Full paper Laboratory Animal Science

Protective effects of Corni Fructus Extract in mice with Potassium Oxonate–induced Hyperuricemia

Chih-Chiang Wang 1), You-Liang Li2), Po-Yen Chiu2), Chun Chen3), Hung-Che Chen2), Fu-An Chen2)*

1) Department of Medicine, Kaohsiung Armed Forces General Hospital, No.2, Zhongzheng 1st Rd, Lingya Dist, Kaohsiung City, 802, Taiwan

2) Department of Pharmacy and Master Program, Tajen University, Pingtung, Taiwan

3) School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung City 807, Taiwan

* Corresponding author. Department of Pharmacy and Master Program, Tajen University, 20 Weixin Road, Yanpu, Pingtung 907, Taiwan. E-mail addresses: fachen@tajen.edu.tw (F.-A. Chen). Tel : +886-87624002#2617

Running head
Corni Fructus reduces hyperuricemia
Abstract

Corni fructus is consumed as food and herbal medicine in Chinese culture. Studies have revealed that corni fructus exhibits potent antioxidant activity; however, few studies have investigated the ability of corni fructus to lower uric acid concentrations. In this study, the xanthine oxidase (XO) inhibition and uric acid–lowering effect of corni fructus extract (CFE) were evaluated in mice with potassium oxonate–induced hyperuricemia. Hyperuricemia is a chronic disease prevalent worldwide and is associated with high recurrence rates. In addition, drugs used to treat hyperuricemia induce side effects that discourage patient compliance. Hyperuricemia induces metabolic imbalances resulting in accumulative uric acid deposition in the joints and soft tissues. Hyperuricemia not only induces gout but also interrupts hepatic and renal function, thereby trigging severe inflammation and various complications, including obesity, nonalcoholic fatty liver disease, diabetes, and metabolic diseases. In this study, the ethyl acetate fraction (EAF) of CFE resulted in yields of antioxidant photochemical components significantly higher than those of CFEs formed using other substances. The EAF of CFE exhibited high free radical scavenging activity and XO inhibition and effectively lowered uric acid concentrations in the animal model of chemically induced hyperuricemia. The results of this study can serve as a reference for the prevention of preclinical gout as well as for functional food research.

KEYWORDS: corni fructus, hyperuricemia, recurrent hyperuricemia
Hyperuricemia refers to an abnormally high serum uric acid (SUA) concentration. The concentration of urate in the body depends on the amount of purine ingested, synthesized, and excreted. Hyperuricemia results from a dysregulation of metabolism [18] and has attracted much attention, particularly from genome-wide association studies (GWASs). Generally, in such studies, the blood or saliva of numerous individuals with and without hyperuricemia is analyzed and genetically sequenced. After sequencing, researchers often refer to literature on biology, genomics, and medicine to determine which genes may affect the disease. GWASs have been used to identify the effects of urate reabsorption–associated transporters—including urate transporter 1, organic anion transporter 1 (OAT1), OAT3, and OAT4, glucose transporter 9, and ATP binding cassette subfamily G member 2—and their respective genes on kidney tissue [53]. Genetic variations identified through GWASs explain less than 10% of the variation in SUA concentration; the remaining variation can be explained by the influences of environmental and genetic characteristics contributing to gout [41].

An abnormally high rate of uric acid production can lead to the formation of uric acid crystals, which stimulate the secretion of inflammatory factors by macrophages and neutrophils, causing an autoimmune response [40, 46]. Allopurinol, febuxostat, and benzbromarone are the first-line drugs for reducing uric acid levels; among these, allopurinol and febuxostat are similar to purines in structure and therefore exhibit competitive xanthine oxidase (XO) inhibition [9, 15-17, 30, 34, 43, 50, 57]. In addition, benzbromarone can regulate renal transfer proteins, such as GLUT9, OAT1, and OAT3, to reduce uric acid reabsorption and promote renal uric acid excretion [34]. However, the side effects of uric acid–lowering drugs are considerable: allopurinol can cause Stevenson disease, febuxostat can cause cardiovascular disease, and benzbromarone is hepatotoxic [1, 11, 22, 34, 37]. Generally, hyperuricemia-induced gouty arthritis causes severe pain, which is often treated with anti-inflammatory drugs, but such drugs carry the potential side effects of gastrointestinal tract damage, hypertension, and nephrotoxicity [7, 8, 10, 27, 51].
The prevalence of gout in advanced countries exceeds 1%, and many patients require medication to control their hyperuricemia. However, the low rate of compliance with uric acid–lowering drugs leads to a high recurrence rate of gout, in turn leading to a high rate of anti-inflammatory drug use [6, 19]. Therefore, a natural Chinese medicine treatment that can reduce uric acid is desirable.

