**Hibiscus sabdariffa** L. extracts reduce serum uric acid levels in oxonate-induced rats

Chih-Yi Kuo\(\textsuperscript{a,b}\), Erl-Shyh Kao\(\textsuperscript{d,1}\), Kuei-Chuan Chan\(\textsuperscript{e,f}\), Huei-Jane Lee\(\textsuperscript{b}\), Tsai-Feng Huang\(\textsuperscript{b}\), Chau-Jong Wang\(\textsuperscript{b,c,*}\)

\(\textsuperscript{a}\)Department of Clinical Laboratory, Tai-An Hospital, Taichung, Taiwan, ROC
\(\textsuperscript{b}\)Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan, ROC
\(\textsuperscript{c}\)Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan, ROC
\(\textsuperscript{d}\)Department of Beauty Science, College of Living Technology, Chienkuo Technology University, Changhua, Taiwan, ROC
\(\textsuperscript{e}\)School of Medicine, Chung-Shan Medical University, Taichung, Taiwan, ROC
\(\textsuperscript{f}\)Department of Internal Medicine, Chung-Shan Medical University Hospital, Taichung, Taiwan, ROC

**ABSTRACT**

Elevated serum levels of uric acid are associated with an increased risk for hyperuricemia, gout, hypertension, cardiovascular disease, and renal failure. Recent attention has focused on the bioactive properties of edible plants in preventing disease. **Hibiscus sabdariffa** L., a local soft drink material and medicinal herb in Taiwan, is used effectively in native medicines against hypertension, pyrexia, and liver disorders. We investigated the effects of the **Hibiscus sabdariffa** extract (HSE) on oxonic acid (OA)-induced hyperuricemia in rats. The HSE affected serum uric acid levels and urate enzymes such as uricase and xanthine oxidase (XO). We treated rats intraperitoneally with normal saline and oxonate solution for 1 week and with or without feeding allopurinol (an XO inhibitor) or HSE (1%, 2%, and 5%) for 5 weeks. We observed that treatment with HSE inhibited OA-induced hyperuricemia, with a greater uric acid lowering effect than allopurinol treatment. Our results showed that HSE effectively inhibited OA-induced hyperuricemia by decreasing uric acid and increasing uricase activity, but not by affecting XO activity.

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**1. Introduction**

Uric acid is produced by purine metabolism. When adenine is catabolized, adenosine is converted to inosine, and then to hypoxanthine, which is in turn oxidized by xanthine oxidase (XO) to generate uric acid. The catabolism of guanine starts with conversion to xanthine and then to uric acid, with the second step also being catalyzed by XO (Cleland, Hill, & James, 1995). In many mammals, uricase is able to convert uric acid into allantoin, which has higher solubility and no obvious adverse effect (Sherman, Saifer, & Perez-Ruiz, 2008).

Hyperuricemia is a key risk factor for the development of gout, and has been linked to renal dysfunction, cardiovascular diseases, hypertension, diabetes and metabolic syndrome (Choi & Ford, 2007; Johnson, Titte, Cade, Rideout, & Oliver, 2005; Short & Tuttle, 2005). Hyperuricemia occurs as a result of overproduction of uric acid and impaired renal uric acid excretion (Terkeltaub, 2003). Xanthine oxidase (XO) catalyzes...
the oxidation of hypoxanthine and xanthine to uric acid in
the purine catabolic pathway (Ramallo, Zacchino, & Furlan, 2006). Inhibitors of xanthine oxidase are widely used to treat hyperuricemia. Allopurinol is the most commonly used xanthine oxidase inhibitor prescribed clinically for the treatment of gout. Allopurinol blocks the synthesis of uric acid and it also prevents the formation of ROS protecting against post-ischemic reperfusion injury (Riegersperger, Covic, & Goldsmith, 2011). Allopurinol remains to be a dominant urate-lowering agent. However, adverse effects limit its therapy including Stevens–Johnson syndrome, renal toxicity and even fatal liver necrosis (Wallach, 1998). The use of traditional medicine plants in the treatment of hyperuricemia and gout is suffered from the lack of scientific evidences (Kong, Cai, Huang, & Goldsmith, 2011). Allopurinol remains to be a dominant urate-lowering agent. However, adverse effects limit its therapy including Stevens–Johnson syndrome, renal toxicity and even fatal liver necrosis (Wallach, 1998). The use of traditional medicine plants in the treatment of hyperuricemia and gout is suffered from the lack of scientific evidences (Kong, Cai, Huang, & Goldsmith, 2011).

