Original Article J. Clin. Biochem. Nutr., **45**, 285–291, November 2009

Orange Juice and Hesperetin Supplementation to Hyperuricemic Rats Alter Oxidative Stress Markers and Xanthine Oxidoreductase Activity

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Received 13 February, 2009; Accepted 20 March, 2009

Summary **Our objective was to examine the effect of orange juice and hesperetin on serum total antioxidant capacity (TAC), lipid peroxidation (MDA), uric acid and hepatic xanthine oxidase (XO) and xanthine dehydrogenase (XDH) activity in hyperuricemic rats. Experimentally hyperuricemia in rats was induced by intraperitoneal injection of potassium oxonate (250 mg/kg). Orange juice (5 ml/kg) and hesperetin (5 mg/kg) was given by oral gavage to rats for 2 weeks and biochemical data was measured. Data showed that orange juice supplementation increased serum TAC and decreased MDA concentration ($p \leq 0.05$). Orange juice also inhibited hepatic XO and XDH activity and decreased serum uric acid levels. Hesperetin, which is the main flavanone constituent in orange juice, also exhibited antioxidative and antihyperuricemic properties, but its effect was weaker than that of orange juice. Although the hypouricemic effect of allopurinol (5 mg/kg), as a positive control, was much higher than that of orange juice and hesperetin, it could not significantly change biomarkers of oxidative stress. These features of orange juice and hesperetin make them an attractive candidate for the prophylactic treatment of hyperuricaemia, particularly if these compounds are to be taken on a long-term basis.**

Key Words: **antioxidant, flavonoids, uric acid, xanthine oxidase**

Introduction

Hyperuricemia, characterized by abnormal high levels of uric acid, is a common metabolic disorder with a worldwide distribution [*1*]. It has been considered as an important risk factor for gout and may be associated with oxidative stress conditions [*2*]. That is way the control of uric acid production has been widely considered as a key factor in the prevention and treatment of these diseases [*3*]. Xanthine oxidoreductase (XOR), a molybdenum-containing enzyme, is the key enzyme in the catabolism of purines; that catalyses the oxidative hydroxylation of hypoxanthine to xanthine and xanthine to the terminal catabolite uric acid [*4*]. It is worth noting that XOR occurs in two different forms. Xanthine dehydrogenase (XDH) is the prevalent operative form under physiological conditions and has greater affinity for oxidized nicotinamide adenine dinucleotide (NAD^+) compared to oxygen, as the electron acceptor. Under pathological conditions, however, in parallel to the degradation of ATP into

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adenine and xanthine, an extensive conversion of XDH to xanthine oxidase (XO) takes place. The latter uses molecular oxygen in place of NAD^+ as electron acceptor and leads to the formation of superoxide anion and hydrogen peroxide in parallel with uric acid production. Therefore, XOR can act as a source of reactive oxygen species (ROS) [*5*]. ROS may be involved in the pathogenesis of various degenerative diseases because they induce damage to biological macromolecules such as proteins, fats and DNA [*6*]. That is way the inhibition of XOR activity may be decrease the level of uric acid and ROS production, and results in anti-hyperuricemic and antioxidative effects.

Allopurinol is the sole XOR inhibitor under the clinical application and has served as a dominant uric acid-lowering agent in the past three decades [*7*]. However, some severe adverse effects such as hepatitis, nephropathy and allergic reactions limit the clinical use of allopurinol and it would be highly desired to search for new XOR inhibitors, in particular from natural sources, as alternatives for allopurinol [*2*, *4*, *8*].

Flavonoids are non-nutrient polyphenolic compounds that occur in edible plants and consist of six major classes based on specific structural differences: flavonols, flavones, flavanones, catechins, anthocyanidins, and isoflavones. Flavonoids possess a wide range of biochemical and pharmacological effects and have been recommended as chemopreventive agents or nutritional supplements [*6*, *9*]. The predominant mechanism of their biological actions is thought to result from antioxidant activity, enzyme inhibition, and the capacity to scavenge free radicals [*10*, *11*]. XOR is one of the most important enzymes that are inhibited by some flavonoids [*12*].

Hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone), which occurs as hesperidin (its glycoside form) in nature, belongs to flavanone subclass of flavonoids and is mainly found in citrus fruits such as orange [*13*, *14*]. There is significant interest in the direct antioxidant activities of citrus flavanones hesperidin and hesperetin, due to associations between consumption of flavanone-rich fruits, such as orange and decreased incidence of oxidative stress related disease [*15–17*]. However, indirect antioxidant action, such as the inhibition of ROS-producing enzymes, may be equally relevant to health benefits through a general reduction in oxidative stress *in vivo*. Although hesperetin produced from hesperidin is actually bioactive molecule in the body, only a few *in vitro* and *in vivo* studies have assessed hesperetin, rather than hesperidin.

In this study, the effects of orange juice and hesperetin on the *in vivo* activity of XO and XDH and serum uric acid levels were assessed in oxonate-induced hyperuricemic rats, many for the first time. The possible role of those in reducing biomarkers of oxidative stress was also investigated.

Materials and Methods

Chemicals

Hesperetin, potassium oxonate, xanthine, NAD^+, uric acid, allopurinol, tetraethoxypropane (TEP), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), bicinchoninic acid kit and 6-mercaptopurine were purchased from Sigma-Aldrich Chemical Co. (Steinheim, Germany). All other reagents used were from of analytical grades. Orange (*Citrus sinensis* L.) was purchased from a wholesale market.

Test compound preparation

Orange is commonly peeled and squeezed for its juice. Hesperetin was first dissolved in propylene glycol and then added 0.9% saline (1:20 V:V). Allopurinol used as a positive control, was prepared in 0.9% saline.

Animals

A total of 30 male Wistar rats (body weights: 180–200 g) were obtained from the animal house of Tabriz University of Medical Sciences, Iran. They were fed with a commercial laboratory diet and allowed food and water ad libitum for an acclimatization period of 1 week prior to the experiment. All animals were maintained on a 12 h/12 h light/dark cycle and the temperature and humidity were kept at 18 ± 1°C and 50%, respectively. They were handled according to the recommendation of the local and national ethic committees.

Animal model of hyperuricemia in rats

Experimentally-induced hyperuricemia in rats (due to inhibition of uricase with potassium oxonate) was used to study anti-hyperuricemic and antioxidant effects of test compounds [*18*]. Briefly, 250 mg/kg potassium oxonate (PO) dissolved in 0.9% saline solution was administrated intraperitonially to each animal 1 h before oral administration of test compounds.

Experimental design

This study was conducted from April 2008 to Oct 2008. Rats were randomly divided into five equal groups (6 rats per group). In group 1, the normal group, each animal received only water as vehicle. In group 2, the hyperurecemic control group, uricase inhibitor PO (250 mg/kg) was administrated intraperitoneally. In groups 3, 4 and 5, each animal received the same dose of PO 1 h before administration of 5 ml/kg orange juice, 5 mg/kg hesperetin and 5 mg/kg allopurinol, respectively. The freshly prepared samples were administrated to corresponding groups by oral gavage once a day for 2 weeks.

Sample preparation

At the end of the experiment, rats were anesthetized between 09.00 and 10.00 am. Blood was taken from the

abdominal aorta 1 h after the test compound administration. Serum was obtained by centrifuging blood sample at 6000 g for 10 min. For the high performance liquid chromatography (HPLC) analysis, the serum was filtered using SPARTAN 13/0.45 RC, Watman (USA). The sera were stored at −20°C until use.

After blood sampling, livers were removed, weighed and then rapidly washed in 0.9% cold saline and placed in ice-cold isotonic potassium chloride solution (1.15% KCl w/v) containing 0.1 mM EDTA. The livers were then chopped into 4–5 volumes of 50 mM phosphate buffer (pH 7.4) and homogenized by a homogenizer fitted with a Teflon pestle. The homogenate was then centrifuged at 3000 g for 10 min, the lipid layer was carefully removed, and the resulting supernatant fraction was further centrifuged at 15,000 g for 60 min at 4°C. The supernatant stored at −80°C until use.