_Cornus officinalis_ has long been used in traditional Chinese medicine because it contains several active compounds, including morroniside, cornuside, loganin, sweroside, oleanolic acid, and ursolic acid [14]. _C. officinalis_ has been reported to have potential antioxidant, anti-inflammatory, and antidiabetic properties and to provide renal protection [35, 36, 44, 54]. In this study, we demonstrated that corni fructus extract (CFE) could be used to reduce uric acid concentrations.

Materials and methods

2.1 Materials

_C. officinalis_ was purchased from Qingfeng Pharmacy, an herbal store in Taiwan, in 2019. Methyl alcohol, grain alcohol, acetone, phosphoric acid, and hydrochloric acid (HCl) were purchased from Echo Chemical. Other chemical agents, namely, _n_-hexane, ethyl acetate, and _n_-butanol, were obtained from Extra Pure Scientific Co. (Nihon Shiyaku Reagent, Taichung, Taiwan) Allopurinol, potassium oxonate (PO), Folin–Ciocalteu phenol reagent, xanthine, XO, gallic acid, tocopherol, potassium dihydrogen phosphate, sodium dihydrogen phosphate, ethylenediaminetetraacetic acid, sodium chloride, potassium chloride, sodium carbonate, dimethyl sulfoxide (DMSO), carboxymethyl cellulose, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich (Merck KGaA, Darmstadt, Germany).

2.2 Preparation of CFE

Powder was obtained by extraction with a 10-fold excess volume of 95% EtOH (w/v %) for 3
hr using a reflux extraction apparatus (Angu, Kaohsiung, Taiwan). The extraction solution was filtered through paper in a funnel. Crude CFE (CFE95E), which exhibits XO-inhibitory activity, underwent liquid-phase extraction with hexane, ethyl acetate, n-butanol, and pure water fractions to purify its constituent compounds (Supplementary Fig. 1). The solvents were removed through concentration under freeze-drying procedures, and stored for the following experiments.

2.3 Analysis of total polyphenol content of CFE

The total polyphenol content of CFE was estimated spectrophotometrically through colorimetric redox reaction with Folin–Ciocalteu reagent. In brief, 10 mg of serial CFE was dissolved in 10 mL of acetone solution (acetone:H₂O = 6:4). Subsequently, 0.2 mL of the CFE solution mixed with 7.5% Na₂CO₃ and 1.0 mL of Folin–Ciocalteu reagent was added to each well of a 96-well microplate (Jet Biofil, FEP-000-096, Guangzhou, China) and mixed thoroughly. After 30 min of incubation at room temperature, the color change was measured with a spectrophotometer at 765 nm using an enzyme-linked immunosorbent assay (ELISA) reader (BMG Labtech, SPECTROstar Nano, Ortenberg, Germany). The total polyphenol content was determined in micrograms of gallic acid equivalents, as previously described [22].

2.4 Analysis of total flavonoid content of CFE

To determine the total flavonoid content of CFE, the formation of chelatic colorimetrable compounds when the solution reacted with aluminum chloride was evaluated. A CFE (1 mg/mL) stock solution was prepared and diluted with methanol to create sample solutions with various concentrations. In brief, 0.5 mL of diluted CFE solution was prepared by adding 10 mL of 80% methanolic solution to 0.1 g of CFE. The solution was mixed with 0.15 mL of 10% aluminum chloride and 2 mL of 4% sodium hydroxide, and the volume was adjusted to 5 mL with sterilized water. After the mixture stood for 15 min at room temperature, the color
change was measured with the ELISA reader at 510 nm. The total flavonoid content was determined in milligrams per gram of rutin equivalent, as previously described [22].

