Hypouricemic and Anti-oxidant Effects of *Chrysanthemum indicum* L. and *Cornus officinalis* *In Vitro* and *In Vivo* Models

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Research

Keywords: hyperuricemia, uric acid, *Chrysanthemum indicum* L., *Cornus officinalis* Siebold & Zucc, functional foods

Posted Date: July 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-723565/v1>

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Abstract

Background: Hyperuricemia, abnormally excess accumulation of uric acid, is caused by an imbalance between the production and excretion of uric acid and is a major cause of gout. We compared the effects of extracts from *Chrysanthemum indicum* L. (Ci) and *Cornus officinalis* Siebold & Zucc (Co) on hyperuricemia, both individually and in combination (FSU-CC),

Methods: We used hypoxanthine-treated human liver cancer (HepG2) cells and primary mouse renal proximal tubule cells for *in vitro* model, and potassium oxonate-induced hyperuricemic mice for *in vivo* model.

Results: We found that treatment of Ci, Co, and FSU-CC suppressed the activity of xanthine oxidase and mRNA expression of xanthine dehydrogenase, while inducing an increase in the expression levels of the organic anion transporter 1 and organic anion transporter 3 proteins and a decrease in the expression levels of glucose transporter 9 and urate transporter 1 proteins. Particularly, treatment and supplementation with FSU-CC showed stronger effects than those of supplementation with either Ci or Co alone. We observed that the excretion of creatinine and uric acid in the combination of Ci and Co was higher than that observed in their individual supplementations and was similar to that of the normal group.

Conclusions: Therefore, our data suggest that a combination of Ci and Co may potentially be used for the development of effective natural anti-hyperuricemic functional foods.

Background

Purines play important roles as the precursors of nucleic acids, DNA and RNA, promoting the growth, proliferation, and survival of all cells. Purine metabolism is regulated and maintained by the synthesis and degradation of purines, with uric acid being the final compound in purine catabolism [1]. Abnormally excess accumulation of uric acid, known as hyperuricemia, is caused by an imbalance between the production and excretion of uric acid [2]. Consumption of purine-rich and protein-rich foods and alcohol is directly associated with hyperuricemia, which is a major cause of gout due to the deposition of urate crystals in the soft tissues and joints [3]. Gout affects < 1–6.8% of the population and the prevalence of hyperuricemia and gout has been increasing over the years [4].

Uric acid is produced by the activities of xanthine oxidase and xanthine dehydrogenase, together referred as “xanthine oxidoreductase” in the liver. Xanthine oxidoreductase catalyzes the oxidation of hypoxanthine to xanthine and later to uric acid [5, 6]. Xanthine dehydrogenase is initially synthesized and can be converted to xanthine oxidase by reversible sulfhydryl oxidation or irreversible proteolysis [7]. During the production of uric acid, xanthine oxidase delivers electrons directly to molecular oxygen, thus generating the reactive oxygen species (ROS), including the superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which can further induce oxidative stress [8]. Approximately 75% of uric acid excretion occurs in the kidneys and approximately 25% in the intestines. The circulating uric acid is filtered in the

kidneys and about 90% of the filtered load is usually reabsorbed in the nephrons [9]. Uric acid reabsorption occurs in the renal proximal tubules by transporters, such as glucose transporter 9 (GLUT9), urate anion transporter 1 (URAT1), and organic anion transporter 4 (OAT4), while uric acid excretion is facilitated by transporters, such as organic anion transporter 1 (OAT1) and organic anion transporter 3 (OAT3). Maintaining the function of these uric acid transporters is important to prevent hyperuricemia [10, 11].

In this study, we investigated the effects of *Chrysanthemum indicum* L. and *Cornus officinalis* Siebold & Zucc on hyperuricemia in *in vitro* and *in vivo* models. *C. indicum* L., called Indian chrysanthemum, a member of the Compositae family and *C. officinalis* Siebold & Zucc, a member of the Cornaceae family, have been reported to exhibit anti-inflammatory and anticancer effects and possess antioxidant properties [12–14]. The flowers of *C. indicum* L. and fruits of *C. officinalis* Siebold & Zucc are widely known for their health benefits as traditional tea with accepted for use as food in China and Korea [15, 16]. We compared the effects of treatment of extracts from *C. indicum* L. and *C. officinalis* Siebold & Zucc, both individually and in combination, on hypoxanthine-treated human liver cancer cells, primary mouse renal proximal tubule cells, and potassium oxonate-induced hyperuricemic mice to develop agents for the prevention of hyperuricemia.

