Rosehip inhibits xanthine oxidase activity and reduces serum urate levels in a mouse model of hyperuricemia

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Received February 16, 2017; Accepted March 23, 2017

DOI: 10.3892/br.2017.888

Abstract. Rosehip, the fruit of *Rosa canina* L., has traditionally been used to treat urate metabolism disorders; however, its effects on such disorders have not been characterized in detail. Therefore, the present study investigated the effects of hot water, ethanol and ethyl acetate extracts of rosehip on xanthine oxidase (XO) activity *in vitro*. In addition, the serum urate lowering effects of the rosehip hot water extract in a mouse model of hyperuricemia (male ddY mice, which were intraperitoneally injected with potassium oxonate) were investigated. Furthermore, the influence of rosehip hot water extract on CYP3A4 activity, which is the most important drug-metabolizing enzyme from a herb-drug interaction perspective, was investigated. Rosehip extracts of hot water, ethanol and ethyl acetate inhibited XO activity [half maximal inhibitory concentration (IC_{50}) values: 259.6±50.6, 242.5±46.2 and 1,462.8±544.2 μg/ml, respectively]. Furthermore, the administration of 1X rosehip hot water extract significantly reduced the levels of serum urate at 8 h, which was similar when compared with the administration of 1 mg/kg allopurinol. Rosehip hot water extract only marginally affected CYP3A4 activity (IC_{50} value, >1 mg/ml). These findings indicate that rosehip hot water extract may present as a functional food for individuals with a high urate level, and as a therapeutic reagent for hyperuricemic patients.

Introduction

Certain types of food exert beneficial effects on human health; however, the effects are not explained by the nutritional content, such as macronutrients, vitamins and minerals (1). These types of food, termed functional foods, are food-derived products that enhance normal physiological or cognitive functions, or prevent the abnormal function that underlies disease (1). These food types also contribute to the promotion of self-medication, i.e., the use of over-the-counter medicines, including herbal and traditional products, to treat self-recognized illnesses or symptoms (1,2). Of particular interest is the biological activity and safety of natural products, including food, traditional herbs, kampo and their phytochemicals (3-13).

Rosehip is the fruit of rose plants within the genus Rosa, in particular *Rosa canina* L., also termed dog rose. Rosehip has a particularly high vitamin C content compared with other fruits and vegetables (14,15), and contains other vitamins, minerals, sugars, fatty acids and flavonoids (14). Rosehip has traditionally been administered for the treatment of colds, infectious diseases and inflammatory diseases (16). In support of its traditional uses, various studies have reported that rosehip exhibited bioactivity, including antioxidant (17,18), anti-inflammatory (19-22), hepatoprotective (23), anti-diabetic (17) and anti-obesity (24) effects. Therefore, rosehip may be considered a functional food that promotes health. Although rosehip has traditionally been administered for treating uric acid (urate) metabolism disorders (16), its effects have not been characterized in detail.

In humans, urate is the end product of purine metabolism and is delivered from hypoxanthine following double enzyme catalysis by xanthine oxidase (XO) in the liver (25). Serum urate production is regulated by the endogenous (de novo purine synthesis and tissue catabolism under normal circumstances) and exogenous (diet including animal protein) precursor proteins delivered to the liver; whereas its excretion is controlled by the kidneys through renal plasma flow, glomerular filtration and proximal tubular exchange (26,27). The imbalance of its production and excretion induces hyperuricemia, which also develops into gout and kidney stones, and accelerates the progression of renal and cardiovascular

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Abbreviations: CYP, cytochrome P450; XO, xanthine oxidase; β-$NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized form; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; ODS, octadecylsilyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; PO, potassium oxonate

Key words: rosehip extract, xanthine oxidase activity, CYP3A4 activity, hyperuricemic mice, functional foods, self-medication

diseases (28,29). Potassium oxonate (PO)-treated mice generally serve as a model of urate overproduction (hyperuricemia), as its intraperitoneal injection induces the overproduction of urate in mice (30-33). Indeed, a previous study using PO-treated ABCG2-knockout mice reported that a decrease of extra-renal urate excretion was one of the most common causes of hyperuricemia (33).