2. Materials and methods

2.1. Chemicals

H. sabdariffa L. was obtained from the Taitung District Farm Association, Taiwan. Allopurinol, potassium oxonate/oxonic acid, xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Assay kits for serum uric acid, creatinine, aspartate aminotransferase (AST), and alanine transaminase (ALT) were from Boscogen (Irvine, CA, USA). The amplex-red xanthine/xanthine oxidase assay kit and amplex-red uric acid/uricase assay kit were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

2.2. Preparing H. sabdariffa extract (HSE)

The HSE was prepared from H. sabdariffa L. Briefly, the H. sabdariffa (150 g) was macerated with hot water (95 °C, 6000 ml) for 2 h and the aqueous extract was evaporated under vacuum at –85 °C. The extracted solution was filtered and then lyophilized to obtain approximately 75 g of HSE, which was stored at –20 °C before use. The concentration of total phenols was analyzed according to the Folin–Ciocalteau method (Lakenbrink, Lapczynski, Maiwald, & Engelhardt, 2000). HSE (0.1 mg) was dissolved in 1 mL of distilled water and Folin–Ciocalteau reagent (1 M, 0.5 ml) was added and mixed in thoroughly. After an interval of 3 min, 3 ml of 2% Na2CO3 solution was added, and the mixture was incubated for 15 min with intermittent mixing. The absorbance of the mixture at 750 nm was measured on a Hitachi U-3210 spectrophotometer (Tokyo, Japan) with gallic acid as a standard. Total anthocyanin content was determined by the method of Fuleki and Francis (Fuleki & Francis, 1968). Ten milliliters of HSE were diluted to 50 ml with buffer at pH 1.0 and 4.5, respectively. The optical density (OD) of the samples at 535 nm was measured with distilled water as blank. The OD difference was obtained by subtracting the total OD at pH 4.5 from the total OD at pH 1.0. Both values were calculated from the OD readings using the appropriate dilution and calculation factors. Total flavonoid content was determined as described by Jia et al. (Jia, Tang, & Wu, 1999). First, 0.5 ml of 1 mg/ml HSE was diluted with 1.25 ml of distilled water. Seventy five microliter of 50 g/L NaNO2 solution were added. After 6 min, 150 μL of 10% AlCl3 6H2O solution were added and the mixture was allowed to stand for a further 5 min. Second, 0.5 ml of 1 M NaOH was added and the volume was made up to 2.5 mL with distilled water. The solution was mixed well and its absorbance at 510 nm was read immediately.

2.3. Preparing H. sabdariffa L. polyphenolic extract (HPE)

H. sabdariffa (100 g) was extracted three times with 300 mL of methanol at 50 °C for 3 h and the samples were filtered after each extraction. Solvent was removed from the combined extract with 200 mL hexane to remove some of the pigments. The aqueous phase was extracted three times with 180 mL.

Table 1 – Functional components content of HSE.

<table>
<thead>
<tr>
<th>The components of HSE</th>
<th>HSE (%)</th>
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<tr>
<td>Flavonoid (Jia method)</td>
<td>13.9 ± 2.1</td>
</tr>
<tr>
<td>Anthocyanin (Fuleki and Francis method)</td>
<td>24.8 ± 2.6</td>
</tr>
<tr>
<td>Polyphenol (Folin–Ciocalteau method)</td>
<td>16.7 ± 1.6</td>
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ethyl acetate, and the ethyl acetate was evaporated under reduced pressure. The residue was redissolved in 250 mL water and was lyophilized to obtain approximately 2 g of HPE. The components of HPE were analyzed by HPLC (Table 2).

2.4. HPLC Assay for HPE

The components of HPE were determined by HPLC analysis using a Hewlett-Packard Vectra 436/33N system with a diode array detector. The HPLC method employed a 5 μM RP-18 column (4.6 x 150 mm i.d.). The HPE were filtered through a 0.45 μM filter disk and 20 μM were injected onto the column. The chromatography was monitored at 280 nm, and UV spectra were collected to confirm peak purity. The mobile phase contained two solvents (A, formic acid/water = 10:90; B, formic acid/water/acetonitrile = 10:60:30) run by a linear gradient method at room temperature as follows: from 20% B to 85% B (flow rate = 0.8 μL/min) over 55 min.

2.5. Animals and experimental design

All animal experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of the Chung Shan Medical University (IACUC, CSMU), Taichung, Taiwan. Male Sprague-Dawley (SD) rats (150 ± 20 g) used in the studies was purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). All animals were housed in laboratory conditions for 1 week before experiments were performed. Rats were allowed free access to drinking water and food and were maintained at a constant temperature of 22 °C and 50–60% relative humidity conditions with an automatic 12 h light and 12 h dark cycle.