Uric acid determination

The serum uric acid levels were analyzed by HPLC method using a system supplied by Waters Associates (Northwich, Cheshire) which consisted of a Waters 515 pump, Waters 717 plus Autosampler, Waters 2487, Dual λ Absorbance Detector. The mobile phase was a mixture of 100 mM KH_2PO_4 (pH 3.5): Methanol (97:3, v/v). Separations were performed on a C-18 column (Perfectsil Target ODS-3 (5 μm), 250 × 4.6 mm) with a C-18 guard column (Perfectsil Target ODS-3 (5 μm), 10 × 4 mm). The effluent was monitored by UV detection at 290 nm at a flow rate of 1.0 ml/min. Standard solutions of uric acid in the range of 10 to 1000 μM/L were prepared in mobile phase. Serum uric acid concentrations were expressed as μM/L. 6-Mercaptopurine (1 mM) was used as the internal standard. The percentage decrease in serum uric acid levels was calculated as [(*C*C–*C*T)/(*C*C–*C*N)] *100, where *C*C, *C*N, and *C*T are the uric acid concentrations (μM/L) of control (PO treated), normal (without PO treatment), and test (PO + test compounds treated) groups.

XO and XDH activities determination

The XO and XDH activities were measured spectrophotometrically by monitoring the production of uric acid from xanthine according to Prajda and Weber's method [*19*]. In the case of XDH, the assay mixture consisted of 50 μM xanthine, 50 μM phosphate buffer (pH 7.4), 200 μM NAD^+, and 100 μl of the enzyme solution. After preincubation at 37°C for 15 min, the reaction was initiated by the addition of the substrate solution. After 30 min, the reaction was terminated by adding 0.5 ml HCl (0.6 M), and the absorbance was measured at 290 nm using a Shimadzu 2550 UV/VIS spectrophotometer which was controlled by the Shimadzu UV Probe personal software package including kinetics software (Shimadzu, Kyoto, Japan). The instrument was connected to a Shimadzu cell temperature control unit. XO activity was measured using a similar method described for XDH with the difference being that molecular oxygen was used in place of NAD^+ as electron acceptor. One unit (U) of activity was defined as 1 nM of uric acid formed per minute at 37°C, pH 7.4.

Protein determination

Protein concentration was determined spectrophotometrically by bicinchoninic acid kit using bovine serum albumin as the standard.

Total antioxidant capacity (TAC) assay

Total serum antioxidant capacity assay was performed using TPTZ (tripyridyltriazine) reagent. This method measures the ability of the antioxidants contained in the sample to reduce ferrictripiridyltriazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}) which absorbs light at 593 nm. TAC of serum was calculated by plotting a standard curve of absorbance against μM/L concentration of Fe^{2+} standard solution [*20*].

Lipid peroxide determination

Lipid peroxide in the serum was measured according to the method of Yoshioka *et al.* [*21*].

Statistical analysis

All the samples and standards were run in duplicate and the results were expressed as means ± standard deviation (SD). The statistical comparison of each experimental group with control group was performed by Independent-sample *t* test using SPSS computer program. The probabilities of 5% or less ($p \leq 0.05$) were considered significant.

Results

As shown in Table 1, potassium oxonate administration can lead to a decrease in serum total antioxidant capacity in hyperuricemic control rats compared to normal rats. As it is also obvious in Table 1, a significant increase ($p \leq 0.05$) in serum total antioxidant capacity after orange juice administration compared to hyperuricemic control group (280 ± 30.16 vs 213 ± 65.92 μM/L) was observed.

As also shown in Table 1, in hyperuricemic control rats, the level of serum malondialdehyde (MDA), as a biomarker of lipid peroxidation, was statistically higher than that of normal rats ($p \leq 0.05$). Orange juice administration was also significantly able to decrease MDA concentration compared to hyperuricemic control rats ($p \leq 0.05$). Hesperetin and Allopurinol treatment could not significantly increase serum total antioxidant capacity or decrease MDA concentration in this study ($p \geq 0.05$).

The effects of the orally administered orange juice and hesperetin on hepatic XO and XDH *activity* are shown

Table 1. Effect of orange juice, hesperetin and allopurinol on serum TAC and MDA

Treatment	TAC (μmol/L)	MDA (μmol/L)
Normal animal		
Vehicle	275 ± 78.67*	3.41 ± 0.57
Hyperuricemic animal		
PO + vehicle	213 ± 65.92$^{\#}$	5.45 ± 0.89$^{\#\#\#}$
PO + orange juice (5 ml/kg)	280 ± 30.16*	4.04 ± 0.86*
PO + hesperetin (5 mg/kg)	271 ± 52.31	4.95 ± 0.71$^{\#\#}$
PO + allopurinol (5 mg/kg)	244 ± 57.27	5.00 ± 0.92$^{\#\#}$

