Hypouricemic Effects of Acacetin and 4,5-O-Dicaffeoylquinic Acid Methyl Ester on Serum Uric Acid Levels in Potassium Oxonate-Pretreated Rats

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The effects of acacetin (1) and 4,5-O-dicaffeoylquinic acid methyl ester (2), compounds contained in the flowers of Chrysanthemum sinense SABINE, on the serum uric acid level were investigated using the rats pretreated with the uricase inhibitor potassium oxonate as an animal model for hyperuricemia. When administered per orally at doses of 20 and 50 mg/kg, 1 reduced the serum uric acid level by 49.9 and 63.9%, respectively and 2 reduced the level by 31.2 and 44.4%, respectively. On the other hand, when the same doses were given intraperitoneally, both of compounds also exhibited a dose-dependent and more marked reduction of the serum uric acid level (% reduction at 20 and 50 mg/kg were 63.0 and 95.1% in 1, respectively and 66.9 and 86.5% in 2, respectively). Furthermore, the compounds 1 and 2 inhibited the rat liver xanthine oxidase activity with IC50 values of 2.22 μM and 5.27 μM, respectively. These results demonstrated the hypouricemic action of 1 and 2, which may be attributable to their xanthine oxidase inhibitory activity.

Key words hypouricemia; uric acid; acacetin; 4,5-O-dicaffeoylquinic acid methyl ester; xanthine oxidase inhibition

Gout is a common disease with a worldwide distribution and is mainly caused by deposition of monosodium urate crystals in joints and other tissues as a result of extracellular urate supersaturation.1) This disease has been associated with hyperuricemia which results from the overproduction and/or underexcretion of uric acid and is greatly influenced by a high dietary intake of nucleic acids.1—3) In the purine metabolism, xanthine and hypoxanthine are oxidized into uric acid by the activity of xanthine oxidase (XO).4) The compounds with ability to enhance the urinary excretion of uric acid or to inhibit uric acid biosynthesis have been generally employed for the treatment of gout.5) Allopurinol, a XO inhibitor which is used in clinically in the treatment of gout,6) has been confirmed by TLC and 1H-NMR spectral observation as the tail tip 2 h after the test drug administration. Serum blood was taken by cutaneous bleeding from the tail tip 2 h after the test drug administration. Serum was obtained by centrifugation blood sample for 10 min and then was ultrafiltered using Ultrafree-MC centrifugal filter units (Millipore Corp., Bedford, MA, U.S.A.) at 7500 rpm for 1 h to remove proteins before analysis. The sera were stored at −30°C until use.

Measurement of Uric Acid The serum uric acid levels were analyzed by the high performance liquid chromatography (HPLC) method with reversed-phase C-18 column (Shim-pack CLC-ODS(M), 150×4.6 mm i.d., Shimadzu), a 100μm NaH2PO4 (pH 3.5) mobile phase, and UV detector was 290 nm. The percentage decrease in serum uric acid levels was calculated as [(Cc–Ct)/(Cc–Cs)]×100, where Cc, Ct, and Cs are the uric acid concentrations (mg/dl) of control (PO treated), normal (without PO treatment), and test (PO + test compounds treated) groups.

MATERIALS AND METHODS

Chemicals 4,5-O-Dicaffeoylquinic acid methyl ester was isolated from the flowers of C. sinense, and its purity was confirmed by TLC and 1H-NMR spectral observation as reported previously.10) Acacetin, allopurinol, and uric acid were obtained from Wako Pure Chemicals Industry (Osaka, Japan), and potassium oxonic acid was from Acros Organics (Geel, Belgium).

ANIMALS Male Sprague–Dawley rats (6 weeks old, n=8) obtained from Sankyo Labo Service (Tokyo, Japan) were maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled room for 1 week prior to the experiment. The animals were fed with a laboratory pellet chow (CE-2; CLEA Japan Inc., Tokyo, Japan) and water ad libitum during the experiment. This study was conducted in accordance with the standards established by the Guide for the care and use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Hypouricemic Effect in Potassium Oxonate-Treated Rats An animal model of hyperuricemia induced by the uricase inhibitor potassium oxonate has been used to study in vivo anti-hyperuricemia effects of drugs.5,6) Briefly, rats were treated with the uricase inhibitor potassium oxonate (PO) (250 mg/kg, i.p.) 1 h before oral (p.o) or intraperitoneal (i.p) administration of test compounds. Blood was taken by cutting the tail tip 2 h after the test drug administration. Serum was obtained by centrifuging blood sample for 10 min and then was ultrafiltered using Ultrafree-MC centrifugal filter units (Millipore Corp., Bedford, MA, U.S.A.) at 7500 rpm for 1 h to remove proteins before analysis. The sera were stored at −30°C until use.

