

Hypouricemic and nephroprotective effects of total flavonoids from the residue of supercritical CO₂ extraction of *Humulus lupulus* in potassium oxonate-induced mice

Zhen Jing Li, Zhuan Li, Xiao Ying Dong, Lai Feng Lu and Chang Lu Wang*

Key Laboratory of Food Nutrition and Safety, Ministry of Education, School of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin, PR China

Abstract: Total flavonoids of *Humulus lupulus* (TFHL) were prepared using ethanol extraction, liquid-liquid partition and purification with polyamide resin. Different dose of TFHL were orally administered to normal and hyperuricemic mice for 7 days. The xanthine oxidase (XOD) inhibitory activity and hypouricemic effects of TFHL on potassium oxonate-induced hyperuricemic mice were examined. The TFHL showed very potent XOD inhibitory activity with IC₅₀=66.8 µg/mL. At a single oral dose of 100mg/kg TFHL, the serum uric acid levels of hyperuricemic mice significantly decreased ($P<0.01$) compared with a hyperuricemic control group, and the XOD activity was inhibited by 22%. Moreover, TFHL has a protective role against potassium oxonate-induced renal damage in mice. The results suggested that TFHL could be used as a promising drug or ingredient for treatment of hyperuricemia and gout.

Keywords: Total flavonoids, *Humulus lupulus*, xanthine oxidase, hyperuricemia.

INTRODUCTION

Hyperuricemia, one of the most common and extensive metabolic diseases in populations, characterized by high uric acid level in the blood, causing deposition of urate crystals in the joints and kidneys, and is greatly influenced by a high dietary intake of foods rich in nucleic acids. And, several studies have shown that alcohol is also a risk factor for hyperuricemia (Siener and Hesse 2003). The prevalence of gout among hyperuricemia population was 7.5%. Additionally, hyperuricemia is also associated with atherosclerosis, hypertension, diabetes and insulin resistance (So and Thorens 2010). The prevalence of hyperuricemia has been increasing in recent years in developed and developing countries, along with the development of their economies. A study in Beijing has shown that the incidence of hyperuricemia in males and females is 13.8% and 6.0%, respectively (Fang *et al.* 2006).

Xanthine oxidase (XOD), which catalyzes the oxidation of hypoxanthine and xanthine to uric acid, is a key enzyme in the formation of uric acid, and is the major target for the treatment of hyperuricemia. And, screening of XOD inhibitors has become one of the main strategies for the treatment of hyperuricemia. Allopurinol, a kind of chemical synthetic drugs, is the most commonly used XOD inhibitor in the past decades. However, undesirable side effects such as hypersensitivity, limit its therapy (Stamp *et al.* 2012). Therefore, developing new XOD inhibitors from natural plants is urgently necessary. The methanolic extract from the twig of *Cinnamomum cassia* is the most potent XOD inhibitor among the 122

traditional Chinese medicines (Kong *et al.* 2000). Studies have shown that quercetin and rutin can effectively lower serum uric acid levels in hyperuricemic mice and XOD and xanthine dehydrogenase activities in mouse liver (Zhu *et al.* 2004).

The female flowers of hop (*Humulus lupulus* L.) are used as flavoring agents in beer brewing. *H. lupulus* has a potential activity attributed to the presence several flavonoids, namely, prenylnaringenin, isoxanthohumol and geranylated flavonoids (Milligan *et al.* 2000; Gerhauser *et al.* 2002; Figard *et al.* 2008). The total flavonoids from the supercritical CO₂ extraction residue of *H. lupulus* reached 68.09 mg/g dry *H. lupulus* (Yang *et al.* 2008). Xanthohumol, the major flavonoid component of *H. lupulus*, is only found in *H. lupulus*. The study group of Oregon State University identified that the xanthohumol can induce three cancer cell lines apoptosis and decrease cell proliferation (Miranda *et al.* 1999). Additionally, xanthohumol potentially influences AIDS and malaria at least *in vitro*. Some experts believe that xanthohumol is a new all-rounder (Gerhauser and Frank 1999). However, it is not reported that hypouricemic and nephroprotective effects of total flavonoids of *H. lupulus* (TFHL) *in vivo*. In this study, we investigated the efficacy of TFHL in inhibiting XOD activity *in vitro*. And, the hypouricemic and nephroprotective effects of TFHL were also examined in hyperuricemic mice induced by potassium oxonate.