Experimental animal model of hyperuricemia induced by oxonic acid (urate inhibitor) has been used to study drug action (Hall, Scoville, Reynolds, Simlot, & Duncan, 1990). Rats were randomly divided into six groups (eight rats per group). Group one: rats were given the same volume injection of sterile saline only. Group two: rats were injected intraperitoneally with oxonic acid (OA; 280 mg/kg) to increase the serum urate level for 1 week; group three: rats were treated with allopurinol (10 mg/kg) and then injected with OA; group four: rats were treated with 1% HSE (mixed with Purina Chow diet) and then injected with OA; group five: rats were treated with 2% HSE (mixed with Purina Chow diet) and then injected with OA; group six: rats were treated with 5% HSE (mixed with Purina Chow diet; Inc., ST. Louis, USA) and then injected with OA. Rats in group 3–6 were fed orally with allopurinol and HSE once a day for 5 week. Whole blood sample were collected to separate plasma for detection of liver function, and liver tissues were collected for further analysis.

2.6. Measuring serum uric acid, creatinine, aspartate transaminase (AST), and alanine transaminase (ALT) levels

Plasma AST, ALT, uric acid, and creatinine levels were measured by enzymatic colorimetric method using an Olympus AU 2700 automatic analysis (Olympus Co., Tokyo, Japan).

2.7. Determination of xanthine oxidase and uricase activity

Rat liver were excised, frozen immediately and stored at −80 °C until analysis. Liver tissue was homogenized in Tris–HCl buffer (5 mM containing 2 mM of EDTA, pH 7.4) to give a 10% (w/v) liver homogenate. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. After centrifugation, the supernatant was used for XO activity and uricase activity assays. Both XO activity and uricase activity were determined spectrophotometrically using Amplex-Red xanthine oxidase assay kit and Amplex-Red uricase assay kit, respectively (Invitrogen, Carlsbad, CA, USA). The liver enzyme activity is expressed as mol/min per g protein (mU/mL).

2.8. Statistical analysis

The experiment was conducted using a completely random design (CRD). Data were analyzed using analysis of variance (ANOVA). A significant difference was considered at the 0.05 probability level and differences between treatments were tested using the least significant difference (LSD) test. All statistical analyses of data were performed using SAS.

3. Results

3.1. Effect of HSE on OA-induced hyperuricemia

Oxonic acid (an uricase inhibitor) treatment caused hyperuricemia in rats, as indicated by drastic increases in serum uric acid levels. As shown in Fig. 1, serum uric acid levels were 0.9 mg/dl and 1.0 mg/ml for 2 and 5 weeks in control rats. The serum uric acid levels of OA-induced group were elevated significantly to 2.6 mg/dl until the end of the experiment. Rats treated with OA and allopurinol, which is a drug that inhibits XO, had reduced serum uric acid levels (1.3 mg/dl) compared with OA-induced rats. Likewise, the serum uric acid levels of hyperuricemic rats treated with high doses of 5% HSE were lowered significantly by 0.9 mg/dl for 2 and 5 weeks.

Table 2 – Composition of the HPE by HPLC analysis.

<table>
<thead>
<tr>
<th>Polyphenolic compounds of HPE</th>
<th>HPE (%)</th>
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<tbody>
<tr>
<td>PCA (protocatechuic acid)</td>
<td>8.62 ± 0.91</td>
</tr>
<tr>
<td>EGCg (Epigallocatechin galla)</td>
<td>20.34 ± 1.64</td>
</tr>
<tr>
<td>Catechin</td>
<td>9.86 ± 1.02</td>
</tr>
<tr>
<td>EGC (Epigallocatechin)</td>
<td>10.11 ± 1.48</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>18.24 ± 0.39</td>
</tr>
</tbody>
</table>
(p < 0.05). However, the effect of HSE was more potent than that of allupurinol in serum uric levels.

3.2. Effect of HSE on body weight

The effect of continuously consuming HSE on health was examined by measuring body weights each week (Fig. 2). The body weights of all the rats gradually increased throughout the experiment, regardless of their treatment group. Since there were not significant differences between the treatment groups, body weight was not influenced by HSE.

3.3. Effect of HSE on liver and renal functions

Many liver enzymes, such as AST and ALT, are released into the bloodstream when the liver is diseased or damaged, so we evaluated liver health by measuring serum levels of these enzymes (Fig. 3A and B). The levels of AST and ALT were similar for untreated rats and for rats treated with OA, HSE, or with the combination of OA and either allopurinol or HSE. These results indicate that liver function remains normal after continuous treatment with HSE.

Creatinine, produced from creatine, is filtered out of the blood by the glomerulus of kidneys and excreted in urine.
and its level in the blood is a marker for renal function. Treatment with different concentrations of HSE (1%, 2%, and 5%) and allopurinol did not affect serum creatinine levels (Fig. 3C), which suggests that renal function was not compromised by these treatments.

3.4. Effect of HSE on xanthine oxidase activity in the blood and liver

The levels of uric acid are controlled by XO, so we measured serum and liver XO activity, since changes in XO activity might alter serum uric acid levels (Fig. 4). Serum XO activity decreased over 80% and liver XO activity increased in rats treated with allopurinol compared with those treated with only OA. Serum and liver XO activity was not affected by HSE at any of the concentrations tested. This indicates that HSE does not lower serum or liver XO activity when it reduces serum uric acid levels.

