



Effects of luteolin-rich chrysanthemum flower extract on purine base absorption and blood uric acid in Japanese subjects

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ABSTRACT

Background and objective: Chrysanthemum flowers are consumed as fresh condiments, herbal teas, and processed foods in Japan and Taiwan. They contain luteolin as a major polyphenol and are traditionally used for eye care. We previously demonstrated that the ingestion of chrysanthemum flower extract (CFE) for 1 month reduced serum uric acid levels. However, the findings obtained were considered to be biased because the study was performed by a CFE manufacturer. Therefore, we herein conducted a clinical trial on CFE on a larger scale and examined its effects on purine base absorption from the intestines, which represents an effective approach for reducing serum uric acid levels.

Methods: Both studies were performed as randomized, double-blind, placebo-controlled trials and CFE (100 mg) containing 10 mg of luteolin was used as the active sample. We enrolled 44 healthy Japanese men and women with 6.0 to 7.9 mg/dL serum uric acid. All subjects were randomly allocated to an active group (n=22) or placebo group (n=22) using a computerized random number generator. In the purine base absorption study, CFE was ingested with a purine

base-rich diet and serum uric acid levels were measured chronologically. In the 12-week consecutive ingestion study, CFE or placebo was administered between January and April 2021. Serum uric acid levels after 12 weeks were assessed as the primary outcome, and uric acid were measured before and after 4 weeks of the intervention as secondary outcomes. Blood, urine and body parameters were examined to evaluate the safety of CFE.

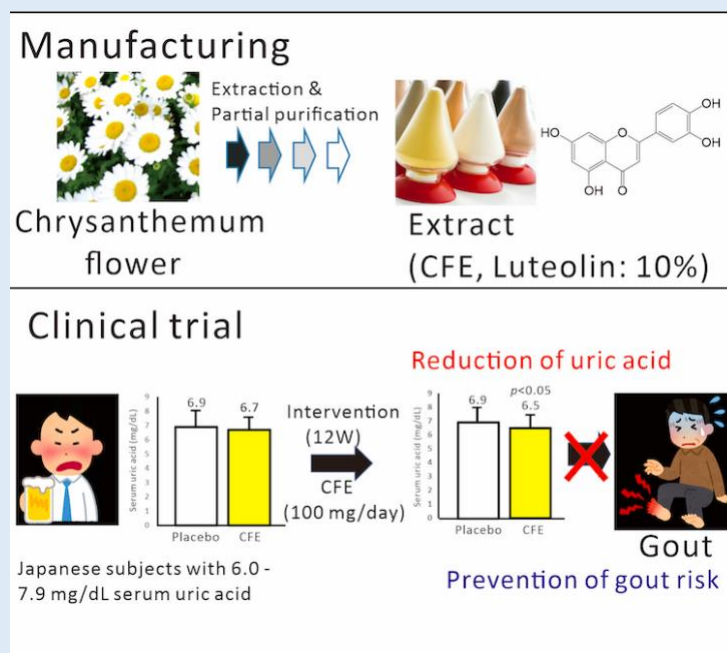
Results: Thirty-nine subjects completed the trial, and the per protocol set comprised 18 and 21 subjects in the active and placebo groups, respectively. In the single dosing study of CFE on subjects loaded by the purine base-rich diet, no significant changes were observed between the CFE and placebo groups. On the other hand, in the 12-week ingestion study, serum uric acid levels were significantly lower in the CFE group than in the placebo group. Laboratory tests revealed no abnormalities to suggest any side effects of CFE.

Conclusions: CFE (100 mg/day) containing 10 mg of luteolin reduced serum uric acid levels. CFE may be beneficial for improving hyperurichemia.

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Keywords: Chrysanthemum, luteolin, uric acid, purine base



INTRODUCTION

Gout mainly affects men, and its underlying cause has been identified as the excessive ingestion of a purine base-rich diet [1]. Purine bases are converted to uric acid, and high blood concentrations of uric acid promote the formation of needle-shaped urate crystals. Particularly at the base of the big toe [1], these crystals accumulate in peripheral tissues and stimulate peripheral nerves, which causes severe pain [1]. The incidence of hyperuricemia in Japan increased from 5% in 1960 to 20% in 1990 [2]. 2016 statistics revealed that millions of people suffered from gout [3]. To reduce the risk of gout, many types of food-derived ingredients that decrease uric acid levels have been examined in mice such as sinapinic acid [4], stevia residue extract [5], and *Camellia japonica* leaf extract [6]. In Japan, anserine [7], a dipeptide from fish meat, was confirmed to reduce serum uric acid levels. Polyphenols, such as monoglycosyl hesperidine [8] and ampelopsin, [9] have been used in dietary supplements to decrease serum uric acid levels. Furthermore, PA-3 lactobacillus [10] and phytic acid [11] have been approved by the Japanese government for use as functional foods to reduce uric acid levels.