Materials And Methods

Extract preparation and HPLC

Flowers of *C. indicum* L. were extracted using water for 8 h at 90°C. The extract was filtered with Whatman paper No. 6 and concentrated in a rotary evaporator under reduced pressure. The concentrate was lyophilized (Ci) and stored at -20°C until further use. Fruits of *C. officinalis* Siebold & Zucc were extracted using water for 8 h at 90°C. The extract was filtered with Whatman paper No. 6 and concentrated in a rotary evaporator under reduced pressure. The extract was dried using hot air with dextrin (50%) (Co) and stored at -20°C until further use. Ci and Co were mixed in a ratio of 1:2 (FSH-CC) and stored at -20°C until further use. And then, we analyzed luteolin and loganin of Ci and Co, and FSH-CC by high-performance liquid chromatography (HPLC) using Agilent 1260 infinity II HPLC system (Santa Clara, CA, USA).

Cell culture and treatments

The human liver cancer (HepG2) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's minimal essential medium (DMEM; Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories), 100 mg/L penicillin-streptomycin, and 2 mmol/L glutamine (Hyclone Laboratories) at 37°C in a humid atmosphere of 5% carbon dioxide (CO₂).

To obtain the primary mouse renal proximal tubule cells, kidney was isolated from Balb/c mice (22–25 g, 6 weeks, male). The kidney was minced using Hank's balanced salt solution (HBSS) containing trypsin, with the addition of 1 mg/mL deoxyribonuclease (DNase) and 2 mg/mL collagenase Type I (Sigma-

Aldrich, USA). After 30 min, the solution was passed through an 80-mesh and 1709-mesh sieve (Fisher Scientific, Pittsburgh, PA) to remove the cell debris and glomeruli. Proximal tubule cells remained on the sieve filter and were collected by washing the sieve filter with HBSS. The proximal tubule cell suspension was centrifuged for 10 min at 1000 revolutions per minute (rpm) at 4°C and the cell pellet was collected.

HepG2 cells and primary mouse renal proximal tubule cells were cultured with Ci, Co, and FSH-CC for 24 h and treated with 4 mM hypoxanthine. After 2 h, assays were performed to measure the activity of xanthine oxidase, mRNA expression of xanthine dehydrogenase, and the expression levels of OAT1, OAT3, GLUT9, and URAT1 proteins.

Animals

The Institutional Animal Care and Use Committee of Kyung Hee University approved the protocol (KHGASP-20-410) for the use of animals in this study. The animals were cared for in accordance with the “Guidelines for Animal Experiments” established by the university.

Six-week-old male C57 black 6 (C57BL6) mice were purchased from SaeRon Bio (Uiwang, Korea) and housed in cages under automatically controlled temperature ($22 \pm 2^\circ\text{C}$), humidity (about 50%), and lighting (12:12-h light-dark cycle) conditions. The mice in the control group with normal diet were fed a commercial pelleted chow (AIN-93G rodent purified diet, Orient Bio, Korea) and water ad libitum. All the mice were randomly divided into eight groups of eight mice per group as follows: normal control (NC), control (C; hyperuricemia-induced mice), positive control (PC; hyperuricemia-induced mice with oral supplementation of allopurinol, xanthine oxidase inhibitor, 10 mg/kg body weight (b.w)), Ci 300 (hyperuricemia-induced mice with oral supplementation of Ci, 300 mg/kg b.w), Co 300 (hyperuricemia-induced mice with oral supplementation of Co, 300 mg/kg b.w), FSH-CC 150 (hyperuricemia-induced mice with oral supplementation of FSH-CC, 150 mg/kg b.w), FSH-CC 300 (hyperuricemia-induced mice with oral supplementation of FSH-CC, 300 mg/kg b.w), and FSH-CC 600 (hyperuricemia-induced mice with oral supplementation of FSH-CC, 600 mg/kg b.w). The extracted samples were orally administered for 21 d. To induce hyperuricemia, an intraperitoneal injection of 200 mg/kg b.w. potassium oxonate (Sigma-Aldrich Co, MO, USA) was given. After 2 h, the mice urine was collected and the mice were anesthetized with isoflurane.

