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Original Article

Summer Savory *(Satureja hortensis)* Extract Inhibits Xanthine Oxidase

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Abstract

Xanthine oxidase plays crucial roles in the production of reactive oxygen species (ROS), and uric acid in blood which may lead to gout, one of the oldest known forms of *arthritis amongst humans*. Summer savory (*Satureja hortensis* L.) a medicinal/spice exhibits antioxidant activity, but unknown effects on xanthine oxidase activity. Here, for the first time, we examined the effects of *S. hortensis* extracts on alleviation of oxidative stress and *in vitro* xanthine oxidase activity. Total phenolic compounds, soluble sugar, carotenoid contents of methanolic extracts of roots, leaves, stems, flowers and seeds as well as their antioxidant activity and potential inhibitory effects on xanthine oxidase were analyzed. Leaves, flowers and fruits of the plant exhibited the highest amounts of phenolic contents. The flavonoid content was highest in the leaves of the vegetative stage and decreased when the plant entered the reproductive stage. In contrast, the anthocyanin and sugar contents as well as the antioxidant capacity were in their highest amounts when the plant entered the reproductive stage. Remarkably, the leaves of the flowering stage displayed significant inhibitory effects on xanthine oxidase activity, which may propose a novel potential role for *S. hortensis* leaves in effective treatment of gout.

Key words: *Satureja hortensis*, Xanthine oxidase, Antioxidant activity, Carotenoid content, gout.

Introduction

Gout, one of the oldest known and most common forms of *arthritis in mankind,* is a crystal deposition disease in which crystals of monosodium urate form in joints and other tissues. The primary risk factor for gout is elevated level of a metabolic by-product called uric acid in blood, which is known as hyperuricemia [1]. Although, reduced renal excretion of urate is the underlying hyperuricemic mechanism in a vast majority of gout patients, the uric acid overproduction in the liver plays an important role as well [2]. The final step in uric acid synthesis, which is the oxidation of hypoxanthine to xanthine and subsequently to uric acid, is catalyzed by xanthine oxidase [3]. During this reaction molecular oxygen acts as electron acceptor, producing superoxide radicals and hydrogen peroxides. Consequently, in addition to increasing the levels of uric acid, xanthine oxidase is considered as an important biological source of reactive oxygen species, which are capable of reacting with membrane lipids, nucleic acids, proteins, enzymes and other small molecules, leading to oxidative stress and cellular damage [4]. Therefore, employment of xanthine oxidase inhibitors can be considered as a therapeutic approach in the treatment of hyperuricemia and oxidative stress [5]. Due to the adverse effects of synthetic drugs, attempts to exploit natural products, particularly phytochemicals, have been increased.

Satureja hortensis L. (Summer savory) is an annual, herbaceous plant that belongs to the Lamiaceae family. The aerial parts of *Satureja* L. species have been widely used in different kinds of food as a flavor component and in traditional medication to treat various ailments, such as

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cramps, muscle pains, nausea, indigestion, diarrhea and infectious diseases [6-8]. There exist a few studies about agricultural and pharmaceutical properties of *S. hortensis*, however, its potential effect on xanthine oxidase activity has not yet been thoroughly investigated. Here we examine *in vitro* the potential inhibitory effects of *S. hortensis* extracts on xanthine oxidase activity as well as oxidative stress.

Material and Methods

Plant collection and extraction

S. hortensis were collected from Khaf Township, located in Northeast of Iran (34º 34' 40.48" N, and 60º 08' 29.7" E.). Then different parts of the plants (leaves, stems, roots, flower and seeds) were separatedand air dried in shade for one week. One gram of each sample was extracted in 30 ml of 80% methanol (v/v) for 48 hours at room temperature and filtered through Whatman paper (3M). Methanol was evaporated by a rotary evaporator at 40 ˚C and the remaining solutions were kept at 4 ˚C for further analysis.

Total phenol and flavonoid measurement

Total phenol content was determined by the Folin-Ciocalteu method, as described by Lin and Tang [9] with slight modifications. In brief, 100 µl of *S. hortensis* extract was mixed with 2.8 ml of distilled water and 100 µl of the Folin-Ciocalteu stock reagent (50%) as well as 2 ml of 2% calcium carbonate reagent (v/v). Absorbance of the solution was measured at 720 nm by spectrometer (Shimatzu, UV 160 A). The amount of total phenol in various extracts was calculated from a standard curve of Gallic acid and the results were expressed in milligrams of Gallic acid equivalents per gram dry weight (DW).