All values are expressed as mean ± SD (n = 6). Independent-sample t test was used for statistical significance assessment. * indicates $p \leq 0.05$ vs hyperuricemic control group, $^{\#}$ indicates $p \leq 0.05$, $^{\#\#}$ indicates $p \leq 0.01$ and $^{\#\#\#}$ indicates $p \leq 0.001$ vs normal control group.

in Table 2. In oxonate-pretreated rats, orange juice and hesperetin resulted in significant inhibition on both hepatic XO and XDH activity. The inhibitory effect of orange juice on XO and XDH activity was 24.49% ($p \leq 0.05$) and 30.89% ($p \leq 0.001$), respectively. Hesperetin treatment caused 23.69% ($p \leq 0.05$) and 20.68% ($p \leq 0.05$) inhibition on XO and XDH activity, respectively. Allopurinol, as a positive control, significantly decreased the mean activity of XO and XDH, respectively by 57.83% ($p \leq 0.001$) and 66.75% ($p \leq 0.001$).

As shown in Fig. 1, intraperitoneal injection of potassium oxonate (250 mg/kg) to control group markedly increased the serum uric acid levels, and reached to 214.36 ± 26.42 (μM/L) at the end of the experiment. The uric acid level in normal rats was only 99.87 ± 17.44 (μM/L).

As also shown in Fig. 1, oral administration of orange juice and hesperetin significantly decreased the serum uric acid levels in hyperuricemic rats. The extents of reduction for orange juice and hesperetin were 38.76% and 24.93%, respectively. The reference drug allopurinol substantially reduced the uric acid level by 124%. It is worth to note that in present study, we found a positive correlation between serum uric acid levels and MDA concentration (Fig. 2).

Discussion

Hyperuricemia is a metabolic disorder which may play an important role in the development of gout and oxidative stress related disease such as cancer and cardiovascular diseases [*2*, *22*]. At the present, allopurinol, a purine analogue, which causes inhibition on XOR, is the only drug with clinical application to lower uric acid production [*7*]. Due to some serious side effects of this drug, many attempts are made to find a safer alternative for allopurinol particularly from natural sources [*2*, *23–25*].

Positive healthy effects of flavonoids and diets rich in these compounds have been reported in several diseases. Flavonoids have been described as health-promoting, disease-preventing dietary supplements because they are extremely safe and associates with low toxicity, making them to be excellent candidates for agents [*26*]. In the present *in vivo* investigation, the effects of orange juice (5 ml/kg) and hesperetin (5 mg/kg) on biomarkers of oxidative stress, serum uric acid levels and hepatic XO and XDH activity in oxonate-induced hyperuricemic rats were assessed, many for the first time.

Following treatment of the hyperuricemic rats with orange juice a significant increase ($p \leq 0.05$) in serum total antioxidant capacity compared to hyperuricemic control was found. Orange juice administration was also significantly able to decrease serum MDA levels, a marker of lipid peroxidation, compared to hyperuricemic control rats ($p \leq 0.05$). Flavonoids presenting in orange juice possess antioxidant properties and suppress destructive oxygen free radicals. An overabundance of free radicals can damage all components of the cell, including proteins, fats and DNA,

Table 2. Effect of orange juice, hesperetin and allopurinol on hepatic XO and XDH activity

Treatment	Activity (U/mg protein)		Inhibition %	
	XO	XDH	XO	XDH
Normal animal				
Vehicle	2.50 ± 0.63	3.80 ± 0.38	—	—
Hyperuricemic animal				
PO + vehicle	2.49 ± 0.52	3.82 ± 0.62	—	—
PO + orange juice (5 ml/kg)	1.88 ± 0.37*,$^{\#}$	2.64 ± 0.20***,$^{\#\#\#}$	24.49	30.89
PO + hesperetin (5 mg/kg)	1.90 ± 0.37*,$^{\#}$	3.03 ± 0.29*,$^{\#\#}$	23.69	20.68
PO + allopurinol (5 mg/kg)	1.05 ± 0.18***,$^{\#\#\#}$	1.27 ± 0.33***,$^{\#\#\#}$	57.83	66.75

All values are expressed as mean ± SD (n = 6). Independent-sample t test was used for statistical significance assessment. * indicates $p \leq 0.05$, and *** indicates $p \leq 0.001$ vs hyperuricemic control group, $^{\#}$ indicates $p \leq 0.05$, $^{\#\#}$ indicates $p \leq 0.01$ and $^{\#\#\#}$ indicates $p \leq 0.001$ vs normal control group.