Levels of uric acid and creatinine in the urine and serum

Blood was centrifuged at 3000 rpm for 10 min and the serum was separated. The levels of uric acid in the urine and serum were determined using the uric acid assay kits (BioVision Inc., CA, USA), while the levels of creatinine in the urine and serum were determined using the creatinine assay kit (BioVision Inc., CA, USA).

Activity of xanthine oxidase

The activity of xanthine oxidase was determined from the HepG2 cells in the culture medium and serum from mice using the Xanthine Oxidase Activity Assay Kit (Sigma-Aldrich Co, MO, USA).

mRNA expression of xanthine dehydrogenase

mRNA was extracted from the HepG2 cells and liver tissues using the RNeasy Mini Kit (QIAGEN, MD, USA). Synthesis of complementary DNA (cDNA) using mRNA was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Polymerase chain reaction (PCR) consisted of 40 cycles of denaturation (95°C for 15 s), annealing (58°C for 15 s), and extension (72°C for 30 s) using the SYBR Green PCR Master Mix (iQ SYBR Green Supermix; Bio-Rad Laboratories, Inc.) and the following primers: GAPDH (H) forward primer 5'-CCC CAC ACA CAT GCA CTT ACC-3', reverse primer 5'-TTG CCA AGT TGC CTG TCC TT-3'; xanthine dehydrogenase (H) forward primer 5'-ATT GGT GCT GTG GTT GCT-3', reverse primer 5'-TGT GAT AAT GGC TGG TAG TTC TTC; GAPDH (R) forward primer 5'-CAT GGC CTT CCG TGT TCC TA-3', reverse primer 5'-GCG GCA CGT CAG ATC CA-3'; xanthine dehydrogenase (R) forward primer 5'-TGC GAA GGA TGA GGT TAC T-3', reverse primer 5'-GGA TTG TGA TAA TGG CTG GAA-3'. The cDNA was amplified using the real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and data analysis was performed using the CFX Maestro™ Analysis Software (Bio-Rad Laboratories, Inc.).

Serum triglycerides, cholesterol, aspartate transaminase (AST), and alanine transaminase (ALT)

Concentrations of total triglycerides (TG), total cholesterol (TC), very-low-density lipoprotein (VLDL)/low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, aspartate transaminase (AST), and alanine transaminase (ALT) in serum were determined by enzyme-linked colorimetric methods using commercial kits (BioVision, CA, USA).

Antioxidant enzyme activity in the liver

The liver tissues were lysed using the CellLytic™ MT lysis reagent (Sigma) and the antioxidant enzyme activity was measured using the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) assay kits (Biomax Inc., Seoul). Malondialdehyde (MDA), a lipid peroxidation marker, was measured using MDA assay kits (BioVision Inc., Mountain View, CA, USA).

Expression levels of OAT1, OAT3, GLUT9, and URAT1 proteins

The primary mouse renal tubular epithelial cells and kidney tissues were lysed using the CellLytic™ MT lysis reagent. Equal amounts of proteins (100 µg/lane) were separated by electrophoresis using 10% Mini-PROTEAN® TGXTM Precast Gels (Bio-Rad Laboratories, Inc.) and transferred to polyvinylidene difluoride membranes using Trans-Blot® Turbo™ Transfer system (Bio-Rad Laboratories, Inc.). The membranes were incubated for 1 h in a blocking solution containing 5% non-fat milk in Tris-buffered saline and further incubated for 12 h at 4°C with the antibodies recognizing OAT1 (1:500; MyBioSource), OAT3 (1:1000; Santa Cruz), GLUT9 (1:800; Invitrogen), URAT1 (1:800; MyBioSource), and β-actin (1:1000; Cell Signaling Technology). Thereafter, the membranes were incubated with secondary antibodies (anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked antibody, 1:3000; Cell Signaling Technology, Inc.) for 1 h at room temperature. The immunoreactive protein bands were detected using

EzWestLumi plus (ATTO, Tokyo, Japan) and analyzed using Ez-Capture $\boxtimes$ (ATTO) and CS Analyzer v.3.0 (ATTO).

Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). The data were statistically evaluated using Duncan's multiple range tests after one-way analysis of variance (ANOVA) using SPSS statistical procedures (SPSS PASW Statistic v.23.0, SPSS Inc., Chicago, IL, USA). When the data were subjected to prior investigations before analysis, parametric assumptions including homoscedasticity and normality of observations were satisfied. Differences were considered to be statistically significant at $p < 0.05$ level.

Results

Luteolin and loganin of Ci and Co, and FSH-CC

The HPLC analysis of the Ci and Co, and FSH-CC revealed three peaks matching those of the commercial standards luteolin (Fig. 1A) and loganin (Fig. 1B), The Ci contained 7.62 mg/g luteolin, Co contained 4.90 mg/g loganin, and FSH-CC contained 3.95 mg/g luteolin and 2.48 mg/g loganin.

The combination of Ci and Co suppressed the xanthine oxidase activity and xanthine dehydrogenase mRNA expression in liver cells more than their individual treatments

We found that hypoxanthine treatment (C) increased the activity of xanthine oxidase and mRNA expression of xanthine dehydrogenase as compared with that in the normal control (NC). However, Ci and Co treatment revealed a significant decrease in the activity of xanthine oxidase and mRNA expression of xanthine dehydrogenase as compared with that in the control group. In addition, the combination of Ci and Co (FSU-CC) suppressed xanthine oxidase activity and xanthine dehydrogenase mRNA expression more than the individual treatment of either Ci or Co alone. Moreover, FSU-CC 300 treatment resulted in the most significant reduction of the xanthine oxidase activity and xanthine dehydrogenase mRNA expression among all the hypoxanthine-treated HepG2 cells ($p < 0.05$) (Fig. 2).

The combination of Ci and Co increased the expression levels of OAT1 and OAT3 and suppressed the expression levels of GLUT9 and URAT1 in renal proximal tubule cells more than their individual treatments

We confirmed the uric acid excretion transporters, OAT1 and OAT3, as well as the uric acid reabsorption transporters, GLUT9 and URAT1, in the primary mouse renal proximal tubule cells. Hypoxanthine treatment in these cells induced a decrease in the expression levels of OAT1 and OAT3 compared with that in the normal control group, while Ci and Co treatment groups increased the expression levels of OAT1 and OAT3 compared with that in the hypoxanthine control group. Moreover, the combination of Ci and Co (FSU-CC) increased the expression levels of OAT1 and OAT3 more than the individual treatment of either Ci or Co alone ($p < 0.05$) (Fig. 3B,C).

Compared to the normal control group, hypoxanthine treatment induced an increase in the expression levels of GLUT9 and URAT1 in the primary mouse renal proximal tubule cells, while Ci and Co treatment groups significantly decreased the expression levels of GLUT9 and URAT1 compared with that in the hypoxanthine treatment control group. Moreover, the combination of Ci and Co (FSU-CC) decreased expression levels of GLUT9 and URAT1 more than the individual treatment of either Ci or Co alone ($p < 0.05$) (Fig. 3D,E).

The combination of Ci and Co increased the excretion of creatinine and uric acid in hyperuricemia-induced mice more than their individual treatments

We investigated the effects of Ci and Co supplementation on hyperuricemia-induced mice and found that Ci and Co supplementation did not affect the change in the levels of serum ALT, AST, triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol in hyperuricemia-induced mice (Table 1). We measured the levels of creatinine and uric acid in the serum and urine in hyperuricemia-induced mice to confirm whether Ci and Co supplementation affects the excretion of creatinine and uric acid in them. Compared to the normal control, hyperuricemia-induced mice showed a significant increase in the levels of creatinine and uric acid in the serum and a significant decrease in the levels of creatinine and uric acid in the urine. Ci 300 and Co 300 supplementation groups significantly decreased the levels of creatinine and uric acid in the serum and increased the levels of creatinine and uric acid in the urine as compared with that in the control group. FSU-CC 300 decreased the levels of creatinine and uric acid in the serum and increased the levels of creatinine and uric acid in the urine than the individual supplementation of either Ci 300 or Co 300 alone ($p < 0.05$) (Fig. 4).

Table 1

Changes in the levels of serum alanine transaminase (ALT), aspartate transaminase (AST), triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol in the hyperuricemia-induced mice supplemented with and without Ci, Co, or FSU-CC.