Chrysanthemum flowers are consumed as herbal teas in Japan and Taiwan [12] and garnishes of Japanese food such as tempura and sushi. They contain luteolin as the principal flavonoid, which exerts a number of beneficial effects [13]. Luteolin has been shown to inhibit the activity of xanthine oxidase, which is a uric acid synthase [14]. In our previous clinical trial, we demonstrated that the consumption of luteolin-rich chrysanthemum flower extract (CFE) for 4 weeks decreased serum uric acid levels in Japanese men [15]. Since CFE did not suppress purine base absorption from the intestines [15], its effects appeared to depend on xanthine oxidase. However, we only enrolled male subjects; therefore, the effects of CFE on females remain unknown. Furthermore, the subjects examined were employees of a CFE manufacturing company and, thus, the findings obtained might have been biased. Moreover, the effects of CFE ingestion for a longer period on serum uric acid levels remain unknown. Therefore, the present study investigated the effects of CFE

(100 mg/day, 10 mg/day as luteolin) on uric acid levels.

MATERIALS AND METHODS

CFE: Kiku Flower Extract-P (Lot. N-001) manufactured by Oryza Oil & Fat Chemical Co., Ltd. was used as CFE. It is composed of 50% partially purified CFE and 50% dextrin. The luteolin content in Kiku Flower Extract-P is 10%, as confirmed by HPLC.

Subjects and grouping: All subjects were recruited between November 2 and December 19, 2020, through the Go106 website (<https://www.go106.jp/>) operated by ORTHOMEDICO Inc. (Tokyo, Japan). Inclusion criteria were healthy Japanese male and female adults (20 years or older). Exclusion criteria were as follows:

1. Current or previous history of cancer, heart failure, or myocardial infarction.
2. Subjects with a cardiac pacemaker or implantable cardioverter defibrillator
3. Currently receiving treatment for arrhythmia, hepatitis, nephritis, rheumatoid arthritis, cerebrovascular disease, diabetes, hyperlipidemia, hypertension, or other chronic diseases.
4. Current use of medications or dietary supplements/beverages.
5. Subjects with allergic reactions to medicines and foods containing chrysanthemum flowers.
6. Pregnancy, lactation, or expected/planned pregnancy during the study period.
7. Subjects currently participating in another clinical trial or who had participated within the previous 3 months.
8. Subjects with gout.
9. Subjects who work irregularly, such as night shift work
10. Subjects who may eat or drink excessively during the winter holidays.
11. Subjects considered to be inappropriate for the present study for other reasons by the attending physician.

Selection criteria were individuals with a serum uric acid level of 6.0 to 7.9 mg/dL including 22 or more subjects with 6.0 to 7.0 mg/dL of serum uric acid. These subjects were considered to be appropriate for the present study by the attending physician.

Forty-four subjects were asked to consume the test samples in a designated manner, with the aim of ingesting more than 80% of capsules and avoiding excessive eating or drinking, particularly during the winter vacation. Subjects were also requested to refrain from taking dietary supplements/beverages and to maintain a regular lifestyle during the study period. One day before testing, subjects were required to avoid the excessive consumption of alcohol and intensive exercise and fasted for 6 hours prior to blood collection, except for drinking water. The above excessive ingestion levels of subjects were defined by themselves.

Test samples and allocation: Test samples were indistinguishable, brown capsules containing either CFE or placebo, and were provided by Oryza Oil & Fat Chemical Co., Ltd. as hard capsules. Active (CFE) capsules contained 100 mg of Kiku Flower Extract-P (consisting of 10 mg of luteolin) and 100 mg of dextrin. Placebo capsules contained 200 mg of dextrin.

Oryza Oil & Fat Chemical Co., Ltd. provided the test samples with red or blue markings on the packages. Sample information was strictly concealed until the study period was completed. The number of subjects were assigned based on the maximum number of attendees within our budget. When the number of registered subjects reached 44, an allocation controller in ORTHOMEDICO Inc. generated an allocation sequence for test capsules according to the identification markers provided and made an allocation sheet and emergency key. Statlight #11 (Ver. 2.10, Yukms Inc.) was used to prepare a random number for the allocation sheet. The allocation sheet was only provided to test sample distributors and was then strictly concealed with the emergency key by the allocation controller. Test capsules were then allocated by class

randomization to equalize the allocation ratio (1:1). Allocation was required in a manner to prevent significant differences in the means and standard deviations (SD) of serum uric acid levels, age, and sex between groups. Allocation information was not disclosed to any other party until the subjects for analysis were selected at a clinical conference after study completion.

Study protocol (purine base-loading study and consecutive ingestion study): This randomized, placebo-controlled, double-blind, parallel-group study was performed at Takara Clinic (Medical Corporation Seishinkai, Tokyo, Japan), and statistical analyses were conducted by ORTHOMEDICO Inc. The protocol was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN000042327). Subjects attended the purine base absorption test followed by the consecutive ingestion study. The serum level of uric acid after the 12-week intervention was selected as the primary outcome. Other parameters included serum uric acid at the 4-week period and uric acid values in the purine base-loading test.