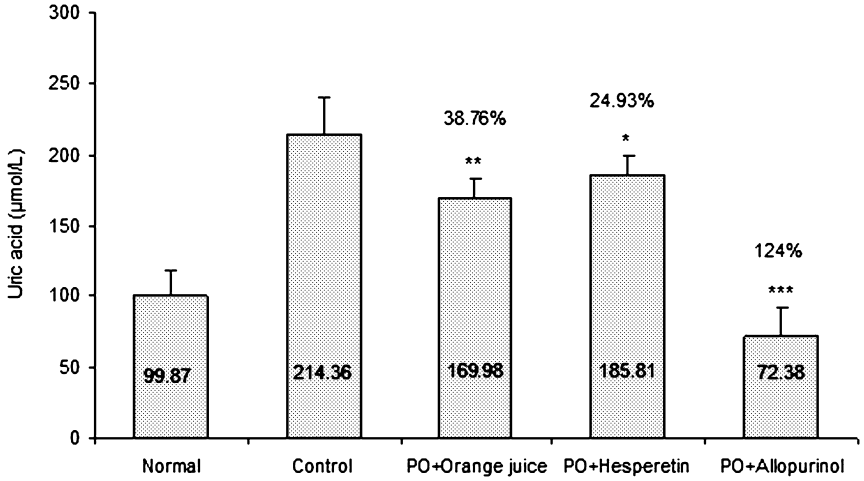

Fig. 1. Effect of orange juice, hesperetin and allopurinol on serum uric acid. All values are expressed as mean ± SD ($n = 6$). Independent-sample t test was used for statistical significance assessment. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$ vs hyperuricemic control group. The number above bars means the percent of uric acid reduction vs hyperuricemic control group.

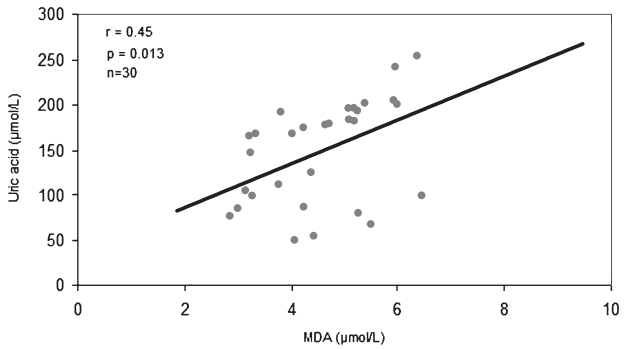

Fig. 2. Linear regression between serum uric acid levels and MDA concentration

contributing to the development of several pathological conditions [*9*, *27*].

In oxonate-pretreated control rats, a significant increase in serum MDA levels and decrease in serum total antioxidant capacity was found. Potassium oxonate, a selectively competitive uricase inhibitor, blocks the effect of hepatic uricase and produces hyperuricemia in rodents [*18*]. Increased uric acid level has been associated with increased production of oxygen free radicals, due to the conversion of XDH to XO that plays a pivotal role in progression of oxidative stress condition [*5*]. In present study, uric acid was positively correlated with MDA. XO is a source of ROS and may explain the link between hyperuricemia and oxidative stress-induced diseases [*2*, *4*, *5*]. Moreover, serum uric acid elevation may promote oxygenation of LDL-C and facilitate lipid peroxidation [*28*]. This capacity of XO makes the enzyme the focus of interest in many recent biochemical and clinical studies [*5*]. From the above discussion we can assume that serum uric acid through lipid peroxidation, might be working towards the etiopathogenesis of ROS-mediated diseases and its serum level may be a deciding factor for progression of the disease.

