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Green Tea Extract (*Camellia sinensis* L.) Effects on Uric Acid Levels on Hyperuricemia Rats (*Rattus norvegicus*)

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ABSTRACT

Uric acid is the end product of purine degradation. When uric acid levels exceed normal limits, it will build up and cause hyperuricemia. Allopurinol is one of the most effective and common medicine for hyperuricemia, but it brings serious side effects, therefore it is needed alternative therapy for hyperuricemia. One plant that may be expected to low uric acid levels is green tea (*Camellia sinensis L.*), that contains many antioxidants polyphenols, especially flavonoids. Flavonoid has strong antioxidant properties, act as free radical and metal scavengers, and also xanthine oxidase (XOD) inhibitors. This study investigates the potential of green tea using various doses of 150 mg/kg, 300 mg/kg, and 600 mg/kg of body weight in 24 white male rats (*Rattus norvegicus*) Wistar strain that has been received high purine diet in 60 consecutive days. This study used DHBSA methods to measure uric acid levels in blood serum and urine that excreted 8 hours before surgery. Green tea extract that contains polyphenol can inhibit XOD activities, therefore, it leads to decrease uric acid level in blood and increase the excretion through urine by modulating urate gene transporter. A therapy with 600 mg/kg body weight of GTE is the most effective dose to decrease uric acid levels in serum and to increase excretion of exceeding uric acid significantly ($p < 0.01$), from One Way ANOVA and Tukey analysis.

Keywords: hyperuricemia, uric acid, green tea extract, uric acid levels

INTRODUCTION

In the body, normal amount of uric acid (UA) levels are 3.4 – 7.0 mg/dL in men, and 2.4 – 6 mg/dL in women. As many as 75% of the veins will be secreted through urine [1]. The ionized uric acid becomes urate that will dominate in extracellular plasma and synovial fluid as monosodium urate at pH 7.4, in this condition blood plasma will be saturated. At higher concentrations, blood plasma becomes supersaturated, thus, uric acid will precipitate and uric crystals formed. Normally, UA is formed by the body as much as 400 mg/day, while uric acid obtained from food reaches 300 mg/day [2]. However, when UA levels in the body exceed normal limits, it tends to build up and leads to hyperuricemia (HUA).

HUA is a disease associated with kidney disease and often used as a marker of renal dysfunction [3]. The symptoms is joint pain due to chronic systemic inflammation of the joints caused by disposition of monosodium urate crystals in connective tissue in extracellular fluids [4], such as knees, armpits, wrists and feet and areas joint with pain and stiffness of redness and swelling that are not caused by a collision or accident, known as gout [5]. If this condition is neglected, HUA may cause impaired renal function, such as nephrolithiasis, urate nephropathy, and may further lead to gout arthritis and lead to cardiovascular disease [6]. In

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the other side, HUA leads to overproduction of ROS, which is a major contributor of oxidative stress formation [7] as a result of xanthine oxidase (XOD) over activity.

To date, allopurinol (ALP) is the most widely used drug since it is considered to be most effective in inhibiting UA formation by inhibiting xanthine oxidase activity [8]. However, its use can lead to some serious side effects, such as nephropathy, allergic reactions, and indigestion [9]. Side effects caused by chemical drugs make some people choose to use medicinal plants that are considered able to cure HUA with minimal side effects. Some medicinal plants have been tested, such as cat's whiskers, *rosella* [10], and coffee [11]. However, the results obtained cannot exceed the effectiveness of ALP.

One plant that is thought to reduce UA levels is green tea (*Camellia sinensis*). Green tea extract (GTE) contains many antioxidants polyphenols, especially flavonoids. GTE has strong antioxidant properties by performing several mechanisms, such as anti-lipid peroxidase, free radical scavengers, and inhibitors of several enzymes including xanthine oxidase [12]. Previous studies have shown that Chinese GTE at appropriate doses can decrease UA levels and decrease xanthine oxidase activity, URAT1 expression, and increase OAT1 and OAT3 expression in mice's renal with hyperuricemia [13].

GTE contains several substances, one of them is caffeine, contained as much as 8-30 mg per cup (240 mL) [14]. Caffeine is one type of alkaloid with a chemical formula 1,3,7-trimehylxanthine ($C_8H_{10}O_2N_4$) which is diuretic. Although some studies suggest that caffeine consumption may trigger excessive UA formation, however, in moderate consumption, caffeine may provide some benefits, such as headache treatment, increased alertness, and muscle relaxation [15], [16]. In GTE, caffeine activity is inhibited because of the presence of L-theanine amino acids which provide relaxing effect on the brain [16] and caffeine antagonist, therefore can compensate caffeine activity in the body [15]. Thus, in this study green tea decaffeination did not perform.