	AST (mU/mL)	ALT (mU/mL)	Triglycerides (µg/mL)	Total cholesterol (µg/mL)	HDL (µg/mL)	LDL (µg/mL)
NC	22.79 ± 3.34 ^{ns}	3.37 ± 0.41 ^{ns}	2.60 ± 0.39 ^{ns}	7.58 ± 0.72 ^{ns}	1.72 ± 0.24 ^{ns}	1.63 ± 0.89 ^{ns}
C	23.02 ± 2.26	2.91 ± 0.64	2.26 ± 0.14	8.06 ± 0.81	1.89 ± 0.29	1.74 ± 0.84
PC	21.83 ± 2.49	2.91 ± 0.35	2.62 ± 0.26	7.75 ± 0.83	1.70 ± 0.34	1.30 ± 0.91
Ci 300	23.66 ± 2.13	3.06 ± 0.77	2.63 ± 0.37	7.56 ± 0.97	1.74 ± 0.32	1.61 ± 0.75
Co 300	23.39 ± 2.18	3.13 ± 0.48	2.44 ± 0.33	8.33 ± 0.56	1.75 ± 0.19	1.55 ± 0.92
FSU-CC 150	22.77 ± 1.29	2.79 ± 0.32	2.47 ± 0.42	7.60 ± 0.76	1.80 ± 0.33	1.30 ± 1.55
FSU-CC 300	24.08 ± 6.82	3.25 ± 0.58	2.43 ± 0.14	7.72 ± 1.46	1.57 ± 0.22	1.38 ± 1.32
FSU-CC 600	22.85 ± 1.36	3.24 ± 0.78	2.61 ± 0.19	7.11 ± 0.56	1.64 ± 0.27	1.24 ± 0.79

^{ns} not significant

NC : normal control, C : hyperuricemia-induced mice, PC : hyperuricemia-induced mice with oral supplementation of allopurinol 10 mg/kg b.w, Ci 300 : hyperuricemia-induced mice with oral supplementation of *C. indicum* L. 300 mg/kg b.w, Co 300 : hyperuricemia-induced mice with oral supplementation of *C. officinalis* Siebold & Zucc. 300 mg/kg b.w, FSU-CC 150 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 150 mg/kg b.w, FSU-CC 300 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 300 mg/kg b.w, FSU-CC 600 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 600 mg/kg b.w. Different letters indicate the significant difference at P < 0.05, as determined by Duncan's multiple range test.

The combination of Ci and Co increased the xanthine oxidase activity and xanthine dehydrogenase mRNA expression and inhibited the oxidative stress in hyperuricemia-induced mice more than their individual treatments

We measured the xanthine oxidase activity, xanthine dehydrogenase mRNA expression, and oxidative stress in the liver of hyperuricemia-induced mice. The xanthine oxidase activity and xanthine dehydrogenase mRNA expression in the liver were significantly increased in the hyperuricemia-induced

mice group compared with that in the normal control group. However, Ci 300 and Co 300 supplementation suppressed the xanthine oxidase activity and xanthine dehydrogenase mRNA expression in the liver of hyperuricemia-induced mice. FSU-CC 300 supplementation significantly decreased the xanthine oxidase activity and xanthine dehydrogenase mRNA expression in the liver of hyperuricemia-induced mice more than the individual supplementation of either Ci 300 or Co 300 alone ($p < 0.05$) (Fig. 5A,B).

We found that hyperuricemia-induced mice caused an increase in the MDA levels and a decrease in the activities of antioxidant enzymes, including SOD, CAT, and GPx compared with that in the normal control group. Ci 300, Co 300, and FSU-CC supplementation induced a decrease in the MDA levels and an increase in the antioxidant enzyme activities compared with that in the control group ($p < 0.05$) (Fig. 5C-F).

The combination of Ci and Co increased the expression levels of OAT1 and OAT3 and suppressed the expression levels of GLUT9 and URAT1 in hyperuricemia-induced mice than their individual treatments

Hyperuricemia-induced mice showed a decrease in the expression levels of OAT1 and OAT3 in the kidney compared with that in the normal control group. Ci 300 and Co 300 supplementation increased the expression levels of OAT1 and OAT3 in the kidney of hyperuricemia-induced mice compared with that in the control group. In addition, FSU-CC 300 supplementation significantly increased the expression levels of OAT1 and OAT3 in the kidney more than the individual supplementation of either Ci 300 or Co 300 alone ($p < 0.05$) (Fig. 6B,C).