Flavonoids were quantified using aluminum chloride reagent ($AlCl_3$) as described by Chang, Yang [10]and expressed as quercetin equivalents. In brief, 500 µl of samples were dissolved in 1500 µl of methanol. 100 µl of 10% (w/v) $AlCl_3$ and 100 µl of 1M potassium acetate were added and incubated for 40 min at room temperature, before measurement of the absorbance at 415 nm by spectrophotometer (Shimatzu, UV 160 A).

Total anthocyanin and carotenoid measurement

Total anthocyanin was determined as described by Mita, Murano [11].Briefly, 0.02 g of dried plant tissue and 4 ml of 1% HCl in methanol (v/v) was pulverized in a porcelain mortar. The mixture was kept at 4 ºC for 24 hours and centrifuged for 10 min at 13000 g. The absorption of the supernatant was measured at 530 and 657 nm against the blank by a spectrophotometer (Shimatzu, UV 160 A). The anthocyanin level for each extract was calculated using the following equation and reported as $mg.gr^{-1}DW$:

$$A=A_{530} - (0.25\times A_{657}) \quad (1)$$

Carotenoids were measured as described by Lichtenthaler [12].0.05 gram of dried plant tissue and 5 ml of acetone were pulverized in a porcelain mortar. 1 gram of sodium sulfate was added to the mixture and filtered. The solution was centrifuged for 10 min at 13000g. Absorption of the supernatant was measured at 470, 645 and 662 nm against the blank by a spectrophotometer (Shimatzu, UV 160 A). Total Carotenoid content for each extract was calculated using the following equations:

$$Chl_a = 11.24\ A_{662} - 2.04\ A_{645} \quad (2)$$

$$Chl_b = 20.13\ A_{645} - 4.19\ A_{662} \quad (3)$$

$$C_t = (1000\ A_{470} - 1.9\ C_a - 63.14\ C_b)/214 \quad (4)$$

(C_a:chlorophyll a content, C_b:chlorophyll b content, Ct:total carotenoid content)

Measurement of Soluble Sugar Content

Total soluble sugar was measured using phenol-sulfuric acid method, as described by Kochert [13]. Briefly, 0.05g of the plants dried powder was extracted with 5ml of 70% ethanol (v/v) at 4 ºC for one week. The extract was centrifuged for 15 min in 10000 g at room temperature and the supernatant was 4 times diluted. 5 ml of concentrated sulfuric acid and 1 ml of 5% phenol were added to 2 ml of the diluted plant extract. The mixture was well stirred and kept at room temperature for 30 min. Absorption was measured at 485nm against the blank by a spectrophotometer (Shimatzu, UV 160 A). Glucose was used as control for drawing the standard curve.

Ferric-Reducing Antioxidant Power Assay (FRAP) and antioxidant activity

The FRAP assay was carried out according to the procedure described by Benzene and Strain [14]. The FRAP reagent was prepared fresh and warmed to 37 ºC prior to use. 150 µl of the sample was added to 4.5 ml of the FRAP reagent and the absorbance of the reaction mixture was recorded at 593 nm after 4 min using a spectrophotometer

(Shimatzu, UV 160 A). The results were expressed as mmol Fe (II) per gram DW of plant material.

Antioxidant activity of the samples was also determined by β-carotene bleaching method (BCB) based on measuring the inhibition of volatile organic compounds and the conjugated dienehydroperoxides arising from linoleic acid oxidation, as described by Kumazawa, Taniguchi [15]. The reaction mixture was incubated for 1hour at 50 °C, after which its absorbance was measured at 470 nm using a spectrophotometer (Shimatzu, UV-160).

The β-carotene bleaching rate (R) was calculated according to equation 5:

$R = \ln (a/b)/ t$ (5)

Where, ln = natural log, a = absorbance at time t (0), b = absorbance at time t (60 min).