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Preparation of Test Samples  Potassium oxonate was dissolved in 0.9% saline solution. Acacetin and 4,5-O-dicaffeoylquinic acid methyl ester were suspended in 5% gum Arabic solution, and given at a constant volume of 2 ml/kg. Allopurinol used as a positive control, was prepared in the same way as test compounds.

Xanthine Oxidase Inhibitory Assay  Rat liver was excised and homogenized in 50 mM phosphate buffer (pH 7.4). The homogenate was then centrifuged at 3000×g for 10 min, the lipid layer was carefully removed, and resulting supernatant fraction was further centrifuged at 10000×g for 60 min at 4°C. The supernatant was used for enzyme assays.6,11)

The XO inhibitory activity was assayed spectrophotometrically under aerobic conditions, according to method reported by Noro et al.,12) with slight modification using 96-well plates. Briefly, the assay mixture consisting of 50 μl of test solution, 35 μl of 70 mM phosphate buffer (pH 7.5), and 30 μl of enzyme solution. After preincubation at 25°C for 15 min, the reaction was initiated by the addition of 60 μl of substrate solution (150 μM xanthine in the same buffer). The assay mixture was incubated at 25°C for 30 min. The reaction was terminated by adding 25 μl of 1 N HCl, and the absorbance at 290 nm was measured with a Perkin-Elmer HTS-7000 Bio Assay Reader (Norwalk, CT, U.S.A.). A blank was prepared in the same way, but the enzyme was added to the assay mixture after adding 1 n HCl. One unit of XO was defined as the amount of enzyme required to produce 1 μmol of uric acid/min at 25°C. XO inhibitory activity was expressed as the percentage inhibition of XO in the above assay system, calculated as \((1 - B/A) \times 100\), where \(A\) and \(B\) are the activities of the enzyme without and with test material, respectively.

The assay was carried out at five different concentrations ranging from 0.2—100 μM. IC_{50} values were calculated from the mean values of data from four independent determinations. Allopurinol, a known inhibitor of XO, was used as a positive control.

Statistics  The statistical analysis was performed using Student’s t-test. Data are presented as means±S.E. Significant difference was accepted with \(p<0.05\).

RESULTS AND DISCUSSION

Gout is an inherited metabolic disease and results from hyperuricemia, an elevation of serum uric acid level. Control of hyperuricemia is most often achieved by reducing uric acid production with an inhibitor of XO, the enzyme catalyzing the two terminal reactions in uric acid synthesis, or, less frequently, by employing uricosuric agents to increase renal clearance of uric acid.1,13) To date, the only commercially available XO inhibitor is allopurinol, a purine analogue in clinical use for more than 30 years.14) However, it has been observed that allopurinol induces side effects such as hepatitis, nephropathy, and allergic reactions.6,14) Thus, new alternatives with an increased therapeutic activity and less side effects are desired.

In our previous study, we reported the in vitro XO inhibitory activity of the methanolic extract of the flowers of C. sinense and its constituents.9) Our results suggested that flavonoids and caffeoylquinic acid derivatives are the active XO inhibitory constituents in the flowers of C. sinense. Flavonoids and caffeoylquinic acid derivatives are groups of natural products with varied biological and pharmacological activities. Flavonoids are well known anti-oxidants and attracted a tremendous interest among researchers as possible therapeutic agents for diseases mediated by free radicals.15) Among them, some studies on the structure–activity relationship of different chemical classes of flavonoids as potential inhibitors of XO in vitro, have been reported.16,17) While caffeoylquinic acid derivatives have also been reported to possess hepatoprotective, radical scavenging, and XO inhibitory activities.10,18) In present study, two potent XO inhibitors in our in vitro experiment, acacetin (1) and 4,5-O-dicaffeoylquinic acid methyl ester (2) (Fig. 1), were further tested for in vivo hypouricemic effect in rat model.