Numerous studies have demonstrated that the simultaneous administration of a food or beverage that inhibits drug-metabolizing enzymes, such as cytochrome P450 (CYP), and a drug that is metabolized by said enzyme alters blood concentrations, although occasionally with adverse effects (34). A phenomenon similar to this food-drug interaction can be generated between food and drugs. Indeed, it has previously been demonstrated that beverages and food, such as beer, red wine, black and herbal tea, garlic, spices, mace, nutmeg, fruit and fruit juice, tomato juice, and licorice root inhibited enzyme-mediated drug metabolism (9,10,35-39). Although rosehip has been used as a food and as a traditional medicine (16), to the best of our knowledge, there is no evidence of an interaction between rosehip and CYP3A4-metabolizing drugs.

In the present study, the effects of hot water, ethanol and ethyl acetate extracts of rosehip on XO activity were investigated in an *in vitro* assay. In addition, the effect of rosehip hot water extract on urate metabolism was evaluated according to the level of serum urate in hyperuricemia model mice. Furthermore, whether rosehip hot water extract inhibits CYP3A4 activity *in vitro* was investigated.

Materials and methods

Materials. Unless otherwise stated, the various reagents and the Urate C-test Wako kit were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rosehip was obtained from the Tree of Life Co. (Tokyo, Japan). Xanthine oxidase (from buttermilk), nicotinamide adenine dinucleotide phosphate oxidized form (β-$NADP^+$), glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase (G-6-PDH) were purchased from Oriental Yeast, Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) and 11α-hydroxyprogesterone were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Human CYP3A4R Bactosomes (recombinant CYP3A4) were purchased from Cypex Ltd. (Scotland, UK). Mightysil RP-18 GP 250-4.6 (5 μm) [Octadecylsilyl (ODS) column] was purchased from Kanto Chemical, Co., Inc. (Tokyo, Japan).

Preparation of rosehip hot water extract. The extract was prepared from triturated powder (1 g) with 20 ml MilliQ-water at room temperature and decocted at 100˚C for 30 min. The extract was cooled, filtered and evaporated using a freeze dryer, after which the dried sample (yield, 65.2%) was weighed and dissolved at a concentration of 50 mg/ml in MilliQ-water.

Preparation of rosehip ethanol or ethyl acetate extracts. The extracts were prepared from triturated powder (1 g) with 20 ml ethanol or ethyl acetate and agitated at room temperature for 2 h. The extracts, with ethanol or ethyl acetate, were subsequently filtered and evaporated, after which the dried samples were weighed (yields, 5.66 and 0.85%, respectively) and prepared at a concentration of 50 or 100 mg/ml in DMSO, respectively.

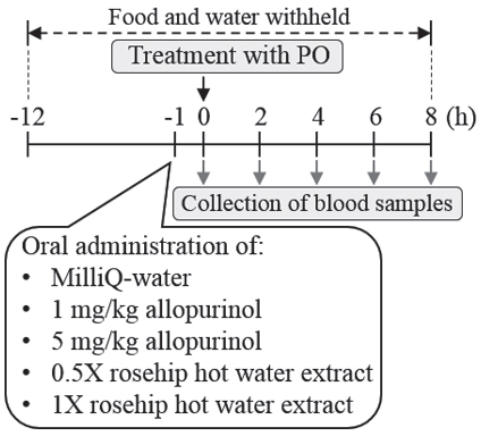

Figure 1. Diagram of the timeline of treatment with rosehip hot water extracts and allopurinol in the hyperuricemia model mice.

Measurement of XO activity in vitro. The measurement of XO activity was performed in accordance with previously published methods with modifications (40). Briefly, 151.5 μl Tris-HCl buffer (pH 7.5; 100 mM), 7.5 μl XO (0.4 U/m) in 50 mM Tris-HCl buffer (pH 7.5), and 9 μl rosehip extracts [concentrations, 10, 20, 50 and 100 mg/ml (final concentrations, 500, 1,000, 2,500 and 5,000 μg/ml, respectively) in the ethyl acetate extract, and 0.5, 1, 5, 10, 50 mg/ml (final concentrations, 25, 50, 250, 500 and 2,500 μg/ml, respectively) in the hot water and ethanol extracts] were mixed in 1.5-ml tubes. These tubes were pre-incubated in a heat-block at 37˚C for 5 min. Subsequently, 12 μl xanthine (80 μM) in 25 mM NaOH was added and incubated at 37˚C for 30 min. The tubes were incubated at 100˚C for 1 min to terminate the reaction. To measure the quantities of urate production, the Urate C-test Wako kit was used according to the manufacturer's protocol. The relative XO activity was expressed as a ratio of the absorbance of each rosehip extract group to that of the corresponding vehicle control group (MilliQ-water or DMSO). The half maximal inhibitory concentration (IC_{50}) values were calculated from the XO activity curves.