The antioxidant activity (AA) was calculated as the percent inhibition relative to the control using equation 6:

$AA = [(R_{control}-R_{sample}) /R_{control}] \times 100$ (6)

Xanthine Oxidase Activity

Xanthine oxidase activity was measured according to Noro, Oda [16] with slight modifications. 405 µl of potassium phosphate buffer (50 mM, pH 7.5) and 150 µl of xanthine oxidase (0.25 units) with or without 20 µl of the plant extract were mixed and incubated at 25 ˚C for 15 min. Then 300 µl of 300 mM xanthine was added to each sample, as the enzyme substrate, and the tubes were incubated at 25 °C for 30 min. Afterwards, 750 µl of hydrochloric acid (1M) was added to terminate the enzyme activity, and absorbance was measured at 295 nm against the blank using a spectrophotometer (Shimatzu, UV-160). The following equation was used to determine the percentage of xanthine oxidase inhibition (%I) in each sample.

$\%I = (1- B/A) \times 100$ (7)

Where, A and B are the absorbance changes in the absence or presence of plant extract, respectively.

Statistical Analyses

The experiments were performed according to a 3-stage nested design and statistically significant differences at the %5 level were determined using the LSD method. All determinations were conducted at least in three replicates and the results were reported as mean ± standard error (SE).The correlation index was used to confirm the effect of parameters on xanthine oxidase inhibition. The analyses were carried out on SAS and Minitab software packages and the graphs were drawn in Excel.

Results

Phenolic, Flavonoid and Anthocyanin contents

Many plant-extracts that contain phenols and flavonoids exhibit efficient antioxidant properties [17]. Total phenol, flavonoid and anthocyanin content of various organs of summer savory were measured at different stages of growth and the data are depicted in Table 1. These data show that the phenolic content of the plant extracts ranged from about 38 mg quercetin equivalent per gram DW (mgQ-Eq.gr^{-1}) in leaves and flowers, to about 13 mgQ-Eq.gr^{-1} in roots of the flowering stage.

The flavonoid content of the plant extracts ranged from about 6 mg Q-Eq.gr^{-1} in leaves to about 0.3 mg Q-Eq.gr^{-1} in roots, whereas, the anthocyanin content of the plant extracts ranged from 1 mg.gr^{-1} in roots to about 40 mg. gr^{-1} in fruits.

Carotenoid and soluble sugar contents

Carotenoids are pigments which play vital roles in the protection of plants against photo-oxidation, via scavenging various reactive oxygen species [18].Total carotenoid and soluble sugar of various organs of the plant were measured at different stages of growth and the data are shown in Table 2 indicating that the lowest amount of carotenoid was observed in roots while its highest amount was detected in leaves of the flowering stage. Similarly, the soluble sugar content had its highest amounts in the extract of flowering stage, while its lowest amount was observed in the root extracts. Statistical analysis revealed a significant positive correlation between the carotenoid and soluble sugar contents (Pearson correlation: 0.840, $P \leq 0.001$).

Antioxidant capacity of the plant extracts

To identify the reducing capacity of the plant extracts, FRAP and β-carotene bleaching (BCB) assays were employed, as described in Materials and Methods, and the data are presented in Table 3. The FRAP analysis revealed that all analyzed samples demonstrated significant reducing power capacity, while extracts obtained from areal parts of the plant in flowering stage exhibited stronger reducing power than that of the other parts (Table 3).

Table 1 Total phenol, flavonoid and anthocyanin content of summer savory.

Developmental Stage		Phenol (mgQ-Eq.gr^{-1})	Flavonoid (mgQ-Eq.gr^{-1})	Anthocyanin (mg.gr^{-1})
Vegetative	Leave	38.07±0.48	6.05±0.22	5.37±0.44
	Stem	21.29±0.25	2.74±0.44	4.47±028
	Root	33.40±0.36	1.41±0.04	1.41±0.04
Flowering	Flower	38.21±4.06	2.42±0.24	13.55±0.11
	Leave	38.07±1.76	3.94±0.43	12.56±0.05
	Stem	25.81±0.98	1.40±0.02	6.22±0.09
	Root	14.34±0.65	0.69±0.01	5.05±0.12
Fruiting	Fruit	35.26±0.66	2.40±0.12	39.21±0.22
	Root	13.34±0.13	0.34±0.05	0.95±0.04

Based to the data obtained from the BCB method, all areal parts of the plant in various developmental stages, as well as the roots in fruiting stage, performed comparable antioxidant activities (Table 3). The results obtained from FRAP analysis correlated well with the total phenol, anthocyanin, carotenoid and soluble sugar contents (Table 4), suggesting that antioxidant activity of *S. hortensis* might be related to its phenolic, carotenoids and soluble sugar contents.

