best of our knowledge, this is the first study reporting the *in vivo* antihyperuricemic activities of 3,4,5-triCQA and Chal in the well-established animal model. CA exerted weak inhibitory effects on liver XOD activity indicating that the antihyperuricemic effects of CA may not be attributed to a decrease in UA production. 3,4,5-triCQA, Chal, and CA may be the key *in vivo* active principles of *G. affine* for urate-lowering therapy and even promising agents for the treatment of HUA. In addition, UA is associated with immune system activation and inflammation. HUA may play a key role in the development and progression of kidney disease. CA and its derivatives (e.g., caffeoylquinic acids) may be the major components responsible for the beneficial effects of *G. affine* extract on kidney function, which would be confirmed in further studies.

Ethics Statement

The use of animals in the study was approved by the Animal Ethical Committee of the Shanghai Institute of Pharmaceutical Industry, which conformed to the National Institutes of Health Guidelines on Laboratory Research and Guide for the Care and Use of Laboratory Animals.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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