Animals. All experiments and the care and handling of the animals were approved by Josai University (Sakado, Japan) Institutional Animal Care and Use Committee. Thirty male ddY mice (age, 5 weeks), obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) were used. The mice were housed in six cages with five mice per cage. They were exposed to a 12-h light/dark cycle and maintained at a constant temperature of 22±2˚C and humidity of 55±10%. The mice were allowed 1 week to adapt to the laboratory environment prior to the experiments and fed laboratory pellet chow (CE-2; CLEA Japan Inc., Tokyo, Japan) and water ad libitum. All mice were euthanized by the intraperitoneal injection of pentobarbital sodium following completion of the experiments.

Treatment of hyperuricemia model mice with rosehip hot water extracts and allopurinol PO, a uricase inhibitor, was used to establish the hyperuricemia model mice, as described previously (30-33). Briefly, pellet chow and water supplies to the ddY mice (age, 6 weeks; body weight, 31.0±0.37 g) were halted the night before the experiment. The mice were randomly divided into five groups (n=7 in the control group; n=5 in the 5 mg/ml allopurinol group; and n=6 in the 1 mg/ml allopurinol, 0.5X

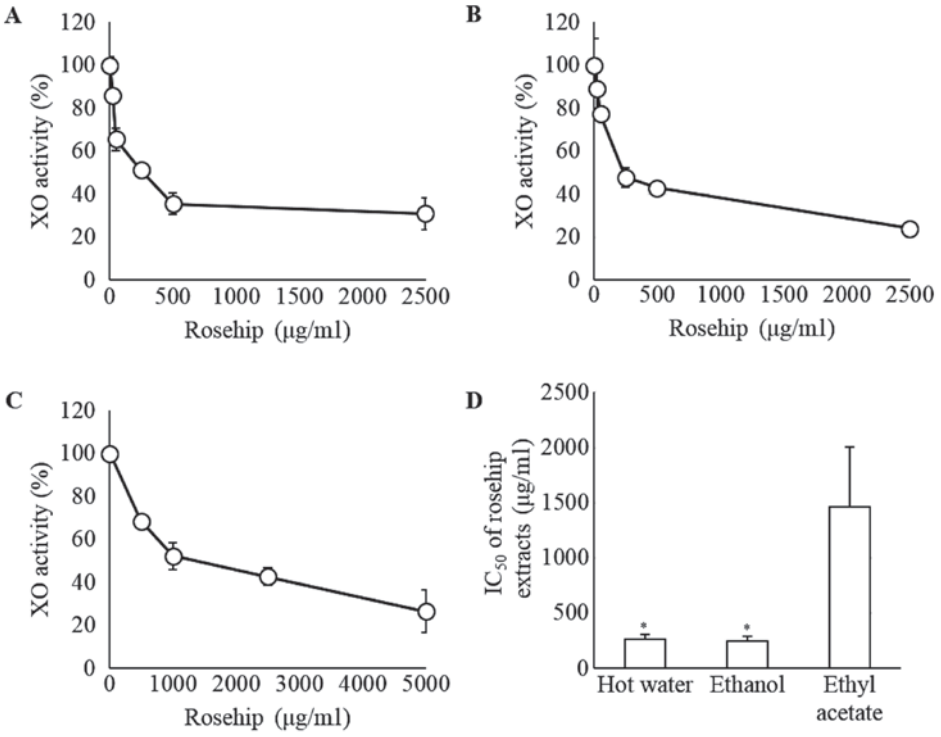

Figure 2. Effect of rosehip extracts on XO activity. (A) Hot water extract, (B) ethanol extract, and (C) ethyl acetate extract of rosehip dose-dependently reduced XO activity. (D) The IC_{50} values of each extract were calculated and compared. Data are reported as means ± standard deviation. *P<0.05 vs. ethyl acetate extraction. XO, xanthine oxidase; IC_{50}, half maximal inhibitory concentration.

or 1X rosehip hot water extract groups. The mice were treated with PO in 0.5% CMC-Na (280 mg/kg, i.p.) 1 h before oral administration of 5 ml/kg MilliQ-water (as the control group), 1 or 5 mg/kg allopurinol, or 5 ml/kg of 0.5X or 1X rosehip hot water extract (~82.5 and ~165 mg/kg, respectively). A diagram of the timeline of the experiment is presented in Fig. 1.