The purine base-loading test was performed on the same day as the baseline test (day 0 of consecutive ingestion study). In the purine base-loading study, subjects ingested a cup of corn soup containing 3.5 g each of sodium 5'-guanylate and sodium 5'-inosinate as the purine base-rich diet under a fasted status. One active or placebo capsule was then ingested with water less than 20 minutes after the consumption of corn soup. Blood samples were collected chronologically from a forearm vein at 1-hr intervals and serum was separated. Serum uric acid levels were measured using L-type Wako UA-M.

In the consecutive ingestion study, subjects took 1 capsule (CFE or placebo) daily after breakfast for 12 weeks. After a 1-month recovery period, blood samples were collected in addition to the intervention period, and blood parameters were analyzed. All subjects recorded a daily report including capsule ingestion, menstruation, and details of drinking such as the type and amount. They also answered a questionnaire by a physician after 4 and 12

weeks. Subjects were also asked to record a Calorie and Nutrition Diary from 3 days before to the day of the screening.

Laboratory tests: Body weight, body mass index (BMI), the body fat ratio, blood pressure, and pulse rate were measured at all test periods. Blood and urine were analyzed by LSI Medience Corporation (Tokyo, Japan). All items were examined at baseline and after 4 and 12 weeks of the intervention. A venous blood sample was collected from an arm vein and the following tests were performed for a safety assessment. Hematology components were as follows: hemoglobin (Hb), hematocrit, red blood cell, leukocyte, platelet, lymphocyte, monocyte, eosinophil, and basophil counts. Biochemical components were as follows: total protein, total bilirubin, urea nitrogen, creatinine, uric acid, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, HbA1c, blood glucose, glycoalbumin, amylase, creatine kinase (CK), aspartate aminotransferase (AST), alanine transaminase (ALT), γ -glutamyltransferase (γ -GTP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), choline esterase, cholecystokinin, Na, K, Cl, Ca, Fe, inorganic phosphorus, and IgE.

Urine samples were collected for a qualitative evaluation including protein, glucose, urobilinogen, bilirubin, ketone bodies, pH, and occult blood.

Ethics, adherence, and compliance: The present study was performed according to the Declaration of Helsinki (2013 revision) and conducted in conformity with ethical considerations. This protocol was approved by the Ethics Committee of Takara Clinic (Medical Corporation Seishinkai, Tokyo, Japan) on October 27, 2020 (Approved ID: 2010-00023-0071-1A-TC), and substantial deviations from the protocol required authorization by the committee. All subjects received a full explanation about the protocol and

purpose of the study before consenting to participate and submitted the signed informed consents as agreement. No subject was part of the sponsoring or funding companies.

Statistical analysis: A per protocol set (PPS) was selected as the analysis dataset for the primary and secondary outcomes. Results were shown as the mean and SD. Data on the screening point and the serum level of uric acid before purine base loading were set as the baseline values. Baseline data were analyzed using the student's *t*-test. After the intervention, actual scores were analyzed using the linear mixed model with baseline data utilized as covariates and time, groups, the baseline–time interaction, group–time interaction, and subjects as factors.

The results of the physical examination and blood tests were indicated as means and SD and safety analysis population (SAF). The student's *t*-test was used to evaluate the significance of differences between the values of the placebo and active groups, except for height. The χ^2 -test was employed for urinalysis parameters, with normal and abnormal values being coded as "1" and "0", respectively. We set the significance level to 5% with no adjustments for multiple comparisons. SPSS (Ver. 23.0, Japan IBM) or Microsoft Excel 2013 was used for statistical evaluations. Missing data was analyzed without storage.

RESULTS

Study performance: The present study was performed between November 9, 2020, and May 29, 2021. During the study period, 2 subjects who did not receive any intervention (Figure 1) were excluded. In addition, 3 subjects whose serum uric acid levels were 2 SD outside of the mean were excluded. After the key was opened, 4 of the eliminated subjects were found to belong to the active group and 1 to the placebo group. Accordingly, 18 subjects (43.9 \pm 12.3 years) were analyzed in the CFE group, and 21 (44.2 \pm 13.5 years) in the placebo group. The physical profiles of subjects included in the analysis are shown in the groups.