EXPERIMENT

Chemicals and instrumentation

The experimental animals used in the study were white male rats (*Rattus norvegicus*) male Wistar strain aged 2-2.5 months with an average body weight of 175-225 g which were purchased from provider of laboratory animals in Bandung, West Java and all the animal procedures have been approved by the ethical acceptance of UB's Research Commission 690-KEP-UB. They had been feed with standard feed (SP) and water.

The chemicals used in this study include uric acid kit (Reiged Diagnostics), dry green tea (Kepala Djenggot), allopurinol (Kimia Farma), sodium chloride 0.9% (Merck), Paraformaldehyde (Sigma Aldrich), azide-Phosphate Buffer Saline (Merck), 70% ethanol (Merck), and aquadest (Hydrobatt).

The instrumentation used in this research include rat cages and wire enclosures, gloves, masks, drinkers, Eppendorf centrifuges (OneMed), analytical balance (Mattler Toledo), a set of glassware (IWAKI), incubator, visible spectrophotometer (Thermo Scientific Genesys 20), water baths, autoclaves, 100 µL micropipette (Biohit Proline), 1000 µL micropipette (Bio Rad), yellow tip (OneMed), blue tip (OneMed), gavage, Eppendorf tube (BioMed), vortex (Thermoline), scissors, spatula, surgical tool and table, vacutainer Non-EDTA (Vaculab), urine pot, mortar and pestle, freezer -20^{o}C, refrigerator, 3 mL syringe (Terumo), cuvette, blood lancet (OneMed) and set of Easy Touch GCU.

Green Tea Leaves Extraction

Commercial dry green tea leaves (50 g) were crushed by blending, and then it sifted by a sieve of 80 mesh. Furthermore, green tea powder was brewed using boiled water (± 95^{o}C) with ratio 1:10 then stirred for 30 minutes. Then, the result of maceration was cooled to a room temperature and filtered by a sheet of cloth and separated between the filtrate and the dregs. The brewing process was repeated up to 3 times. Furthermore, the extract obtained was concentrated by using a vacuum rotary evaporator at a temperature of 85°C and 110 rpm. The GTE's extraction yield was 24.94% (w/w) and kept in a -20^{o}C freezer.

Acclimatization of Experimental Animals

24 rats were placed in a polyethylene cage that filled with wood husks with a dimension of 45 x 35 x 20 cm with wire enclosures. The room temperature was 22 ± 2^{o}C. The rats were acclimated for a week before the experiments with a standard feed and water *ad libitum*.

Induction of High Purine Diet and Drugs Treatment

The 24 rats were divided into 6 groups, which each group had 4 rats: (1) negative control group, (2) positive control group, (3) the medicinal therapy of ALP with 5mg/kg of weight dose, (4) GTE therapy of 150 mg/kg, (5) GTE therapy of 300 mg/kg, and (6) GTE therapy of 600 mg/kg. Each rat was fed a high purine diet, except the negative control group. A high-purine diet consisting of 25 grams of cow's liver, 25 grams of cow's spleen, 25 grams of fried *melinjo*, and 25 grams of fried peanuts blended and dissolved into the water until 150 mL and then the mixture was filtered. The filtrate was introduced was 3 mL/rats, twice a day (at 8:00 and 14:00) daily for 60 consecutive days. The UA level was examined every five days to determine whether rats were suffered from HUA. When UA levels were more than 7 mg/dL, then rats were ready to be treated.

After 5 groups of rats suffered from HUA, rats in) group 3 were administered using 3 mL ALP with dose of 5 mg/kg body weight. Rats in the groups 4, 5, and 6 were treated by 3 mL of GTE with the same dose as mentioned before.. On day 13, all rats were incubated in the metabolite cages to withdraw the urine. The urine was collected on the next day and placed into urine pot. The urine was wrapped and stored in -20^{o}C freezer. On the same day, all rats were sacrificed for organ and blood collection.

Serum collection from blood

After blood coagulated, about 2 hours after collection, blood was centrifuged at 2000 rpm for 10 min, in order to collect blood serum. Serum was moved into Eppendorf tube, then serum was centrifuged at 1000 rpm for 10 minutes. Serum samples were moved into new Eppendorf tube and stored in -20^{o}C freezer until UA determination.