2.5 Determination of DPPH free radical scavenging activity of CFE

The antioxidant activity of CFE was measured with a DPPH free radical scavenging assay. The CFE stock solution (1 mg/mL) was prepared and diluted with methanol to create sample solutions with different concentrations. Aliquots of 50 μL of each dilution were added to the 96-well microplate. Subsequently, 150 μL of a working solution of DPPH (250 μM) was added to each well; after 30 min, the optical density was measured with a microplate reader at 490 nm. The half-maximal inhibitory concentration (EC50) of CFE for scavenging DPPH free radicals was calculated and compared with that of vitamin E, as previously described [22].

2.6 High-performance liquid chromatography

2.6.1 Uric acid

Samples of the CFE stock solution (1 mg/mL) were prepared and diluted with DMSO (DMSO:H2O = 2:8) to different concentrations for an in vitro XO inhibition test. After that, 250-μL test samples or standards of different concentrations were added to test tubes. Subsequently, 175 μL of 50 mM phosphate buffer (pH 7.5) and 150 μL of XO solution (0.1 U/mL containing 50 mM phosphate buffer, pH 7.5) were added at 25°C for 15 min, and 300 μL of 150 mM xanthine reagent (containing 50 mM phosphate buffer, pH 7.5) was added at 25°C for 30 min to induce a reaction. Finally, 125 μL of 1 M HCl was added to stop the reaction. The reaction solution was filtered through a 0.45 μm filter for high-performance liquid chromatography–diode-array detection (HPLC-DAD) to quantify the uric acid production. A Cosmosil 5C18-AR-II (i.d. 4.6 × 250 mm, 5 μm) reversed phase column was used with phosphate buffer-methanol (98:2) as the mobile phase with a sample injection volume of 20 μL at a flow rate of 1.0 mL/min for 15 min for analysis at a wavelength of 280 nm. Allopurinol, an
XO inhibitor, served as a positive control. Each sample was tested three times, and the inhibition rate (%) was calculated with the following formula:

\[
\text{Inhibition of uric acid production (\%)} = \left( 1 - \frac{\text{Uric acid concentration in sample group}}{\text{Uric acid concentration in control group}} \right) \times 100
\]

2.6.2 Multiple compounds

Gallic acid and the fractions of CFE stock solution (1 mg/mL) were prepared and diluted with methanol to 0.1 mg/mL to determine the composition of the fractions. Samples were filtered through a 0.45 μm filter for HPLC-DAD to monitor the component shift in the fractions. A Cosmosil 5C18-AR-II (i.d. 4.6 × 250 mm, 5 μm) reversed phase column was used with 0.05% phosphoric acid:methanol as the mobile phase through gradient elution with a sample injection volume of 20 μL at a flow rate of 1.0 mL/min for 45 min for analysis at a wavelength of 220–700 nm. The gradient elution formula is presented in Suppl2.

2.7 The enzyme kinetics of XO-inhibitory activity

Samples of the CFE and gallic acid stock solution (1 mg/mL) were prepared and diluted with DMSO (DMSO: H2O = 2:8) to different concentrations for an in vitro XO inhibition test. After that, 250-μL test CFE and gallic acid of different concentrations were added to test tubes. Subsequently, 175 μL of 50 mM phosphate buffer (pH 7.5) and 150 μL of XO solution (0.1 U/mL containing 50 mM phosphate buffer, pH 7.5) were added at 25°C for 15 min, and 300 μL of varies concentrations (150.0, 75.0, 37.5, 18.8, 9.4, 4.7 mM) xanthine reagent (containing 50 mM phosphate buffer, pH 7.5) was added at 25°C for 30 min to induce a reaction. Finally, 125 μL of 1 M HCl was added to stop the reaction, as previously described.
The reaction solution was filtered through a 0.45 μm filter for HPLC-DAD to describe the uric acid-producing efficacy. $K_i$ (inhibitory constant) values were calculated with the following formula:

$$K_i = \frac{IC_{50}}{1 + \frac{substrate \text{ concentration}}{K_m}}$$

2.8 Hyperuricemia animal model

A total of 48 male Institute of Cancer Research mice (ICR mice, male, 5 weeks, 25–27g) were purchased from BioLASCO (Taipei, Taiwan) and housed under standard laboratory conditions in a temperature-controlled (22°C ± 2°C) animal facility with a 12-hr/12-hr light/dark cycle. Standard animal feed (LabDiet®, LabDiet 5001, St. Louis, MO, USA) and sterilized water were available ad libitum. The mice were procured 1 week prior to testing to allow them to become acclimated to the laboratory environment and diet before the experiments. The mice were randomly assigned to six groups of six mice each: normal (N), vehicle control (VC), positive control (PC, 10 mg/kg allopurinol), low-dose (L, 250 mg/kg ethyl acetate fraction [EAF]), medium-dose (M, 500 mg/kg EAF), and high-dose (H, 1,000 mg/kg EAF) groups. For hyperuricemia induction, the mice in the VC, PC, L, M, and H groups were administered 250 mg/kg PO through subcutaneous injection every day for 7 days, as previously described [3, 12, 45, 53]: 1 hr later, 250, 500, and 1,000 mg/kg EAF were respectively administered through oral gavage to the L, M, and H groups, and 1 mg/kg allopurinol was administered to the PC group. After 1 week of drug administration, the mice were humanely sacrificed by carbon dioxide inhalation at the end of treatment, and blood was immediately collected from inferior vena cava into a tube without any anticoagulant [31].
After centrifugation, serum was obtained and then subjected to analysis for SUA, blood urea nitrogen (BUN), creatinine (CRE), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT). This study was approved by the appropriate animal care and use committees of Tajen University (Pingtung, Taiwan: approval no. IACUC-107-12).

2.9 Statistical analysis

Data are expressed as means ± standard deviations, and each analysis was performed in triplicate. Statistical comparisons were performed through one-way analysis of variance, and Tukey-HSD multiple comparison tests were conducted using SPSS version 16.0 (SPSS, Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

3.1 Phytochemical composition and free radical scavenging activity of CFE

The phytochemical composition and free radical scavenging activity of CFE are listed in Table 1. Our results indicate that ethyl acetate is optimal for extracting antioxidants, polyphenols, and flavonoids from CFE; the polyphenol and flavonoid content and free radical scavenging activity of the EAF were significantly higher (p < 0.05) than those of the other CFE fractions.

3.2 XO-inhibitory effect of CFE

The results of the evaluation of the XO-inhibitory activity of CFE are presented in Fig. 1. The EAF of CFE at 1000 µg/mL had significantly higher gallic acid content and consequently exhibited greater concentration-dependent XO-inhibitory activity than did CFE95E (Fig. 1A and B). EAF of CFE exhibited a dose-dependent inhibitory effect on XO in vitro.

3.3 Enzyme kinetics of XO-inhibitory activity of CFE and gallic acid
The enzyme kinetic results of the XO inhibition test are presented in Fig. 2. Gallic acid (IC$_{50}$ = 37.54 ± 0.93 μg/mL) provides competitively inhibited xanthine oxidase activity with a $K_i$ value of 2.37 μg/mL. EAF (IC$_{50}$ = 1,277.13 ± 0.43 μg/mL) provides mixed inhibited xanthine oxidase activity with a $K_i$ value of 41.34 μg/mL. Due to, multiple compounds EAF can exhibit different types of inhibition. This type of inhibition is usually caused by the allosteric effect of the inhibitor binding to multiple sites on the enzyme.