Hyperuricemia-induced mice induced an increase in the expression levels of GLUT9 and URAT1 in the kidney as compared to the normal control. Ci 300 and Co 300 supplementation significantly decreased the expression levels of GLUT9 and URAT1 in the kidney compared with that in the control group. Moreover, FSU-CC 300 decreased the expression levels of GLUT9 and URAT1 in the kidney of hyperuricemia-induced mice more than the individual supplementation of either Ci 300 or Co 300 alone ($p < 0.05$) (Fig. 6D,E).

Discussion

Recently, the prevalence of hyperuricemia-induced gout is increasing and the treatment for gout includes the use of non-steroidal anti-inflammatory drugs to relieve the symptoms of the illness and the use of allopurinol and xanthine oxidase inhibitors to reduce the production of uric acid [17]. However, these drugs have side effects, including gastrointestinal toxicity and bleeding, renal toxicity, and hypersensitivity reactions [18]. Therefore, alternative therapies have been explored in an attempt to treat and prevent hyperuricemia [19, 20]. We compared the effects of Ci, Co, and a combination of Ci and Co (FSU-CC) on hyperuricemia-induced HepG2 cells, primary mouse renal proximal tubule cells, and potassium oxonate-induced hyperuricemic mice. The present study aimed to compare the effects of Ci, Co, and a combination and to develop agents for the prevention of hyperuricemia.

Xanthine oxidase, converted from xanthine dehydrogenase, acts as a key enzyme for the oxidation of hypoxanthine and xanthine during the production of uric acid in the liver. It is well known that the mitochondrial ROS are produced during the xanthine oxidase-mediated production of uric acid [7, 8]. We found increase in the xanthine oxidase activity and xanthine dehydrogenase mRNA expression in hypoxanthine-treated HepG2 cells and the liver of potassium oxonate-induced hyperuricemic mice as compared to that in the normal cells and liver of healthy mice. In addition, the potassium oxonate-induced hyperuricemic mice caused oxidative stress in the liver by decreasing the antioxidant enzyme activities and increasing the MDA levels. However, treatment of Ci, Co, and FSU-CC suppressed the xanthine oxidase activity, xanthine dehydrogenase mRNA expression, and oxidative stress in hypoxanthine-treated HepG2 cells and the liver of potassium oxonate-induced hyperuricemic mice. We found that the combination of Ci and Co inhibited the activity of xanthine oxidase more than either of the two given separately.

The study of Nishida [21] has demonstrated the significant positive correlations between the excretion of creatinine and uric acid in urine. Thus, we measured the levels of creatinine and uric acid in the serum and urine to observe the excretion process of creatinine and uric acid. Supplementation of Ci, Co, and FSU-CC increased the excretion of creatinine and uric acid through urine, which was suppressed by potassium oxonate injection in mice. Moreover, the excretion of creatinine and uric acid in the combination of Ci and Co was higher than that of individual supplementation and was similar to that of the normal group. These results indicate that the combination of Ci and Co helps to treat hyperuricemia more than individual dietary supplements.

In order to elucidate the mechanisms of Ci, Co, and FSU-CC that mediate the excretion of uric acid in hyperuricemia, we observed the expression of the transporters involved in uric acid excretion in hypoxanthine-treated primary mouse renal proximal tubule cells and the kidney from potassium oxonate-induced hyperuricemic mice. Previous studies have identified GLUT9 and URAT1, involved in uric acid reabsorption, and OAT1 and OAT3, involved in uric acid excretion, as potential therapeutic targets for hyperuricemia [10, 22, 23]. We have shown in the present study that Ci, Co, and FSU-CC significantly increased the expression levels of OAT1 and OAT3, while decreasing the expression levels of GLUT9 and URAT1 in hypoxanthine-treated primary mouse renal proximal tubule cells and the kidney from potassium oxonate-induced hyperuricemic mice. Moreover, the combination of Ci and Co increased the expression levels of OAT1 and OAT3 and suppressed the expression levels of GLUT9 and URAT1 more than the individual treatments. Therefore, we hypothesize that FSU-CC aids in maintaining the function of these uric acid transporters to prevent hyperuricemia more than the individual treatment of either Ci or Co alone.