Measurement of Uric Acid Levels in Serum and Urine

Uric acid levels were determined based on enzymatic reactions using DHBSA uric acid reagents. The reagent composition comprises of: 2 mM 3.5 DHBS, 4 mM 4-AAP, 150 U/L uricase, 12000 U/L peroxidase, and non-reactive stabilizers with a pH of 7.6 ± 0.2.

The uric acid levels measurement was performed by adding 25 µL serum with 1000 µL reagent, then incubated for 10 minutes at 37 °C. Urine was taken as much as 25 µL and 1000 µL of reagent was added, and was incubated at 37^{o}C for 10 min. After incubation, absorbance reading was performed using spectrophotometer UV-Vis at wavelength of 520 nm. The reagent compositions as follows :

Table 1. Reagent and samples composition for uric acid measurement

	Blank	Standard	Sample
Reagent	1.0 µL	1.0 µL	1.0 µL
Distilled water	25 µL	-	-
Standard	-	25 µL	-
Sample	-	-	25 µL

Statistical Analysis

In this study, the data obtained were analyzed by using data normality test using Shapiro-Wilk statistic and homogeneity in order to determine the normality of data distribution. Effects of treatment on parameters of total UA level was analyzed using ANOVA which was completed by Tukey test with 99% confidence level to know the difference between treatments. Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) 23.0 software. Results were significant when $p < 0.01$.

RESULT AND DISCUSSION

The GTE flavonoid has been characterized using UHPLCMS/MS to determine the compound that present in GTE and to determine the specific compound that can reduce uric acid level in serum and increase uric acid excretion in urine. The result of qualitative analysis of GTE was presented in **Figure 1** and **Table 2.**

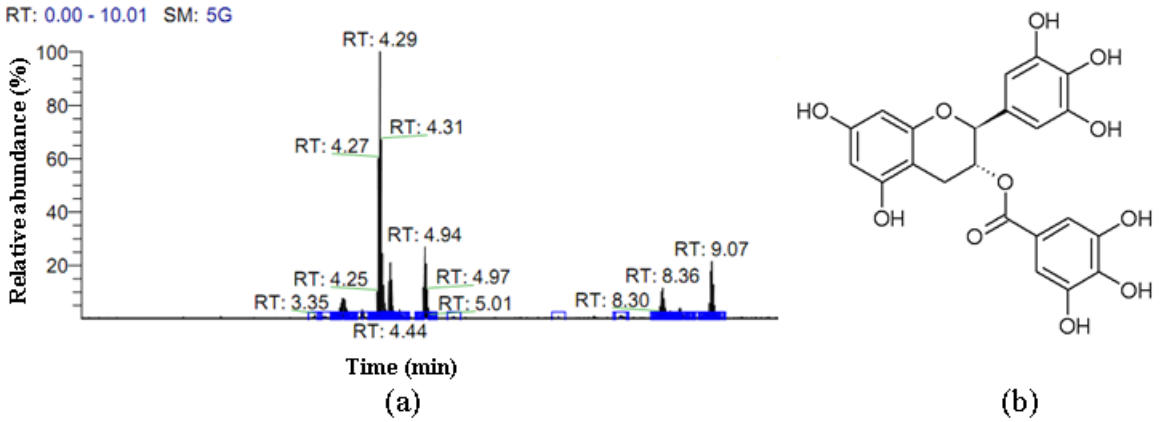

Figure 1. (a) UHPMCMS/MS chromatogram of GTE compounds and (b) epigallocatechin gallate (EGCG) or gallocatechin gallate (GCG) molecular structure

The flavonoid compound from GTE contained approximately eight compounds, as shown at **Table 2**, based on the fragment of molecular ions that formed and the standard fragment. Based on **Figure 1**, the GTE were analyzed qualitatively some peaks are appeared with its retention time. The highest chromatogram peak from UHPLCMS/MS spectra formed at retention time of 4.29 min with molecular ions $[M^+]$ weight of 457 *m/z*. This peak was identified as epigallocatechin gallate (EGCG) or its epimer, gallocatechin gallate (GCG) with molecular formula of $C_{22}H_{18}O_{11}$. The structure of EGCG and GCG has 8 glycosylate groups that can interact with the active side of XOD enzyme, so it can inhibit the formation of UA.