MATERIALS AND METHODS

Chemicals

Potassium oxonate, XOD and xanthine were purchased from Sigma-Aldrich Chemical Company. All other

*Corresponding author: e-mail: clw123@tust.edu.cn

chemicals and reagents used were of the highest commercially available purity.

Animals

Adult male ICR mouse strains weighing 22±3g were purchased from the Academy of Military Medical Sciences (Beijing, China). They were housed at least 1 week to adapt to their environment prior to the experiment. All studies were performed strictly in accordance with the Institutional Animal Care Committee at the Tianjin University of Science and Technology.

Preparation of total flavonoids of *H. lupulus*

Supercritical CO₂ extraction residue of *H. lupulus* (20 kg) was obtained from Gansu TouZhan Company in China. The hop residue was enriched in flavonoids and prepared according to the following methods.

The aqueous hop extract was obtained by macerating 20g of dry powder in 400mL of boiling water for 2h. The ethanol extract (20g) was extracted with 60% ethanol above 70°C for 1.5 h. The extractions were leached in vacuo and the solvents were removed using a rotary evaporator at 55°C to yield dried aqueous extract (7g, brown color) and ethanol extract (6g, green-brown color).

The aqueous and ethanol extracts were reconstituted in water and extracted by n-Butanol, and the upper solution was removed and powder A (brown color) and powder B (green-brown color) were obtained, respectively. Based on the combination of visual appearance for red coloration (indicating the presence of flavonoid) and XOD inhibitory activity, powder B was selected for further purification.

The powder B was reconstituted in water and enriched in flavonoids content using adsorption chromatography on a polyamide resin column. The ethanol extract was adsorbed onto the polyamide resin and eluted with 70% and 90% ethanol. The filtrate was then collected by a droplet collector; each tube volume was 4mL. Thin layer chromatography was carried out using GF₂₅₄ plates to examine the flavonoids. The column was then eluted with ethanol, and the filtrate was combined to obtain a gold extract (TFHL) after solvent removal as described previously.

Determination of total flavonoids content

The total flavonoids content of TFHL was calculated as equivalents of rutin and was determined using ultraviolet (UV) analysis monitoring at 510 nm (the characteristic absorption wavelength of flavonoids) (Jiang *et al.* 2009). Using a six point standard curve (0-50mg/mL), and the total flavonoids purity of TFHL (%) was expressed in percentage (milligram rutin equivalents/milligram dry TFHL powder×100).

XOD inhibitory activity

The XOD inhibitory activity was spectrophotometrically determined by following the increase in the absorbance at

295nm as described in the previous report (Umamaheswari *et al.*, 2007). Allopurinol was used as a positive control. All samples and allopurinol were assayed for XOD inhibitory activity at concentrations of 25, 50, 100 and 200µg/mL. The IC₅₀ values were calculated from the mean.

Hypouricemic effects

Hyperuricemic mice induced by potassium oxonate (uricase inhibitor) has been used to study hypouricemic effects of drug (Zhao *et al.* 2006; Hu *et al.* 2010). After one week of acclimation, 40 mice were randomly divided into 5 groups. Group 1 served as the normal group which received 0.5% sodium carboxymethyl cellulose; group 2 served as the hyperuricemia control, which received 0.5% sodium carboxymethyl cellulose; groups 3 and 4 received oral TFHL (100, 200 mg/kg body weight, respectively); and group 5 orally received allopurinol (10 mg/kg body weight). On the seventh day, the animals from groups 2 to 5 were intraperitoneally (i.p.) injected with potassium oxonate at 1h before drug administration. Two hours after injection, blood samples and livers were collected from mice. The blood was allowed to clot for 1h at room temperature and centrifuged at 3000 rpm for 5 min to obtain serum. The liver was rinsed with cold saline and cleaned by the filter paper. The liver tissue and serum were stored at -20°C until assayed.

STATISTICAL ANALYSIS

All statistical analyses of data were carried out using SPSS. The data were expressed as the mean ± S.D., and analysis of ANOVA and T Test was used to determine the level of significance. Values of *P*<0.05 were considered significant and *P*<0.01 as highly significant.