We showed that Ci contained luteolin, a common flavonoid, and Co contained loganin, an iridoid glycoside. Matsuda *et al.* isolated new flavanone glycosides and phenylbutanoid glycoside from the flowers of *C. indicum* L. and found the inhibitory activity for rat lens aldose reductase [24]. Dong *et al.* showed that the various pharmacological activities of the extract and chemical compounds, including terpenoids, flavonoids and tannin, identified from *C. officinalis* Siebold & Zucc [16]. Several studies demonstrated the phenolic compounds treatment suppressed the development of hyperuricemia *in vitro*,

in vivo, and human clinical trials studies [25–27]. According to these reports and our present results, we can assume that phenolic compounds from Ci and Co can have hypouricemic effects on hyperuricemia-induced HepG2 cells, renal cells, and mice. However, further human clinical trials are needed to fully understand the effects of dietary supplementation on hyperuricemia, with both Ci and Co.

Conclusions

We compared the effects of extracts from Ci, Co, and FSU-CC on hyperuricemia using hypoxanthine-treated HepG2 cells, primary mouse renal proximal tubule cells, and potassium oxonate-induced hyperuricemic mice. We found that the FSU-CC treatment inhibited the production and excretion of uric acid more than the individual treatment of either Ci or Co alone in both *in vitro* and *in vivo* models. We confirmed that treatment with FSU-CC directly inhibited the production of uric acid in hepatocytes and increased the expression of uric acid transporters in renal cells, which are involved in the excretion process. This study provides scientific evidence and describes the underlying mechanisms responsible for the anti-hyperuricemic effects of Ci and Co. Therefore, our data suggest that a combination of Ci and Co may potentially be used for the development of effective natural anti-hyperuricemic agents.

Abbreviations

ROS : reactive oxygen species; GLUT9: glucose transporter 9; URAT : urate anion transporter; OAT: organic anion transporter; DMEM : Dulbecco's minimal essential medium; FBS : fetal bovine serum; HBSS : Hank's balanced salt solution; AST : aspartate transaminase; ALT : alanine transaminase; SOD : superoxide dismutase; CAT : catalase; GPx : glutathione peroxidase; MDA: malondialdehyde

Declarations

Acknowledgements

Not applicable.

Author Contributions

O.K.K and J.L. designed the experiments; O.K.K., J.M.Y., M.L., and D.K. performed the experiments; J.M.Y. and M.L. analyzed the data; O.K.K. and J.L. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

This research was supported by 2020 Collaborative R&BD Program of The Food Industry Promotional Agency of Korea

Availability of data and materials

The datasets used during this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The Institutional Animal Care and Use Committee of Kyung Hee University approved the protocol (KHGASP-20-410) for the use of animals in this study. The animals were cared for in accordance with the “Guidelines for Animal Experiments” established by the university.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Figures

Figure 1

High-performance liquid chromatography analysis of luteolin (A) and loganin (B) levels in Ci and Co, and FSH-CC

Figure 2

Xanthine oxidase activity (A) and xanthine dehydrogenase mRNA expression (B) in hypoxanthine-treated human liver cancer (HepG2) cells, with and without Ci, Co, or FSU-CC. NC : normal control, C : 4 mM hypoxanthine, PC : 4 mM hypoxanthine + 100 μ M Allopurinol, Ci 150 : 4 mM hypoxanthine + 150 μ g/mL *Chrysanthemum indicum* L., Ci 300 : 4 mM hypoxanthine + 300 μ g/mL *C. indicum* L., Co 150 : 4 mM hypoxanthine + 150 μ g/mL *Cornus officinalis* Siebold & Zucc, Co 300 : 4 mM hypoxanthine + 300 μ g/mL *C. officinalis* Siebold & Zucc, FSU-CC 150 : 4 mM hypoxanthine + 150 μ g/mL mixture of *C. indicum* L., and *C. officinalis* Siebold & Zucc (1:2), FSU-CC 300 : 4 mM hypoxanthine + 300 μ g/mL mixture of *C. indicum* L., and *C. officinalis* Siebold & Zucc (1:2). Different letters indicate the significant difference at $p < 0.05$, as determined by Duncan's multiple range test.