Our study also showed that orange juice has a higher potential than hesperetin to improve total antioxidant capacity and to reverse MDA. In orange, hesperidin (hesperetin 7-O-beta-rutinoside) is predominant and it is well known that the bioavailability of hesperidin in orange is higher than that of its aglycon [*29*]. Therefore, the higher efficiency of orange juice compared to hesperetin to improve biomarkers of oxidative stress could be attributed to its higher intestinal absorption. Furthermore, it is possible that the other flavonoids except hesperetin -studied in this investigation- to be responsible for the antioxidative effects of orange juice. In this study, allopurinol could not significantly increase total antioxidant capacity or decrease MDA concentration in hyperuricemic rats. However, the inhibition of XO by allopurinol was previously reported to decrease the level of ROS production and reduce the hepatic injury associated with liver transplantation [*25*].

We also showed that the orally administered orange juice (5 ml/kg) and hesperetin (5 mg/g) could significantly decrease the uric acid levels compared to hyperuricemic control rats, but the mean levels of uric acid in these treated animals were yet higher than that of normal values. In this study, we did not investigate the dose response effect of them. It is speculated that the higher dosage of orange juice and hesperetin may be more effective in this regards.

It is important to note that, orange juice was more potent than hesperetin to attenuate and or to reverse hyperuricemia.

As it is mentioned before, the less bioavailability of hesperetin aglycon than that of hesperetin glycosides representing in orange juice may be responsible for this finding. However, the effect of allopurinol, as a reference drug on reducing uric acid level was even more potent and reached serum uric acid levels to a lower level than that of normal levels (72.38 ± 0.32 vs 99.87 ± 0.29 μM/L). On the other hand, this property of orange juice and hesperetin could be considered as an advantage for them. Although the elevated levels of uric acid in the circulation could give rise to gout and possibly other pathological conditions [*22*], the antioxidant action of uric acid, particularly its ability to inhibit DNA damage, is also well documented [*30*]. Thus, excessive lowering of the uric acid level in the circulation beyond that of the normal range might even be counter productive [*8*]. These data also indicated that orange juice and hesperetin might bring fewer side effects than allopurinol in treatment of hyperuricemia and oxidative stress related diseases.

In oxonate-pretreated rats, orange juice and hesperetin resulted in significant inhibition on both hepatic XO and XDH activity. Several *in vitro* studies confirmed the competitive inhibition of flavonoids such as hesperetin and naringenin on XDH and XO activity. Structure–activity relationships of flavonoids have been proposed for the enzyme inhibitory effect of them. Due to the presence of several hydroxyl moieties on the carbon atoms of the basic skeleton, flavonoids may interact with active site of XDH and XO and inhibit them [*1*, *11*]. Therefore the hypouricemic property of orange juice and hesperetin observed in this study could be explained by the inhibitory effect of them on XDH and XO activity; however, there is not apparently a parallel relationship between the extent of the hypouricemic action and the reduction in the enzyme activity. Therefore, the observed hypouricemic effect can not be entirely attributed to this mechanism. Similar results have been reported by others [*8*, *23*, *24*]. According to these studies, the involvement of other possible mechanisms such as enhanced uric acid clearance or actions on other purine metabolizing enzymes can not be ruled out [*8*, *23*]. This could be further supported by the existence of some hypouricemic compounds including natural products that are devoid of XDH and XO inhibitory activity [*8*, *31*].

In conclusion, orange juice and hesperetin prevented oxidative stress by enhancing total antioxidant capacity and decreasing lipid peroxidation. However, the hypouricemic and XOR inhibitory action of orange juice (5 ml/kg) and hesperetin (5 mg/kg) was significant in this study; but this effect was partially weak and they could not reach the uric acid levels to the normal values in hyperuricemic rats. The higher dosage of orange juice and hesperetin should be investigated later. As orange is the most frequently consumed fruit in almost throughout the world and can be used safely long-term, this natural food could be served as a possible alternative for allopurinol, or at least in combination therapy to minimize the side-effects of allopurinol. Further investigations to explore the effect of other components of orange (*Citrus sinensis* L.) and to define their clinical efficacy would be highly desirable. Furthermore, the search for safe anti-hyperuricemic and antioxidative foods and diets must continue.

Acknowledgment

The authors would like to acknowledge the financial support provided by Tehran University of Medical Sciences. The authors are also grateful for the technical help of School of Pharmacy of Tabriz University of Medical Sciences. This work was supported Financially by a grant from Tehran University of Medical Sciences (NO: 85-04-27-4935).

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