During the last step of purine metabolism, XO catalyzes the conversion of hypoxanthine into xanthine and xanthine into uric acid. In most of the mammals, uric acid is further converted into allantoin, a highly polar and water soluble product, by an enzyme urate oxidase and finally excreted via urea. However, humans lost the enzyme urate oxidase during primate evolution, and uric acid is the final product of purine degradation.6) Uric acid is sparingly soluble in blood plasma and its concentration above 7 mg/dl may lead to a state known as hyperuricemia. During hyperuricemia, uric acid may crystallize and deposits in the joints, the connective tissue, and the kidney.13) Thus humans are predisposed to the development hyperuricemia and gout. In order to study hyperuricemia and gout in an animal model, hepatic urate oxidase must be blocked by a selective inhibitors. Potassium oxonate is a well known inhibitor of urate oxidase, and is most frequently employed to create an animal model of hyperuricemia in medical, toxicological or nutritional investigations, where production accumulation or elimination of uric acid is expected.9) Thus, the present study was designed to evaluate hypouricemic effects of 1 and 2 using a potassium oxonate-pretreated rat model.

As shown in Fig. 2, intraperitoneal injection of potassium oxonate (250 mg/kg) markedly increased the serum uric acid levels, and reached \(C_{\text{max}}\) to 4.16±0.07 mg/dl at 2 h followed by slow decrease in serum uric acid level until 8 h after injection. The uric acid level in normal rats was only 1.48±0.01 mg/dl.

As shown in Fig. 3, when administered orally, the test drugs significantly and dose-dependently decreased the serum uric acid levels in hyperuricemic rats at 2 h after the administration. The extents of reduction at 20 and 50 mg/kg were 49.9 and 63.9%, respectively, for 1 and 31.2% and 44.4%, respectively, for 2. The reference drug allopurinol (10 mg/kg, p.o.), on the other hand, substantially reduced the
uric acid level by 134.6% (Fig. 3).

Generally, bioactivities of drugs in vivo can be influenced by factors involved in absorption, metabolism, and distribution. When administered orally, drugs are susceptible to metabolism by intestinal bacteria flora and various enzymes and in some cases the metabolites are responsible for observed pharmacological activity. For example, allopurinol is converted by XO to its main metabolite, oxipurinol, which is also a potent XO inhibitor and contributes to the hypouricemic action of allopurinol. Like wise, C-ring cleavage of flavonoids by intestinal bacteria has been reported in human. Quercetin has been reported to be converted to phloroglucinol and 3,4-dihydroxyphenylacetic acid by Escherichia coli. Flavonoid metabolite methyl p-hydroxyphenyllactate has been reported to block nuclear estrogen receptor binding and inhibiting the growth of MCF-7 breast cancer cells. Similarly, caffeoylquinic acid derivatives may be metabolized by gut microflora to various aromatic acids including m-coumaric acid, phenylpropionic, and benzoic acid derivatives. Therefore, it is possible that the decrease in serum uric acid level caused by 1 and 2 after oral administration is due to their free forms or conjugated metabolites. To investigate this possibility, we examined if 1 and 2 have effects on the serum uric acid level after i.p. injection using the same animal model.

As illustrated in the Fig. 4, the i.p. injection of these compounds produced a marked reduction of the serum uric acid level in a dose dependent manner. The extents of the reduction at doses of 20 and 50 mg/kg (i.p.) were 66.9 and 95.1% in the rats treated with 1, respectively, and 63.1 and 86.8% in the rats treated with 2, respectively. The hypouricemic effects of 1 and 2 administered i.p. were found to be more potent than those of the compounds administered p.o. (Fig. 3), suggesting that bioavailability of these compounds are different between two routes of administration and that the observed hypouricemic effects of 1 and 2 are due to their original forms rather than their gut flora metabolites.

Compounds 1 and 2 and allopurinol were further tested on inhibitory activity against XO isolated from rat liver. The result indicated that 1, 2, and allopurinol showed potent inhibitory effects on XO with IC50 values of 2.22, 5.27, and 5.02 μM, respectively. Thus, it is likely that these compounds exhibit hypouricemic action via in vivo inhibition of XO activity in rats.

In conclusion, the present results clearly demonstrated the beneficial hypouricemic effect of flavonoids such as acacetin (1) and 4,5-O-dicaffeoylquinic acid methyl ester (2), which could be attributable to their XO inhibitory activity. The plant containing flavonoids and caffeoylquinic acids such as C. sinense should be useful in the treatment hyperuricemia and gout in real clinical situation.

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REFERENCES