Enzyme kinetics analysis was used to study XO-inhibitory effect of CFE. The method involves the interpretation of the intersection of the two curves representing reactions with and without an inhibitor: intersection on the Y-axis indicates competitive inhibition, and intersection on the X-axis indicates noncompetitive inhibition, and parallel lines indicate uncompetitive inhibition. We discovered the intersection point for CFE to be in the second quadrant, indicating inhibition through binding at different binding sites, kinetics similar to those of other XO-inhibitory substances such as allopurinol and febuxostat.

3.4 HPLC analysis of CFE

CFE contains gallic acid: its retention time for gallic acid was 6.3 min. CFE95E and the EAF exhibited peaks after the same retention time (Fig. 3), indicating that gallic acid is an active ingredient in both CFE95E and the EAF. Furthermore, ultraviolet (UV) spectroscopy revealed peaks of CFE95E and the EAF at 6.3 min and a decrease in $\lambda$ that all occurred at 269 nm, further supporting the presence of gallic acid in both substances. The recovery rates as an active ingredient in both substances.

3.5 Effect of CFE on hyperuricemia in animals

We selected the EAF of CFE for application in our animal model of hyperuricemia (Table 2) on the basis of its in vitro inhibition of XO. The growth of animals can reveal whether a
substance is toxic to them. The weights of each group did not change significantly after treatment, indicating that the EAF of CFE has no significant influence on body weight of animals in experimental period.

The hepatic and renal indices CRE, BUN, GOT, and GPT of the mice with PO-induced hyperuricemia revealed no significant hepatic or renal toxicity after EAF administration (Table 2). The SUA results revealed that the EAF reduced chemically induced hyperuricemia in a concentration-dependent manner. Therefore, CFE extracted with ethyl acetate exhibits potential for use as a uric acid–lowering treatment.

Discussion

Although allopurinol, febuxostat, and benztropine [2, 38] are used to treat hyperuricemia, the undesirable side effects of these drugs reduce patient willingness to undergo treatment for an extended period, resulting in poor compliance [19, 20, 39]. To address the difficulties associated with uric acid–lowering therapies, studies have evaluated the use of dietary interventions to lower uric acid concentrations, and the consumption of cherry juice and lychee as a complementary therapy was demonstrated to reduce uric acid concentrations and the risks of recurrent gout and inflammation [56]. Therefore, nontoxic and inexpensive dietary supplements that can reduce uric acid concentrations, are desirable. In vivo trials have indicated that the EAF of CFE can reduce SUA and protect the liver and kidneys; in vitro trials have suggested that polyphenols, especially flavonoids, are the main components of CFE that inhibit XO through hydrophobic interaction [13, 33].

Phenolic compounds such as flavonoids inhibit XO [23, 24]. The ability of polyphenols to inhibit XO depends on the structures of the enzymes’ hydrophobic amino acids, which induce competitive or noncompetitive XO-inhibitory activity [33]. A similar trend was observed among the CFEs with rich polyphenol and flavonoid contents in the present study.

In the present study, the EAF of CFE reduced CRE, GOT, and GPT levels, indicating that
**C. officinalis** has hepatoprotective and renoprotective effects in the mice with potassium oxonate-induced hyperuricemia. Flavonoids can regulate superoxide dismutase, glutathione S-transferase, glutathione peroxidase, heme oxygenase, cytochrome P450, and telomerase levels; prevent liver and kidney damage and complications of oxidative stress and inflammation; and reduce tumor necrosis factor-α, interferon-γ, interleukin-6, and cyclooxygenase-2 levels to reduce the inflammatory response and protect the liver and kidneys [5, 47, 48]. Oxidative stress reflects a bodily imbalance of free radicals and antioxidants. Intracellular xanthine is converted to uric acid by XO, and the resultant excessive oxidative stress promotes apoptosis [25], which leads to the processing and secretion of caspase-1 and interleukin-1β and thus to a series of inflammatory responses. Chronic inflammation increases uric acid production and leads to a vicious cycle of hyperuricemia and gout; therefore, antioxidants are crucial in preventing such a cycle. Fortunately, CFE exhibits high antioxidant and uric acid–lowering activity [28, 42].