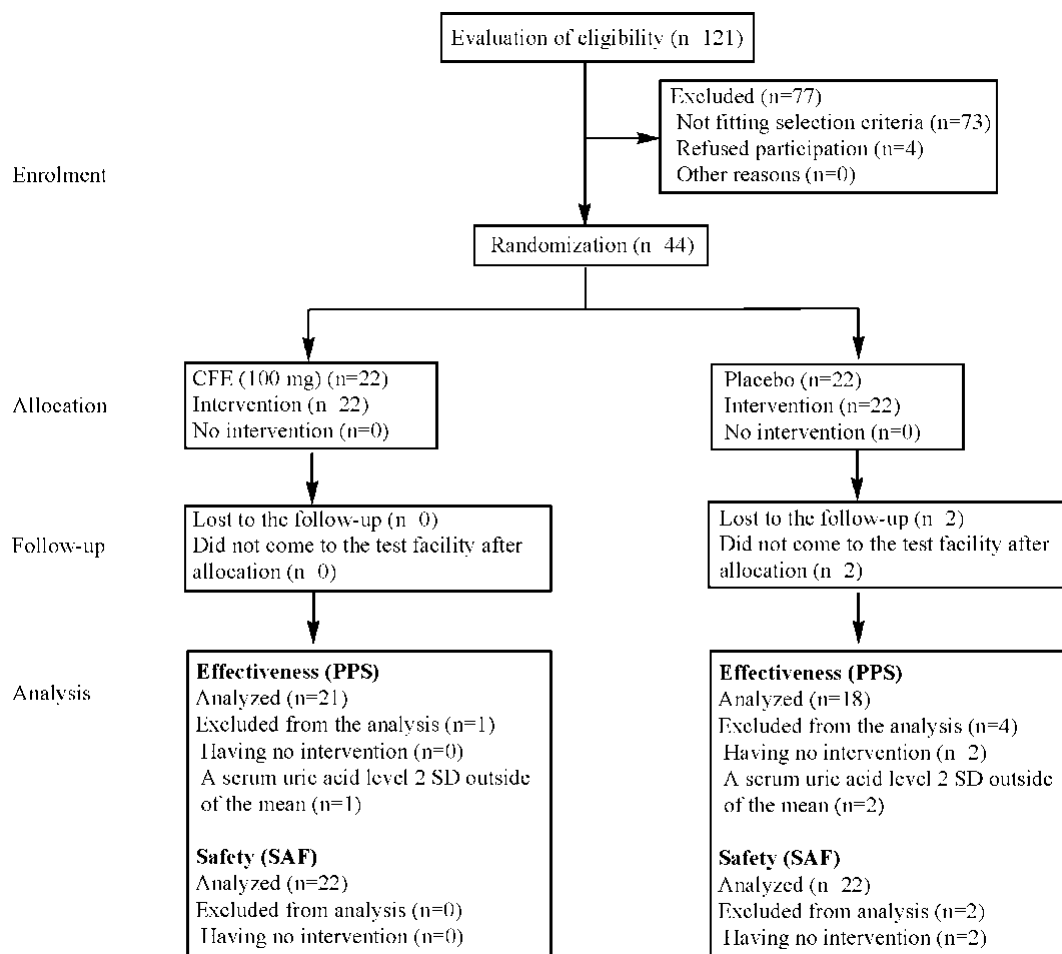


Figure 1. Flowchart of subject characteristics

Table 1. Subject profiles

	Baseline		12 W	
	Placebo	CFE	Placebo	CFE
Age	43.9±12.3	43.9±12.9	–	–
Height (cm)	170.2±7.1	169.2±5.9	–	–
Body weight (kg)	69.6±14.2	67.5±9.4	68.9±14.5	67.4±10.2
BMI (kg/m²)	23.9±3.9	23.6±3.6	23.7±4.0	23.6±3.8
Body fat ratio (%)	22.4±7.2	22.3±8.0	21.8±7.2	21.6±7.9
Systolic blood pressure (mmHg)	125.6±12.6	128.5±13.9	122.1±11.5	125.6±11.9
Diastolic blood pressure (mmHg)	83.1±8.9	83.2±9.0	81.7±9.2	82.5±8.1
Pulse rate (bpm)	73.0±10.8	68.7±12.5	69.4±9.9	69.0±10.8
Non-specific IgE (IU/mL)	136±174	280±834	–	–

Data are shown as means ± SD (n=22 for the placebo, n=20 for CFE). The student’s t-test was used to assess the significance of differences, except for age (the χ^2 -test). No significant differences were detected between the placebo and CFE groups.

Purine base-loading study: Figure 2 shows changes in serum uric acid levels after the consumption of a purine base-rich diet. One CFE or placebo capsule was taken after the ingestion of the purine base-rich diet. No significant differences were observed in changes in serum uric acid level between the groups. Areas under the curve (AUC) were 7.7 ± 3.1 mg/dL-hr for the placebo group and 9.2 ± 2.9 mg/dL-hr for the CFE group. Cmax were 9.4 ± 1.2 mg/dL for

the placebo group and 9.6 ± 1.0 mg/dL for the CFE group.

Consecutive ingestion study: As the primary outcome of this study, serum uric acid levels were significantly lower in the CFE group than in the placebo group after 12 weeks of the intervention (Table 2). No significant differences were observed after 4 weeks. Furthermore, urinary uric acid levels did not significantly differ between the groups.

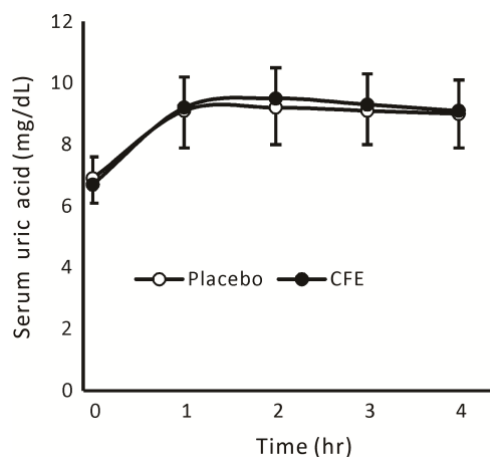


Figure 2. Changes in serum uric acid levels after the ingestion of a purine base-rich diet. Each value represents the mean \pm SD (n=22 for placebo, n=20 for CFE). No significant differences were observed between the groups.