Measurement of serum urate. Blood samples (0.1 ml) were collected into 0.6-ml tubes sequentially at 2, 4, 6 and 8 h via a small incision in the tail vein using a razor blade. The blood samples were incubated for 1 h at room temperature and centrifuged at 800 x g at 4˚C for 15 min. The supernatant (~20 μl) from each blood sample was collected as serum samples and stored at -20˚C until use. The quantity of serum urate in 3.3 μl of each serum sample was measured using the Urate C-test Wako kit, according to the manufacturer's protocol.

Measurement of CYP3A4 activity in vitro. Measurement of CYP3A4 activity was performed according to a previously described method (10). Briefly, 125 μl NADPH regenerating system [2.6 mM β-$NADP^+$, 6.6 mM G-6-P and 6.6 mM $MgCl_2$ in 400 mM potassium phosphate buffer (pH 7.4)], 12.5 μl G-6-PDH (4 U/ml) in 100 mM Tris-HCl buffer (pH 7.4), 2.5 μl recombinant CYP3A4 (1.0 nmol/ml), 3.75 μl rosehip hot water extracts [concentrations, 0.8, 7.4, 22.2, 44.4 and 66.7 mg/ml (final concentrations, 12, 111, 333, 666 and 1,000 μg/ml, respectively)], and 105 μl MilliQ-water were mixed and preincubated at 37˚C for 10 min. The reaction was initiated by the addition of 1.25 μl testosterone (60 mM). The reaction was terminated by the addition of 500 μl 11α-hydroxyprogesterone (10 μM) to the ethyl acetate after 15 min. Following centrifugation (15,000 x g for 5 min), 400 μl supernatant was collected, dried and suspended in 200 μl methanol. Analyses of the metabolite, 6β-hydroxytestosterone, were performed using a high-performance liquid chromatography (HPLC) system (PU-2089, UV-2075 and AS-2057; JASCO Corp., Tokyo, Japan) equipped with an ODS column. The mobile phase consisted of 70% (v/v) methanol and the metabolites were separated at a flow rate of 1.0 ml/min. Quantification of the metabolite was performed by comparing the HPLC peak area at 254 nm to that of 11α-hydroxyprogesterone, the internal standard. The retention times for 6β-hydroxytestosterone, 11α-hydroxyprogesterone and testosterone were ~4.3, ~6.2 and ~8.6 min, respectively. The relative CYP3A4 activity was expressed as the ratio of the HPLC peak area of each rosehip extract group to that of the corresponding vehicle (MilliQ-water) control group.

Statistical analysis. Statistical analysis was performed using the software BellCurve for Excel Ver. 2.1 (Social Survey Research Information Co., Ltd., Tokyo, Japan). After applying a rejection test, data were analyzed using Student's t-test and P<0.05 was considered to indicate a statistically significant difference. Data are reported as means ± standard deviation *in vitro* and as means ± standard error of the mean *in vivo*.

Results

Dose-dependent effects of rosehip extracts on XO activities. To investigate the XO inhibitory effects of rosehip extracts, the XO activity was assessed *in vitro*. Significant decreases in XO activity were observed in a dose-dependent manner for the hot water, ethanol and ethyl acetate extracts (Fig. 2A-C,