Table 2. Parameters in qualitative analysis using UHPLCMS/MS of GTE flavonoid

RT Peak	Fragment Ions (m/z)	Standard fragment ions (*m/z*)	Molecular ions $[M]^+$ (*m/z*)	Prediction of Flavonoid Compounds
4.44	204.5-205.5	205	289	(+)-catechin (C), (–)-epicatechin (EC)
3.72	136.5-137.5	137	305	(–)-gallocatechin (GC), (–)-epigallocatechin (EGC)
4.29	168.5-169.5	169	457	(–)-epigallocatechin gallate (ECGC), (–)-gallocatechin gallate (GCG)
4.94	168.5-169.5	169	441	(–)-epicatechingallate (ECG), (–)-catechingallate (CG)

GTE effects on uric acid levels in serum

After the rats treated, it is necessary to determine the level of uric acid to know the effect of green tea extract in reducing UA levels after induced with high purine diet. The test was performed by DHSBA method, the enzymatic-colorimetric method that converts UA into allantoin with uricase enzyme, afterward allantoin reacts with DHBS and peroxidase enzymes into peach quinoneimine complex compounds. The color formed is measured by a spectrophotometer at a wavelength of 520 nm. Results from the statistical test using one way ANOVA analysis showed that green tea treatments with 3 different doses decreased UA levels in blood serum as shown in **Figure 2**. Differences between treatments were highly significant ($p < 0.01$).

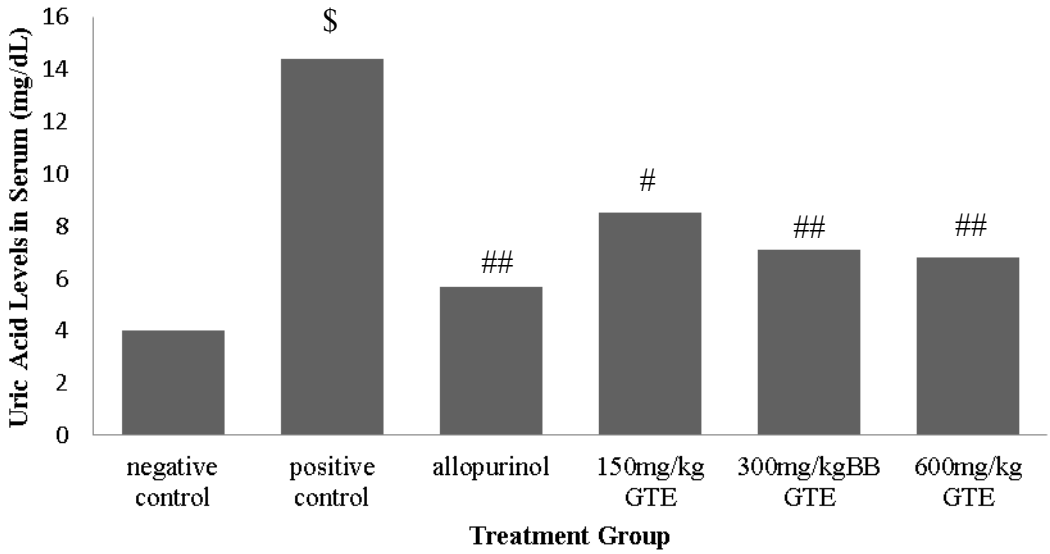

Figure 2. Effects of GTE on UA levels in serum of HUA rats. Data were expressed as mean ± SD (n=4). $^{\$}p < 0.01$ compared to negative control group. $^{\#}p < 0.05$ compared to positive control group. $^{\#\#}p < 0.01$ compared to positive control group.

As shown in **Figure 2**, the lowest serum UA levels were in negative control group at 3.95 ± 1.01 mg/dL. This group is group of rats that were not received any treatment, only standard feed and water, therefore UA levels in serum was in normal range, 3.4-7.0 mg/dL. This group was used as a reference, to compare increasing or decreasing of UA levels in serum due to treatments, both high purine diet induction or green tea therapy.

Positive control group was group that received high purine diet, thus, the UA levels reached 14.29 ± 2.89 mg/dL. These numbers are above normal level range of UA, as a result this group was suffered from HUA. [17], [18]. After 60 days of high purine diet, there was a

significant increase approximately 262 % in UA levels between mean positive control group levels compared with negative control group. This suggests that a high purine diet increased uric acid levels ($p < 0.01$).