RESULTS

Total flavonoids purity was 62.2% by reference to a standard curve ($Y=1.1735X-0.0035$, $R^2=0.9995$). The extracts demonstrated XOD activity at 25, 50, 100 and 200µg/mL *in vitro*. The activity of the ethanol extract with IC₅₀=136.9µg/mL was better than that of aqueous extract, which showed a slight inhibitory activity (IC₅₀>200µg/mL). TFHL with IC₅₀=66.7µg/mL was the most potent extract. Allopurinol was a better inhibitors with IC₅₀=0.6µg/mL used as control (table 1).

As shown in table 2, the i.p. injection of potassium oxonate significantly increased the serum uric acid levels in hyperuricemic mice, which reached 296.4±22.1 µmol/L at 2 h after potassium oxalate injection. Administration of an oral dose of 100 and 200mg/kg TFHL to hyperuricemic mice reduced the serum uric acid levels to 197.0±12.0µmol/L and 246.6±26.8 µmol/L, respectively. In the same treatment, the serum uric acid levels of mice decreased to 114.6±24.7 µmol/L when allopurinol was used at a dose of 10 mg/kg.

Table 1: Xanthine oxidase (XOD) inhibitory activity *in vitro*

Extracts	Percentage of XOD inhibition (µg/mL)				IC ₅₀ (µg/mL)
	200	100	50	25	
Aqueous extract	42.2±0.3	33.6±0.2	21.7±0.2	-	>200
Ethanol extract	62.3±0.2	41.7±0.1	31.5±0.2	29.2±0.1	136.9
TFHL	57.0±0.2	54.8±0.1	47.4±0.1	43.7±0.01	66.7
Allopurinol	72.5±0.2	93.9±0.0	89.8±0.1	86.8±0.1	0.6

* The data represented the values of mean ± S.D. of three parallel measurements

Table 2: The effects of total flavonoids of *H. lupulus* (TFHL) and allopurinol on serum uric acid levels and liver XOD activity in hyperuricemic mice induced by potassium oxonate

Treatment	Dose (mg/kg)	Serum uric acid levels (µmol/L)	XOD activity (U/gprot)	XOD inhibition (%)
Normal	-	233.1±24.1 ^a	23.2±4.8 ^a	-
Hyperuricemia	-	296.4±22.1	28.9±3.9	-
Allopurinol	10	114.6±24.7 ^b	17.9±1.8 ^b	38
TFHL	100	197.0±12.0 ^b	22.5±3.2 ^a	22
	200	246.6±26.8 ^a	24.6±3.6	15

* The data represented the values of mean ± S.D. for 8 mice. (a, $P<0.05$; b, $P<0.01$ compared with the hyperuricemic control group)

Table 3: The effects of total flavonoids of *H. lupulus* (TFHL) and allopurinol on the levels of serum creatinine and serum urea nitrogen in hyperuricemic mice induced by potassium oxonate

Treatment	Dose (mg/kg)	Serum creatinine (µmol/L)	Serum urea nitrogen (µmol/L)
Normal	-	57.5±7.7 ^a	7.17±1.26 ^b
Hyperuricemia	-	67.8±4.5	15.79±2.15
Allopurinol	10	90.8±4.0 ^b	38.56±2.98 ^b
TFHL	100	61.5±14.9	9.50±1.09 ^b
	200	50.4±9.9 ^b	8.81±0.79 ^b

*The data represented the values of mean ± S.D. for 8 mice. (a, $P<0.05$; b, $P<0.01$ compared with the hyperuricemic control group)

TFHL also showed good XOD inhibitory activity in mouse liver. Administration mice with TFHL at a daily dose of 100 and 200 mg/kg for a week caused 22% and 15% inhibition to XOD in mouse liver, compared with a hyperuricemic control group. Allopurinol exhibited the highest activity with inhibition of 38% at a dose of 10 mg/kg.