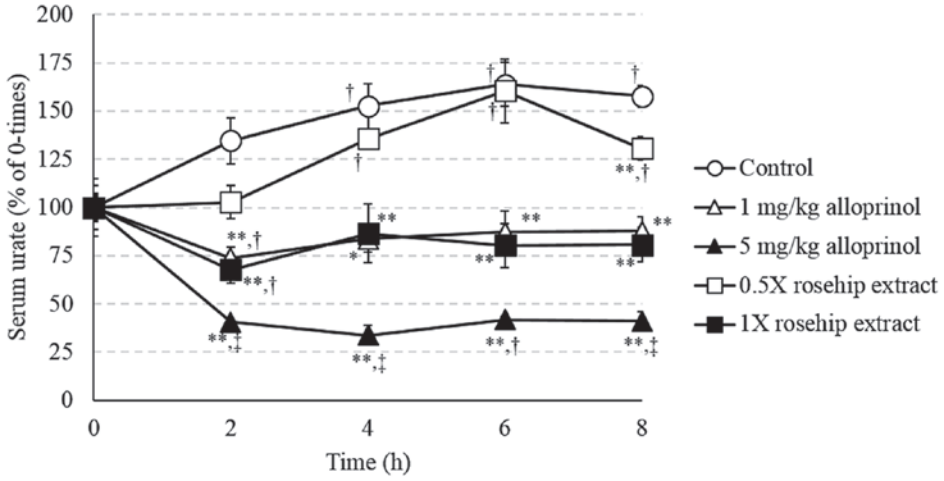

Figure 3. Levels of serum urate in hyperuricemic mice treated with vehicle, 1 mg/ml allopurinol, 5 mg/ml allopurinol, 0.5X rosehip extract, and 1X rosehip extract. Data are presented as means ± standard error of the mean. *P<0.05 and **P<0.01 vs. control group; †P<0.05 and ‡P<0.01 vs. each case at time 0.

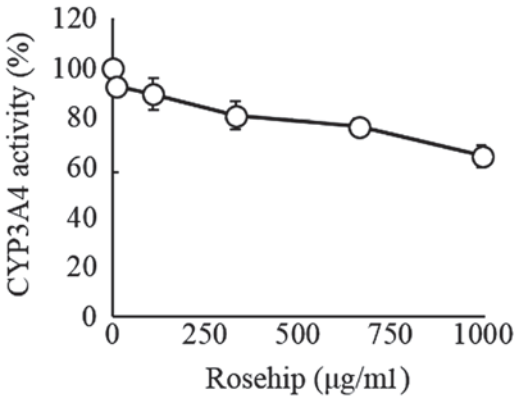

Figure 4. Effect of rosehip extracts on CYP3A4 activity. Data are presented as means ± standard deviation.

respectively). As shown in Fig. 2D, the IC_{50} values were 259.6±50.6, 242.5±46.2 and 1,462.8±544.2 μg/ml, respectively. These values differed significantly between the ethyl acetate extract, and the hot water or ethanol extract groups (P<0.05; Fig. 2D).

Effects of rosehip hot water extract on serum urate levels in hyperuricemic mice. All mice were healthy prior to the experiment and adverse events were not detected during the experiment itself. Treatment with PO, a uricase inhibitor, caused hyperuricemia in the mice, as indicated by a significant increase in serum urate levels at 4, 6, and 8 h (P<0.05; Fig. 3). The levels of serum urate upon administration of 5 mg/kg allopurinol (the 5 mg/kg allopurinol group) were significantly lower when compared with time 0 (P<0.05 at 6 h; P<0.01 at the other time-points) and compared with the levels in the control groups at each time-point (P<0.01). The levels of serum urate upon administration of 1 mg/kg allopurinol (the 1 mg/kg allopurinol group) and 1X rosehip hot water extract (1X rosehip extract group) were significantly lower at 2 h than at time 0 (P<0.05), however, did not change at 4, 6 and 8 h. Furthermore, these levels were significantly lower than the levels in the control group (P<0.01), although there was no difference in these levels at each point between the 1 mg/kg allopurinol group and the 1X rosehip extract group (P>0.05). The levels of serum urate upon administration of 0.5X rosehip hot water extract (the 0.5X rosehip extract group) were significantly higher at 4, 6 and 8 h than at time 0 (P<0.05). Furthermore, these levels were significantly lower when compared with the levels in the control group at 8 h (P<0.01).

Effects of rosehip hot water extract on CYP3A4 activities. The addition of rosehip hot water extract tended to decrease the CYP3A4 activity in a dose-dependent manner, although this decrease was not significant (Fig. 4). Furthermore, at the highest examined concentration (1 mg/ml), rosehip hot water extract inhibited only 40% of CYP3A4 activity (IC_{50} value, >1 mg/ml).