The drug therapy group using ALP was used as a comparative control to compare how effective green tea is in lowering UA levels in the blood. ALP is the most commonly used XOD inhibitor for many years [9]. This suggests that the target for treatment is XOD because after high purine diets, there was significant increase in XOD activity in the rats liver. ALP is more effective in reducing UA levels in blood serum than GTE. This has been shown by UA levels reached 5.45 ± 1.06 mg/dL, or decreased UA levels up to 61.88% ($p < 0.01$). These results are in agreement with previous studies [13], [19] which suggest that ALP is potent inhibitor in inhibiting XOD activity. This is due to the analog molecular structure of ALP and xanthine as XOD substrates in UA, therefore ALP is a competitor substrate to xanthine and is able to occupy active sides of XOD, and inhibit enzyme activity irreversibly. This inhibited activity prevents UA synthesis, and lowers UA levels in the blood.

Based on preliminary studies [13], the GTE doses for the therapy used in this study were 150 mg/kg, 300 mg/kg, and 600 mg/kg. At therapy doses of 150 mg/kg, 300 mg/kg, and 600 mg/kg, UA levels in serum were 8.53 ± 0.45 mg/dL, 6.63 ± 0.55 mg/dL, and 6.40 ± 0.47 mg/dL, respectively. These results indicate that GTE with those doses decreased UA levels with decreases of 28.73% ($p < 0.05$), 53.60% ($p < 0.01$), and 55.25% ($p < 0.01$), respectively. These results were in agreement with previous studies [13], [20], [21] which reported that green tea extract can lower UA levels by inhibiting activity of the XOD enzyme and modulating the urate transporter in the kidneys thus increasing UA excretion. The molecular structure of flavonoid in ring A has many analogies to xanthine and ALP, thus, flavonoid can be act as suicide substrate for XOD [85].

Previous *in vitro* studies [22], [23] have shown that flavones and flavonols that do not have glycosylate groups are strong XOD inhibitors because the glycosylate group is too bulky and prevents flavones from binding to the enzyme. Methylation of aromatic hydroxyl groups can also decrease the interaction between flavonoids and XOD. The C-glycosylate group in C-6 and C-8 greatly decreases the effect of the inhibitor because of the steric interaction occurs. A previous study [87] found that hydroxyl groups in C-5 and C-7 and double bonds in C-2 and C-3 may be a major factor inhibiting XOD enzyme activity. Previous studies [88] suggest that the planar molecular structure has a greater inhibitory potential than non-planar molecular structure, due to a conjugation with 3 C rings on the flavone structure. This has been proven by C and EC having low inhibitory power, while ECG and EGCG have the most galloyl groups that have higher ability to interact and inhibit XOD activity, then reducing the synthesis of UA in the body. This study showed the potential for flavonol inhibition on EGC, ECG, and EGCG with IC_{50} values of more than 100 µM, 48.5 µM, and 44.7 µM, respectively.

GTE effects on uric acid levels in urine

Results from statistical tests using one-way (One Way ANOVA) analysis showed that green tea with 3 different doses of 150 mg/kg, 300 mg/kg, and 600 mg/kg increased uric acid clearance through kidneys with highly significant difference between treatments ($p < 0.01$).

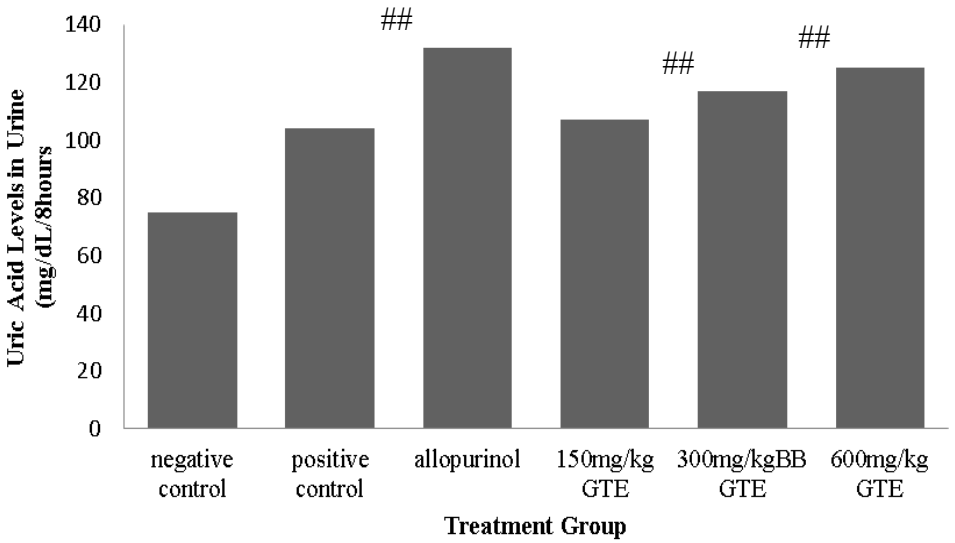

Figure 3. Effects of GTE on UA levels that excreted in the urine of HUA rats. Data were expressed as mean ± SD (n=4). $^{##}p < 0.01$ compared to negative control group.