Table 2. Changes in serum and urinary uric acid levels

	Baseline		4 W		12 W		After 4 W recovery	
	Placebo	CFE	Placebo	CFE	Placebo	CFE	Placebo	CFE
Serum uric acid (mg/dL)	6.9 \pm 0.8	6.7 \pm 0.9	6.6 \pm 0.7 (-0.1 \pm 0.6)	6.7 \pm 0.8 (-0.1 \pm 0.7)	6.9 \pm 0.6 (0.2 \pm 0.7)	6.5 \pm 0.7* (-0.3 \pm 0.7*)	6.8 \pm 0.8	6.9 \pm 0.8
Urinary uric acid (mg/dL)	43.4 \pm 23.4	47.8 \pm 24.4	40.8 \pm 22.4	39.5 \pm 25.4	44.7 \pm 31.2	41.1 \pm 24.6	40.0 \pm 20.4	39.5 \pm 25.4

Actual scores and changes from baseline (in parentheses) are shown as means \pm SD (n=21 for placebo and n=18 for CFE). Baseline data were analyzed using the student's *t*-test. After intervention data had been obtained, actual scores were analyzed using the linear mixed model with baseline data utilized as covariates and time, groups, the baseline–time interaction, group–time interaction, and subjects as factors. Changes from baseline were examined using the linear mixed model with time, groups, the group–time interaction, and subjects as factors. Serum uric acid levels at 12 W showed significant differences between the groups (**P* < 0.05 vs. the placebo group). No significant differences were observed in urinary uric acid levels between the placebo and CFE groups.

Laboratory data and adverse effects: Blood pressure and pulse rate are shown in Table 1 and blood hematology parameters in Table 3. No significant changes were observed between the two groups. Biochemical parameters are shown in Table 4. After 12 weeks of the

intervention, no significant differences were noted between the two groups. Any changes observed were all within reference ranges. Urinalysis parameters did not significantly change in either group (Table 5).

Table 3. Changes in hematology parameters

	Baseline		12 W		Standard value
	Placebo	CFE	Placebo	CFE	
Red blood cells ($\times 10^4$ cells/ μ L)	487 \pm 40	497 \pm 30	480 \pm 39	495 \pm 28	Men: 430-570; Women: 380-500
Leukocytes (cells/ μ L)	5172 \pm 1237	5370 \pm 1361	5740 \pm 1913	5665 \pm 1267	3300-9000
Hemoglobin (g/dL)	15.1 \pm 1.1	15.2 \pm 1.1	14.8 \pm 1.1	14.9 \pm 1.0	Men: 13.5-17.5; Women: 11.5-15.0
Hematocrit (%)	46.7 \pm 2.8	46.9 \pm 3.3	45.8 \pm 2.8	46.6 \pm 2.8	Men: 39.7-52.4; Women: 34.8-45.0
Platelets ($\times 10^4$ cells/ μ L)	24.5 \pm 6.1	25.2 \pm 5.8	23.4 \pm 4.9	24.9 \pm 6.8	14.0-34.0
Neutrophils (cells/ μ L)	2920 \pm 921	3152 \pm 1194	3255 \pm 1513	3251 \pm 1029	
Lymphocytes (cells/ μ L)	1760 \pm 441	1698 \pm 417	1970 \pm 522	1882 \pm 507	
Monocytes (cells/ μ L)	318 \pm 124	287 \pm 93	334 \pm 114	301 \pm 75	
Eosinophils (cells/ μ L)	128 \pm 66	186 \pm 163	130 \pm 60	179 \pm 205	
Basophils (cells/ μ L)	46.3 \pm 23.5	46.1 \pm 15.0	49.4 \pm 23.8	49.0 \pm 14.3	

Each value is shown as the mean \pm SD (n=21 for placebo and n=18 for CFE). The student's *t*-test was used for statistical analyses. No significant differences were detected between the placebo and CFE groups.