Discussion

In the present study, it was demonstrated that each of the rosehip extracts obtained using hot water, ethanol, and ethyl acetate inhibited XO activity. The different methods of extracting rosehip also generated different strengths of inhibitory effect, namely, the hot water and ethanol extracts exhibited markedly stronger dose-dependent inhibition of XO activity when compared with the ethyl acetate extract. Numerous studies have suggested that the particular polarity of an extraction solvent affects the biological activity of the extract (20,41,42); for example, the free radical scavenging activities associated with extracts of *Galla chinensis*, a traditional Chinese herb, were in the following order: Ethyl acetate (weak polarity) >ether polarity), because an antioxidant phytochemical, tannin, in this extract is soluble in non-polar or weak polar solvents (41). Certain studies have reported that polyphenols, flavonoids and saponins are potent XO inhibitors (43-46), and rosehip contains numerous phenolic phytochemicals (47). Taking these previous results and the observations of the current study into account, the difference in the inhibitory effect of rosehip extracts on XO activity may be associated with the polarity of the extraction solvent, and rosehip containing phenolic phytochemicals, the principal substances, are more likely to

induce an inhibitory effect. Identification of the XO-inhibiting constituents in rosehip is currently underway.

The end product of purine metabolism varies between species. In the majority of mammals, urate is converted to allantoin, a more soluble product that is easily excreted in the urine, by the enzyme uricase (also termed urate oxidase). However, uricase was lost in hominoids during primate evolution (25). To mimic the purine metabolism of humans who lack uricase, mice were exposed to PO, a uricase inhibitor (30-33). In the current study, the oral administration of hot water extract was selected in hyperuricemic mice, as rosehip has typically been used for herbal tea and this extract had a high yield when compared with extracts from other extraction methods. In addition, allopurinol, an XO inhibitor, was administered to serve as a positive control. The administration of 1 and 5 mg/kg allopurinol in hyperuricemic mice significantly and dose-dependently decreased the level of serum urate at each time-point, indicating that each dose of allopurinol effectively inhibited XO activity in the hyperuricemic mice. Furthermore, the administration of 1X rosehip extract reduced the increased serum urate level to the same extent as the administration of 1 mg/kg allopurinol. This is identical to the effect observed in the 1 mg/kg allopurinol group, suggesting that the inhibitory effect of 1X rosehip hot water extract on XO activity virtually mimicked the effect of 1 mg/kg allopurinol.

CYP3A4 is considered to be the most important drug-metabolizing enzyme, as it metabolizes >50% of all clinical drugs (48). It is necessary to consider herb-drug interactions in order to use herbs safely (9,10,35-39). The present study attempted to determine whether rosehip hot water extract inhibited CYP3A4 activity. In the current study, although 12-1,000 μg/ml rosehip hot water extract tended to exhibit dose-dependent inhibition of CYP3A4 activity, it had a very weak effect. Therefore, the risk of interaction between rosehip hot water extract and CYP3A4 substrate drugs appears to be low. Furthermore, other studies reported that ethyl acetate, n-butanol and ethanol extracts from rosehip did not induce toxicity in an acute toxicity test in mice (20), and favorable results were obtained from clinical trials in osteoarthritis (49-51). Thus, the safety of rosehip has been confirmed by traditional experiences of its use, as well as by safety testing.

In conclusion, the current study has demonstrated for the first time, to the best of our knowledge, that hot water, ethanol and ethyl acetate extracts of rosehip inhibited XO activity *in vitro* and that this inhibitory effect was greater for the hot water and ethanol extracts. In addition, the oral administration of rosehip hot water extract decreased the levels of serum urate in hyperuricemic mice, as a result of the inhibition of XO activity. Notably, rosehip hot water extract exerted little effect on CYP3A4 activity. Collectively, these results indicate that rosehip hot water extract is a promising candidate as a functional food for individuals with a high urate level and as a therapeutic reagent of hyperuricemic patients.

Acknowledgements

The present study was supported by the Japan Medical Herb Association (grant no. FY 2016 for KS). This manuscript was edited for English language, grammar, punctuation and spelling by Enago (Mumbai, India).

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