As shown in table 3, potassium oxonate induced a significant elevation in serum creatinine ($P<0.05$) and serum urea nitrogen ($P<0.01$) compared with normal group. TFHL at 200 mg/kg significantly reduced serum creatinine ($P<0.01$) and serum urea nitrogen ($P<0.01$) compared with the hyperuricemic control group. In addition, at a dose of 100 mg/kg, TFHL did not affect serum creatinine, but restored the elevated serum urea nitrogen ($P<0.01$) in potassium oxonate-treated mice.

DISCUSSION

In recent years, natural or traditional Chinese herbs have become a novel resource of XOD inhibitors to directly cure hyperuricemia, or as lead compounds used for further research. Flavonoids are widely distributed in plants, attracting increasing attentions and interests owing to its various biological activities in the human body

(Ross and Kasum 2002). Coincidentally, many flavonoids are the natural XOD inhibitor in the prevention and treatment of hyperuricemia and gout (Lin *et al.* 2015).

H. lupulus L. is an ingredient in beers and rich in flavonoids, to preserve the beer and give beer its characteristic aroma, bitterness and flavor. China has rich resources of hops, and its total output ranks third in the world. At present, with the development of beer industry, the use amount of hops extract also increased year by year. Supercritical CO₂ is widely used to assist hop extraction, gives excellent identity to their use in the brewing industry. Whereas, flavonoids cannot be fully extracted out from hops by CO₂, leading to large amounts of flavonoids remaining in residues. In this study, the XOD inhibitory activity and hypouricemic effects of TFHL were examined on potassium oxonate-induced hyperuricemic mice, to explore and expanding its potential capability against hyperuricemia.

Flavonoid-rich plant extracts, such as pomegranate, onions, apples, garlic and scallions, are frequently standardized to rutin equivalents by research. The result of the present study demonstrated that flavonoids occupied 62.2% of TFHL and TFHL with IC₅₀=66.7 µg/mL was the most potent extract.

According to table 2, TFHL is a strong XOD activity inhibitor and presented strong hypouricemic effects in potassium oxonate-induced hyperuricemic mice. Interestingly, the potency of TFHL on the inhibition of XOD activity at 100mg/kg was less than that of allopurinol at 10mg/kg, the hypouricemic effects of TFHL in hyperuricemic mice were similar to that of allopurinol action after one week of oral administration. These hypouricemic effects are partly due to the inhibition of XOD activities in mouse liver. Meanwhile, we also found that flavonoids possibly contributes to the beneficial effects of TFHL on the reduction of urate levels and inhibition of enzyme activity.

Potassium oxonate treatments significantly increased serum creatinine ($P<0.05$) and blood urea nitrogen ($P<0.01$) level in hyperuricemic mice compared with normal group (table 3) and allopurinol enhanced above two indexes much more higher than potassium oxonate treatments in mice, suggesting both of potassium oxonate and allopurinol may damage the kidney function in mice, which was in agreement with the renal toxicity of allopurinol reported before (Stamp *et al.* 2012; Richette *et al.* 2013). TFHL at 100mg/kg could only reduce blood urea nitrogen content in hyperuricemic mice, while have no effect on serum creatinine level. When the concentration was increased to 200mg/kg, the TFHL could significantly reduce both levels of serum creatinine and blood urea nitrogen in hyperuricemic mice blood ($P<0.01$). Therefore, TFHL was supposed to play a protective role against renal damage in potassium oxonate-induced hyperuricemic mice.

Uric acid is the end product of purine metabolism in the human body, and 70% of total uric acid is rapidly removed from the human body by the kidneys (Chowalloor *et al.* 2014). More and more evidences show that protecting kidney is very important to hyperuricemia, which was associated with diabetic nephropathy, IgA nephropathy, acute kidney injury, chronic kidney disease and other kidney diseases (Ohno, 2011; Berni *et al.* 2000). Our results provide the evidence that the TFHL can reduce the total content of uric acid through inhibiting the activity of XOD, and also play a protective or repair roles against potassium oxonate-induced renal damage in mice.

CONCLUSION

The present study shows that TFHL could reduce the uric acid levels through inhibiting the activity of XOD in mice's blood, playing a protective or repair roles against potassium oxonate-induced renal damage in mice. These evidences provide more possibilities to TFHL of that it could be used as a promising drug or ingredient for treatment of hyperuricemia and gout. However, to understand the mechanism underlying such phenomena still requires further investigation at the molecular level.

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