Table 4. Changes in biochemical parameters

	Baseline		12 W		Standard value
	Placebo	CFE	Placebo	CFE	
Total protein (g/dL)	7.3 \pm 0.4	7.3 \pm 0.4	7.3 \pm 0.3	7.3 \pm 0.5	6.7-8.3
Total bilirubin (mg/dL)	0.93 \pm 0.24	0.76 \pm 0.29	0.95 \pm 0.41	0.80 \pm 0.22	0.2-1.2
Urea N (mg/dL)	16.6 \pm 4.4	14.3 \pm 3.8	15.5 \pm 4.2	14.3 \pm 3.6	8-20
Creatinine (mg/dL)	0.89 \pm 0.11	0.84 \pm 0.14	0.90 \pm 0.13	0.85 \pm 0.12	0.47-0.79
Total cholesterol (mg/dL)	213 \pm 33	213 \pm 24	210 \pm 31	212 \pm 30	120-219
LDL cholesterol (mg/dL)	129 \pm 35	122 \pm 19	126 \pm 31	121 \pm 29	65-139
HDL cholesterol (mg/dL)	62 \pm 15	67 \pm 15	63 \pm 15	69 \pm 11	Men: 40-85 Women: 40-95
Triglycerides (mg/dL)	102 \pm 51	131 \pm 100	105 \pm 53	109 \pm 67	30-149
HbA1c (%)	5.4 \pm 0.2	5.3 \pm 0.3	5.4 \pm 0.3	5.4 \pm 0.2	4.6-6.2
Glycoalbumin (%)	13.6 \pm 1.1	12.9 \pm 1.4	13.3 \pm 1.1	12.5 \pm 1.1	12.3-16.5
Blood glucose (mg/dL)	84 \pm 9	89 \pm 7	83 \pm 7	87 \pm 7	70-109
Amylase (U/L)	79 \pm 25	80 \pm 20	77 \pm 23	81 \pm 25	40-122
CK (U/L)	144 \pm 72	129 \pm 59	175 \pm 141	138 \pm 85	40-150
AST (U/L)	25 \pm 7	22 \pm 5	22 \pm 6	22 \pm 7	10-40
ALT (U/L)	27 \pm 19	21 \pm 8	22 \pm 10	23 \pm 11	5-45
γ -GTP (U/L)	36 \pm 20	36 \pm 27	31 \pm 12	45 \pm 54	Men: \leq 80 Women: \leq 30
ALP (U/L)	192 \pm 50	213 \pm 48	68 \pm 18	74 \pm 20	100-325 (38-113)*
LAP (U/L)	56 \pm 9	52 \pm 10	54 \pm 6	55 \pm 10	Men: 45-81 Women: 37-61
LDH (U/L)	196 \pm 31	179 \pm 26	195 \pm 28	181 \pm 25	120-240 (124-222)*
Na (mEq/L)	140 \pm 1	141 \pm 1	142 \pm 2	142 \pm 1	137-147
K (mEq/L)	4.2 \pm 0.4	4.2 \pm 0.3	4.2 \pm 0.5	4.2 \pm 0.4	3.5-5.0
Cl (mEq/L)	100 \pm 1	100 \pm 2	101 \pm 1	101 \pm 2	98-108
Ca (mg/dL)	9.5 \pm 0.3	9.5 \pm 0.3	9.5 \pm 0.3	9.6 \pm 0.4	8.4-10.4
Fe (μ g/dL)	126 \pm 52	112 \pm 46	120 \pm 40	109 \pm 34	40-180
Inorganic P (mg/dL)	3.3 \pm 0.6	3.2 \pm 0.6	3.1 \pm 0.5	3.3 \pm 0.6	2.5-4.5

Each value is shown as the mean \pm SD (n=22 for placebo and n=20 for CFE). The student's *t*-test was used for statistical analyses. No significant differences were detected between the placebo and CFE groups. Parentheses with an asterisk show the new standard value after 12W due to a change in the inspection method.

Table 5. Changes in urine parameters

	Week	Placebo	CFE	Standard value
Protein	0	(nor):21, (ab):1	(nor):19, (ab):1	(-)
	12	(nor):21, (ab):1	(nor):19, (ab):1	
Glucose	0	(nor):22, (ab):0	(nor):19, (ab):1	(-)
	12	(nor):22, (ab):0	(nor):19, (ab):1	
Urobilinogen	0	(nor):22, (ab):0	(nor):20, (ab):0	(±)
	12	(nor):20, (ab):2	(nor):20, (ab):0	
Bilirubin	0	(nor):22, (ab):0	(nor):20, (ab):0	(-)
	12	(nor):22, (ab):0	(nor):20, (ab):0	
pH	0	(nor):22, (ab):0	(nor):20, (ab):0	(5.0-7.5)
	12	(nor):22, (ab):0	(nor):20, (ab):0	
Occult blood	0	(nor):21, (ab):1	(nor):19, (ab):1	(-)
	12	(nor):21, (ab):1	(nor):19, (ab):1	
Ketone bodies	0	(nor):22, (ab):0	(nor):20, (ab):0	(-)
	12	(nor):22, (ab):0	(nor):20, (ab):0	

Data are shown as the number of subjects with normal values (nor) or abnormal values (ab). The χ^2 -test was used for urinalysis parameters. No significant differences were detected between the placebo and CFE groups.

DISCUSSION

A long-term high blood uric acid status promotes the painful formation of sodium urate crystals such as in the toes. The inflammatory response of gout occurs when phagocytes, including macrophages and neutrophils, accumulate at urate crystals, secrete pro-inflammatory cytokines and cause inflammation [16]. Therefore, the control of serum uric acid levels to within a healthy range and reductions in the intestinal absorption of purine bases, the precursors of uric acid, are effective strategies for the prevention of gout [17].