The use of natural products is trending in the treatment of hyperuricemia. Lychee has been reported to reduce uric acid levels. *C. officinalis* has the advantage of a glycemic index lower than that of lychee [4, 29, 52]. CFE can potentially attenuate the effects of metabolic diseases, including hyperuricemia, and gout. Cherries, a food source that can lower uric acid concentrations, are mainly produced in Asia and the Americas [26]. Because of geographical factors and shipping costs, corni fructus is cheaper than cherries and thus carries a lower financial burden as a treatment, thereby increasing patient willingness to undergo treatment [49].

The results of our polyphenol content and enzyme kinetics analyzes, HPLC fingerprinting, UV spectroscopy, and hyperuricemia mouse model reveal that gallic acid is the primary active ingredient of CFE. One study on hyperuricemia reported that gallic acid inhibited XO and gout-related proinflammatory cytokines in vitro [32]. In contrast to the current first-line drug allopurinol, gallic acid can reduce uric acid and proinflammatory factors simultaneously, and
its anti-inflammatory effects are similar to those of corticosteroids. Gallic acid can serve a simple alternative medication for gout by effectively reduce the recurrence rate [32]. Identifying new therapeutic sources for the pharmaceutical industry and developing valid, effective, affordable, and easily produced plant extract–based treatments are crucial. In sum, CFE may be applied as a uric acid–lowering treatment in the near future.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

This study is found supported by form Department of Medicine, Kaohsiung Armed Forces General Hospital. Funding for this study was provided by plan 108-2.

References


48. Wan, L. and Jiang, J.-G. 2018. Protective effects of plant-derived flavonoids on hepatic...


**Figure legends**

Fig. 1. Xanthine oxidase (XO)-inhibitory effects of corni fructus extract (CFE) and its fractions.

(A) Inhibitory effects of various CFE extracts—a. crude CFE (CFE95E), b. fraction of water, c. fraction of n-hexane, d. fraction of n-butanol, and e. ethyl acetate fraction (EAF)—and f. allopurinol. (B) Inhibitory effects of various EAF concentrations and gallic acid. Data are expressed as means ± standard deviations (n = 3). A *p* value <0.05 was considered statistically significant compared with CFE95E in one-way analysis of variance, and Tukey-HSD multiple comparison tests.

Fig. 2. Enzyme kinetics of xanthine oxidase (XO)-inhibitory effects of corni fructus extract
(CFE) and gallic acid.

Lineweaver–Burk double reciprocal plots for the inhibition of XO by (A) Gallic acid. (B) ethyl acetate fraction (EAF) of CFE. The demonstration of plots is expressed as $1/\text{velocity}$ vs. $1/\text{xanthine}$.

Fig. 3. HPLC chromatograms of cori fructus extract (CFE) and its fractions.

The black arrow indicates the peak of gallic acid, the following HPLC chromatograms reveal a. gallic acid standard, b. crude CFE (CFE95E), c. fraction of n-hexane, d. ethyl acetate fraction (EAF), e. fraction of n-butanol, f. fraction of water and g. methanol.