3.5. Effect of HSE on uricase activity in the blood and liver

Since uricase catalyzes the oxidation of uric acid to allantoin, which decreases uric acid levels, the uricase activity in the blood and liver were monitored in order to understand the mechanism by which HSE reduced serum uric acid levels (Fig. 5). Rats treated with OA 5 weeks later had decreased serum uricase activity that coincided with the occurrence of hyperuricemia. Rats treated with both allopurinol and OA had significantly increased serum uricase activity compared with rats treated with only OA ($p < 0.0005$). Rats treated with both HSE and OA also had increased serum uricase activity compared with rats treated with only OA ($p < 0.0005$ relative to 2% HSE, $p < 0.05$ relative to 1% HSE and 5% HSE). Rats treated with OA had decreased liver uricase activity compared with control rats, while rats treated with allopurinol had increased liver uricase activity compared with rats treated with only OA (Fig. 5B). Rats treated with 2% or 5% HSE had higher increases in liver uricase activity relative to rats treated with only OA ($p < 0.05$ for 2% HSE, $p < 0.005$ for 5% HSE). These results indicate that augmenting uricase activity in the blood and liver might play a part in reducing serum uric acid levels in rats treated with OA.

4. Discussion

Though hyperuricemia is the most important biochemical basis of gout, it is not synonymous with gout. Hyperuricemia is caused by a purine metabolic disorder and can result in gout. Roughly 10% of hyperuricemia patients (defined as those having serum uric acid levels above 7 mg/dL) have gout. Gout stones appear in the face, ears, elbows, or elsewhere, and affect the appearance. Gout stones in the kidney resulted in
urethra stones or obstruction, and then kidney function is damaged. Most researchers use animal models to study hyperuricemia and gout. Three forms of hyperuricemia animal experiments are currently established: (1) test of long-term induction that induced hyperuricemia with 2% OA in daily diet, and also given allopurinol daily for up to 7 weeks to positively control uric acid (Mazzali et al., 2001); (2) test of long-term exposure to high amount of uric acid that mixed OA and extra uric acid with the daily diet to maintain a high concentration of serum uric acid, which leads to uric acid crystals in the kidney and acute renal failure (Mazzali et al., 2001; Wu et al., 1994); and (3) short-term, acute exposure that is injected OA into the abdominal cavity and collected samples as uric acid levels rise 1–2 h after the injection, with uric acid levels decreasing shortly after that (Yonetani, Ishii, & Iwaki, 1980).

The second model of long-term exposure to high levels of uric acid is based on human clinical symptoms of acute hyperuricemia. Our study’s test model modified the short-term, acute exposure method (Yonetani et al., 1980) to give daily intraperitoneal injections of OA for 1 week, and found that hyperuricemia in rats can be maintained at least 5 weeks (Fig. 1). The uric acid levels of the group treated with OA increased 1.5 times after 2 weeks after the intraperitoneal injection and increased 2.5 times after 5 weeks, compared with the control group.

The enzyme XO exists in most mammalian tissues, including rat plasma, with its highest concentrations in the liver and the intestine (Battelli et al., 1999). Uric acid is generated when ing rat plasma, with its highest concentrations in the liver and 2.5 times after 5 weeks, compared with the control group. The mechanism of action of HSE is not by inhibiting XO or by promoting uricase activity to promote uric acid excretion. Although much literature indicates that flavonoids can inhibit XO activity, some in vitro experiments have found that flavonoids have minimal XO or no impact on XO activity, fail to reduce uric acid as well as allopurinol, and may have limited effectiveness (Huang et al., 2011). This agrees with our studies, the HSE treatment did not show a significant effect on XO inhibition in the serum and liver but did have a significant reduction on uric acid levels in hyperuricemic rats. The reduction of uric acid occurs by increasing uricase activity to improve the decomposition of uric acid and promote excretion of uric acid in HSE-treated groups. The difference in XO inhibition results between studies may be due to differences in experimental conditions, differences in the bioavailability of the falconoid and their extensive metabolism in mice. In addition, the diet and plant itself would not be like the pure chemicals directly producing antioxidant or prooxidant effects towards overt toxicity. However, the clinical XO inhibitor, Allopurinol, significantly inhibited XO activity.

This study used animal models to maintain a high concentration of serum uric acid for a longer period of time than other studies. We found that HSE significantly lowered uric acid by increasing uricase activity to promote uric acid excretion. This result seems independent of the human mechanism to reduce uric acid, the uricase activity suggests possible new treatment options for patients with gout (Christopher & Robert, 2011). The HSE may be able to help improve gouty arthritis efficacy of new therapies.

Conflicts of interest

The authors disclose no conflicts.

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