In our initial studies, we examined the effects of CFE on changes in serum uric acid levels after the single intake of a purine base-rich diet with CFE. The single oral dosing study showed no significant changes in serum uric acid levels between the CFE and placebo groups (Figure 2). This result was consistent with our previous findings [15]. Serum uric acid after 12-week ingestion of CFE, which was set as the primary outcome, was significantly lower in the CFE group than in the placebo group (Table 2). In addition, the changes between the baseline and 12-week intervention were significantly lower in the CFE group than in the placebo group (Table 2). Therefore, CFE reduced uric acid levels when continuously ingested without affecting dietary uric acid absorption.

Uric acid is produced by xanthine oxidase, which oxidizes hypoxanthine to xanthine and xanthine to uric acid [18]. Allopurinol, the first-line treatment to control uric acid levels, is an inhibitor of xanthine oxidase [19]. Therefore, the inhibition of xanthine oxidase is considered to be a useful strategy for controlling serum uric acid levels. In the present study, CFE (100 mg/day) contained 10 mg/day of luteolin, which is a flavonoid that was shown to function as a competitive inhibitor of xanthine oxidase in *in vitro* experiments [14, 20-21]. Furthermore, CFE containing polyphenols such as luteolin inhibited the activity of xanthine oxidase in *in vitro* tests [22]. In addition, serum uric acid levels in mice and rats with hyperuricemia were significantly reduced by the ingestion of luteolin and CFE [21-24]. Previous studies also demonstrated that xanthine oxidase activity in the liver was significantly suppressed by luteolin or CFE [22-23]. In our previous clinical trial, we confirmed that the four-week ingestion of CFE reduced uric acid levels in Japanese male workers at Oryza Oil & Fat Chemical Co., Ltd. [15]. Collectively, these findings indicate that the consecutive ingestion of CFE decreases serum uric acid levels.

One possible reason why CFE did not inhibit purine base absorption is possibly due to the absorption and

metabolism of luteolin in humans. Luteolin and luteolin glucuronide are present in blood after luteolin ingestion [25]. Luteolin glucuronide has been shown to exhibit weaker inhibitory activity against xanthine oxidase than luteolin [21]. Therefore, the inhibitory effects of CFE on xanthine oxidase may not have been sufficient following a single dose due to the lack of an increase in serum luteolin to a sufficient concentration.

In the consecutive ingestion study, serum uric acid levels were lower in the CFE group than in the placebo group after the 12-week intervention. Since luteolin persists in the blood following its absorption [26], this result suggests that the continuous ingestion of CFE containing luteolin suppressed increases in uric acid levels. We previously reported that the continuous ingestion of CFE for 4 weeks reduced uric acid levels [15]; however, the present study observes no significant differences in serum uric acid levels between the groups after 4 weeks of the intervention (Table 2). In the first place, since this study aimed to evaluate the effect of CFE on healthy subjects with normal ranges of serum uric acid, it appeared to be difficult to detect reductive the effect. However, we considered the several factors as follows. The following 4 factors may have contributed to this discrepancy in the effects of CFE. The first difference is the test design. The previous study was designed as a crossover study and compared the effects of interventions on an individual basis to eliminate the effects of individual differences. Therefore, the intervention effect was more accurately evaluated than in the parallel group comparison design employed in the present study. However, a crossover study design has a prolonged study period because participants need to receive two interventions, which increases the likelihood of dropouts. Therefore, differences in subject characteristics may be attributed to this difference between the present and previous studies.

The second factor is the influence of seasons on the test. Serum uric acid levels are generally higher in summer than in winter [27-28]. The present study was conducted between January and March (winter to

spring), while the previous study was performed between September and November (summer to autumn). Since the previous study was conducted at a time when uric acid levels were more likely to be higher than in the present study, the effects of CFE may have been more apparent, even after a four-week intervention.

The third reason for differences is the timing of ingestion of CFE and placebo capsules. CFE capsules were ingested after 1 meal each day, either breakfast, lunch, or dinner, in the previous study, but after breakfast only in the present study. *In vivo* studies reported that the inhibitory effects of quercetin, which is a structurally similar flavonoid to luteolin, on intestinal fat absorption and energy metabolism were stronger when taken in the morning than in the evening [29]. In addition, in a previous study that examined the effects of catechin-containing beverages on postprandial blood glucose levels in healthy young men, the suppression of postprandial blood glucose elevations was more pronounced at dinner than at breakfast [30]. Based on these findings, the time at which CFE is ingested may alter its effects.

Subject backgrounds also differed between the previous and present studies. In the previous study, subjects were recruited from employees of Oryza Oil & Fat Chemical Co., Ltd. On the other hand, the recruitment of subjects for the present study was conducted on the participant recruitment site of ORTOMEDICO Co. Ltd. and there were no work-related restrictions. According to a previous study that investigated the prevalence of hyperuricemia based on occupation, various factors were found to increase serum urate levels such as occupation, job stress, overtime, and personal characteristics [31]. Therefore, occupation-dependent differences among subjects may have affected the data obtained. Moreover, hyperuricemia is classified into 4 different types: the excessive uric acid production type, decreased uric acid excretion type, mixed type, and decreased extrarenal excretion type based on the amount of uric acid produced and the ability to excrete uric acid [32]. However, neither this study nor previous

studies used the above classification. Therefore, it is possible that the subjects having the above different backgrounds of uric acid metabolism had attended the study in different numbers and ratio in both groups and this might have led to the current results.