Supplemental Fig. 1. Preparation of CFE

The liquid-phase extraction with n-hexane, ethyl acetate, n-butanol, and pure water fractions, furthermore, removes solvent via vacuum concentration to prepare its CEF and its fractions, sequentially.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Total polyphenols (mg/g, as gallic acid)</th>
<th>Total flavonoids (mg/g, as rutin)</th>
<th>DPPH scavenging (EC_{50}, mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFE95E</td>
<td>24.84 ± 3.44</td>
<td>37.33 ± 9.02</td>
<td>0.89 ± 0.01</td>
</tr>
<tr>
<td>Fr.Hex</td>
<td>18.44 ± 4.75</td>
<td>167.33 ± 10.07*</td>
<td>1.85 ± 0.01*</td>
</tr>
<tr>
<td>EAF</td>
<td>105.24 ± 6.09*</td>
<td>240.00 ± 1.54*</td>
<td>0.22 ± 0.01#</td>
</tr>
<tr>
<td>Fr.BuOH</td>
<td>18.64 ± 1.12</td>
<td>5.67 ± 2.89#</td>
<td>0.64 ± 0.01#</td>
</tr>
<tr>
<td>Fr.W</td>
<td>10.56 ± 1.21##</td>
<td>1.83 ± 2.08#</td>
<td>2.89 ± 0.17*</td>
</tr>
<tr>
<td>Vit. E</td>
<td>—</td>
<td>—</td>
<td>0.16 ± 0.02#</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD (n=3). Tukey-HSD test was used to identify significant differences (* p < 0.05 as a significant increasing compared with CFE95E, # p < 0.05 as a significant decreasing compared with CFE95E), crude CFE (CFE95E), Fraction of n-hexane (Fr. Hex), fraction of n-butanol (Fr. BuOH), fraction of water (Fr. W), tocopherol (Vit. E), corni fructus extract (CFE).
Table 2. Animal biochemical index and body weight

<table>
<thead>
<tr>
<th>Groups</th>
<th>CRE (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>GOT (U/l)</th>
<th>GPT (U/l)</th>
<th>SUA (mg/dl)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.13 ± 0.02</td>
<td>20.99 ± 2.57</td>
<td>66.43 ± 10.75</td>
<td>29.14 ± 5.27</td>
<td>3.51 ± 0.52</td>
<td>32.33 ± 2.54</td>
</tr>
<tr>
<td>VC</td>
<td>0.18 ± 0.01*</td>
<td>25.72 ± 2.51</td>
<td>291.50 ± 49.68#</td>
<td>47.43 ± 4.69#</td>
<td>5.63 ± 0.56#</td>
<td>32.83 ± 1.36</td>
</tr>
<tr>
<td>PC</td>
<td>0.16 ± 0.02</td>
<td>24.43 ± 1.63</td>
<td>145.17 ± 57.67*</td>
<td>32.89 ± 9.73*</td>
<td>0.41 ± 0.40*</td>
<td>30.75 ± 1.67</td>
</tr>
<tr>
<td>L</td>
<td>0.12 ± 0.02*</td>
<td>24.93 ± 2.25</td>
<td>86.88 ± 30.78*</td>
<td>28.67 ± 9.20*</td>
<td>5.18 ± 0.69</td>
<td>31.75 ± 1.35</td>
</tr>
<tr>
<td>M</td>
<td>0.13 ± 0.03*</td>
<td>24.56 ± 2.37</td>
<td>98.00 ± 28.51*</td>
<td>32.40 ± 5.68*</td>
<td>2.10 ± 0.22*</td>
<td>32.15 ± 2.27</td>
</tr>
<tr>
<td>H</td>
<td>0.13 ± 0.04*</td>
<td>23.96 ± 1.82</td>
<td>136.8 ± 12.77*</td>
<td>34.40 ± 5.73*</td>
<td>1.02 ± 0.62*</td>
<td>33.4 ± 1.03</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD (n=6). Tukey-HSD test was used to identify significant differences (*p < 0.05 as a significant difference compared with normal group, #p < 0.05 as a significant difference compared with VC group). Six groups: Normal (N), Vehicle Control (VC), Positive Control (PC), Low dose (L, EAF 250 mg/kg), Medium dose (M, EAF 500 mg/kg), and High dose (H, EAF 1000 mg/kg). Creatinine (CRE), blood urea nitrogen (BUN), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and serum uric acid (SUA).
Dried CF (100.00g) → 95% EtOH extracted → CFE (60.00g) → Water and n-Hexane (1:1) → fraction of n-hexane (0.83g), Aqueous layer → Water and EtOAc (1:1) → EAF (5.88g), Aqueous layer → Water and n-BuOH (1:1) → fraction of n-butanol (17.99g), fraction of water (19.18g)
Supplementary Table 1. HPLC gradient elution formula

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

A: 0.05% Phosphoric acid, B: Methanol