Levels of UA excreted by urine by negative control group were 74.21 ± 18.48 mg/dL/8 hr. This level was the lowest compared with other groups because it was a group of rats that were not received any treatment, only standard feed and water, therefore the synthesis and excretion of UA were also low. In positive control group, UA levels excreted through urine reached 101.84 ± 8.17 mg/dL/8 h. This level increased compared to in negative control group. High purine diet intake of 14.1 mg per day increased UA synthesis and increased UA excretion as a result. This increased level of 27.13% ($p < 0.01$) was not proportional to the purine intake consumed per day, hence rats of this group had increasing UA levels in the blood, and consequently, rats suffered HUA.

UA clearance depends on the urate transporter, such as URAT1, OAT1, and OAT3 that mediating reabsorption or urate secretion [24]. The kidneys act important role in the homeostatic state of UA because 70% of urate can be filtered freely on the glomerulus, transmitted to the renal tubules and excreted through the kidneys [25]. HUA occurs because of 2 causes, increasing levels of UA in the blood, or decreasing the ability of UA excretion by the kidneys.

Most of the urate transport is reabsorption. Absorption occurs in the small intestine and then excreted through urine. In most mammals, UA is further degraded by the uric oxidase (uricase) to allantoin, which can dissolve easily in the urine and removed from the body. However, mutation process occurs when the initial hominoid evolution, that makes human and apes losing uricase activity, and as a result , UA levels 4-5 times higher than other mammals [26].

In therapy group using allopurinol, urinary acid excretion results in the urine of 131.842 ± 5.177 mg/dL/8 hr with an increase in excretion of 29.46% ($p < 0.01$). Therapy using allopurinol with a dose of 5 mg/kg was more effective in lowering UA levels in the blood and increasing urinary acid excretion in urine than GTE. This is consistent with results of decreased uric acid levels in the blood that shows the greatest decrease, thus, excess UA in the blood can be effectively removed by allopurinol and make the excretion process run well [27], [28].

GTE therapy group using a dose of 150 mg/kg, 300 mg/kg, and 600 mg/kg, UA levels that excreted through urine were 104.210 ± 2.233 mg/dL/8 h, 118.421 ± 7.895 mg/dL/8 h, and 127.105 ± 6.837 mg/dL/8 h, respectively. These suggest that green tea extracts increased

uric acid levels excreted in urine with increases in excretion by 2.23%, 16.28%, and 18.91%. These are in agreement with previous studies [12], [21] which reported that green tea can lower uric acid levels by inhibiting the activity of the XOD enzyme and modulating the urate transporter in the kidneys thus increasing uric acid excretion.

The kidneys metabolize uric acid by a variety of complex methods and processes, such as glomerular filtration, reabsorption, secretion, and secretion, and post-secretion reabsorption in the proximal convoluted tubules. In humans, 90% of filtered urate was reabsorbed in the proximal convoluted tubules. URAT1 is an anion changer in the kidneys and transport the urate through the apical membrane of proximal tubular cells, which is one of the strongest transporters for urate reabsorption [29]. OAT1 is a localized transporter on the basolateral membrane of the proximal convoluted tubules cell. OAT1 plays a role in urate peritubular discharge which is the first step required in urate removal [30]. OAT3 plays a role in the urate removal in the tubular cells and contributes to the urate excretion. In recent years, these three urate transporter were used as therapeutic targets for the treatment of HUA [31]. Previous studies [13] mentioned that HUA affects urinary reabsorption and decreases urinary excretion. Decreased uric acid levels can be carried out through two pathways, suppressing the synthesis of uric acid through the inhibition mechanism of XOD activity in the liver, and accelerated uric acid excretion by modulating the UA gene transporter in kidneys.

CONCLUSION

It can be concluded from this study that GTE significantly decreased uric acid levels in blood serum and increased excretion of uric acid through the kidneys in the form of urine in hyperuricemia rats. GTE significantly inhibited activity of xanthine oxidase enzyme, and increased excretion of uric acid by modulating urate gene transporter. Results of this study proved that green tea extract is a promising alternative treatment for hyperuricemia.

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