In an *in vivo* study using hyperuricemic rats, the ingestion of CFE increased the urinary excretion of uric acid with increases in expression of ATP binding cassette subfamily G member 2 (Abcg2) and solute carrier family 17, member 1 (Slc17a1) [22]. Abcg2 and Slc17a1 are involved in the urinary excretion of uric acid. Therefore, CFE may promote the urinary excretion of uric acid. However, in the present study, CFE did not affect urinary levels of uric acid (Table 2). As discussed above, since no eligibility criteria for uric acid excretion were established in the present study, it was not possible to examine the effects of CFE on urinary uric acid levels. By establishing criteria for the selection of subjects with the lower urinary excretion of uric acid, it may be possible to examine the effects of CFE on its excretion in more detail.

Comparisons of average serum uric acid levels in men and women of all ages revealed higher levels in the former than in the latter [33-34]. The expression of the uric acid transporter urate anion exchanger, which reabsorbs uric acid in humans, is reportedly reduced by estrogen and promotes uric acid excretion, and, thus, is one of factors contributing to sex differences in uric acid levels [33]. A previous study reported that plasma uric acid levels were significantly decreased, whereas urinary uric acid levels were significantly increased in transsexual men receiving estrogen therapy [35]. The general age of menopause is 50 years old [36], and the secretion of estrogen is significantly reduced in menopausal women [34, 37]. Serum uric acid levels were found to continuously increase in Japanese and American women between their 50s and 70s [38-39], and those who underwent spontaneous or surgical menopause had higher serum uric acid levels than premenopausal women. These findings are consistent with the promoting effects of estrogens on uric acid excretion. CFE was found to reduce uric acid levels in the present

study, which included female subjects (Table 2). However, the number of women enrolled in the present study was only 2 out of 22 (9.1%) in the test food group and one out of 22 (4.5%) in the placebo group. Therefore, it currently remains unclear whether CFE is truly effective for women. According to the National Health and Nutrition Survey in Japan, the proportions of individuals older than 20 years with serum uric acid levels between 6.0 and 7.9 mg/dL were 37.0% for men and 9.6% for women [3]. Since serum uric acid levels are affected by sex and menopause, it may be possible to examine the effectiveness of CFE in women in more detail by comparing its effects on men and women as well as before and after menopause.

In the safety study, no side effects were associated with the CFE intervention under the conditions of the present study. Although several side effects developed in some subjects, the physician did not detect any causal relationship with CFE based on the criteria set at the time of study planning. Furthermore, no significant differences were observed in the results of peripheral blood tests (Tables 3 and 4) or the urinalysis (Table 5) between the groups. Therefore, CFE was safe to ingest under the conditions employed in the present study. As further investigation, anti-inflammatory effect of luteolin might contribute to amelioration of acute gout inflammation as it induces macrophage alteration of M1 to M2 macrophages [40]. Also, luteolin suppresses inflammation and recurrence of symptom gout in rabbit knee model [41]. These reports suggest that luteolin might delay acute gout inflammation and pain through the anti-inflammatory effect. To clarify the effect of luteolin on gout pain and inflammation, clinical studies on gout patients are expected.

CONCLUSIONS

The present results demonstrated that CFE (100 mg/day for 12 weeks) containing 10 mg of luteolin decreased serum uric acid levels in healthy Japanese subjects. However, it did not suppress purine base absorption from the intestines in the single purine base-loading test.

Therefore, CFE appears to reduce serum uric acid levels by mechanisms other than the inhibition of purine base absorption such as the suppression of xanthine oxidase. The ingestion of CFE may reduce blood and urinary levels of uric acid, thereby preventing gout.

Abbreviations: Abcg2: ATP-binding cassette subfamily G member 2, ANOVA: one-way analysis of variance, ALP: alkaline phosphatase, ALT: alanine transaminase, AST: aspartate aminotransferase, AUC: area under the curve, BMI: body mass index, CK: creatinine kinase, CFE: chrysanthemum flower extract, Cmax: maximum plasma concentration, GTP: glutamyltransferase, HDL: high-density lipoprotein, Hb: hemoglobin, LAP: leucine aminopeptidase, LDH: lactate dehydrogenase, LDL: low-density lipoprotein, PPS: per protocol set, SAF: safety analysis population, SD: standard deviation, Slc17a1: solute carrier family 17.

Competing Interests: The sponsor of the present study, Oryza Oil & Fat Chemical Co., Ltd., assigned ORTHOMEDICO Inc. to conduct the study. W.Y., S.T., and H.S. (Ph.D.) are affiliated with Oryza Oil & Fat Chemical Co., Ltd., and K.Y., N.S., S.Y., S.I., T.K., and A.B. are members of ORTHOMEDICO Inc. This study was conducted by both Oryza Oil & Fat Chemical Co., Ltd. and ORTHOMEDICO Inc. T.T. (MD) was the principal investigator who monitored all subject conditions and was supported by M.N. (MD).

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