Figure 3

Expression levels of proteins by western blotting (band images; A, relative protein expression; B-E) in the hypoxanthine-treated primary mouse renal proximal tubule cells, with and without Ci, Co, or FSU-CC. NC : normal control, C : 4 mM hypoxanthine, PC : 4 mM hypoxanthine + 100 μ M Allopurinol, Ci 150 : 4 mM hypoxanthine + 150 μ g/mL *C. indicum* L., Ci 300 : 4 mM hypoxanthine + 300 μ g/mL *C. indicum* L., Co 150 : 4 mM hypoxanthine + 150 μ g/mL *C. officinalis* Siebold & Zucc, Co 300 : 4 mM hypoxanthine + 300 μ g/mL *C. officinalis* Siebold & Zucc, FSU-CC 150 : 4 mM hypoxanthine + 150 μ g/mL mixture of *C. indicum* L., and *C. officinalis* Siebold & Zucc (1:2), FSU-CC 300 : 4 mM hypoxanthine + 300 μ g/mL mixture of *C. indicum* L., and *C. officinalis* Siebold & Zucc (1:2). Different letters indicate the significant difference at $p < 0.05$, as determined by Duncan's multiple range test.

Figure 4

Levels of creatinine in serum (A) and urine (B) and uric acid in serum (C) and urine (D) in the hyperuricemia-induced mice supplemented with and without Ci, Co, or FSU-CC. NC : normal control, C : hyperuricemia-induced mice, PC : hyperuricemia-induced mice with oral supplementation of allopurinol 10 mg/kg b.w, Ci 300 : hyperuricemia-induced mice with oral supplementation of *C. indicum* L. 300 mg/kg b.w, Co 300 : hyperuricemia-induced mice with oral supplementation of *C. officinalis* Siebold & Zucc. 300 mg/kg b.w, FSU-CC 150 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 150 mg/kg b.w, FSU-CC 300 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 300 mg/kg b.w, FSU-CC 600 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 600 mg/kg b.w. Different letters indicate the significant difference at $p < 0.05$, as determined by Duncan's multiple range test.

Figure 5

Xanthine oxidase activity (A), xanthine dehydrogenase mRNA expression (B), malondialdehyde (MDA) levels (C), and activities of superoxide dismutase (SOD) (C), catalase (CAT) (E), and glutathione peroxidase (GPx) (F) in the liver from hyperuricemia-induced mice supplemented with and without Ci, Co, or FSU-CC. NC : normal control, C : hyperuricemia-induced mice, PC : hyperuricemia-induced mice with oral supplementation of allopurinol 10 mg/kg b.w, Ci 300 : hyperuricemia-induced mice with oral supplementation of *C. indicum* L. 300 mg/kg b.w, Co 300 : hyperuricemia-induced mice with oral supplementation of *C. officinalis* Siebold & Zucc. 300 mg/kg b.w, FSU-CC 150 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 150 mg/kg b.w, FSU-CC 300 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 300 mg/kg b.w, FSU-CC 600 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 600 mg/kg b.w. Different letters indicate the significant difference at $p < 0.05$, as determined by Duncan's multiple range test.

Figure 6

Expression levels of proteins by western blotting (band images; A, relative protein expression; B-E) in the kidney of hyperuricemia-induced mice supplemented with and without Ci, Co, or FSU-CC. NC : normal control, C : hyperuricemia-induced mice, PC : hyperuricemia-induced mice with oral supplementation of allopurinol 10 mg/kg b.w, Ci 300 : hyperuricemia-induced mice with oral supplementation of *C. indicum* L. 300 mg/kg b.w, Co 300 : hyperuricemia-induced mice with oral supplementation of *C. officinalis* Siebold & Zucc. 300 mg/kg b.w, FSU-CC 150 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 150 mg/kg b.w, FSU-CC 300 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 300 mg/kg b.w, FSU-CC 600 : hyperuricemia-induced mice with oral supplementation of mixture of *C. indicum* L. and *C. officinalis* Siebold & Zucc. (1:2) 600 mg/kg b.w. Different letters indicate the significant difference at $p < 0.05$, as determined by Duncan's multiple range test.