Table 2 Carotenoid and soluble sugar contents of summer savory in several organs at different stages of development.

Developmental Stage		Carotenoid	Soluble sugar
Vegetative	Leave	5.93±0.46	22.71±0.154
	Stem	0.43±0.02	14.19±0.10
	Root	0.16±0.03	3.81±0.14
Flowering	Flower	3.01±0.14	21.30±0.19
	Leave	12.37±0.24	32.60±0.22
	Stem	0.63±0.03	19.36±0.22
	Root	0.78±0.09	6.86±0.19
Fruiting	Fruit	4.57±0.22	15.36±0.19
	Root	0.16±0.01	7.35±0.19

Summer savory extract inhibits xanthine oxidase activity

Several organs of the plant at different developmental stages were examined for their ability to inhibit xanthine oxidase activity, as described in Materials and Methods, and compared to that of the well-known drug Allopurinol. Interestingly, the data presented in Fig 1 show that when plants were in vegetative stage none of the organs performed significant inhibitory effects on the xanthine oxidase activity. In contrast, at flowering stage the leaves and flowers exhibited considerable (30% of Allopurinol) or marginal inhibitory effects on xanthine oxidase activity, respectively.

Table 3 Antioxidant activity of *S. hortensis* extracts obtained from different developmental stages.

Developmental Stage		FRAP (mmol.g^{-1})	BCB
Vegetative	Leave	0.033±0.002	78.405±0.968
	Stem	0.035±0.000	83.9±0.726
	Root	0.025±0.000	20.392±1.200
Flowering	Flower	0.120±0.002	93.88±0.354
	Leave	0.133±0.003	83.719±1.521
	Stem	0.104±0.006	93.1±0.435
	Root	0.038±0.008	30.9±0.669
Fruiting	Fruit	0.088±0.002	97.413±1.440
	Root	0.029±0.001	92.697±0.874

Discussion

Here, we report on careful measurement of phenolic and flavonoids compounds, anthocyanin and carotenoid contents as well as antioxidant and anti-xanthineoxidase activity of summer savory (*S. hortensis*) in order to investigate its potential application in gout treatment.

Our data showed that when the plant entered the reproductive stage, in contrast to its total flavonoid content, its phenolic and total anthocyanin contents increased. It seems that the increased anthocyanin

content in reproductive stage is related to the general role of anthocyanin in attracting pollinators and seed dispersers [19]. In addition, the observed variation of the plant metabolites in different developmental stages may suggest that the time of plant material collection plays an important role in the plantsmedicinal properties.

Compounds with reducing power indeed are electron donors that can reduce many oxidized intermediates of biochemical reactions, therefore, they can act as antioxidants [20]. We employed two methods for measuring the antioxidant activity of summer savory extracts:*(i)* the FRAP assay that determines antioxidant activity based on the extract's ability to reduce ferric (III) to ferrous (II) cations, and *(ii)* the β-carotene bleaching (BCB) method which is based on the ability of the extract to inhibit β-caroteneoxidation.Any correlation between the data revealed by various antioxidant measurements and content of different plant metabolites is neither consistent in all plants nor in different stages of plant growth and development (4, 6,7 and 10). Data presented here suggest that the phenolics, carotenoids and soluble sugar contents of the plant may play important roles in description of summer savory antioxidant activity, which is probably related to its medicinal properties.

Any inhibition of xanthine oxidase activity may result in decreased levels of uric acid, which can be measured by spectrophotometry [16]. Here, we showed that, in contrast to the vegetative stage, leaves, flowers and fruits of the plant were able to inhibit xanthine oxidase activity. A correlations analysis between the inhibitory effects of the plant extracts and their analyzed biochemical properties (Table 4) indicated that the inhibitory effects of the plant extracts correlated well ($P \leq 0.01$) with carotenoid, soluble sugar content, FRAP as well as the BCB tests. These data suggest that in future experiments, the leaves of summer savory at flowering stage, might be thoughtfully explored as a potential novel treatment of gout.

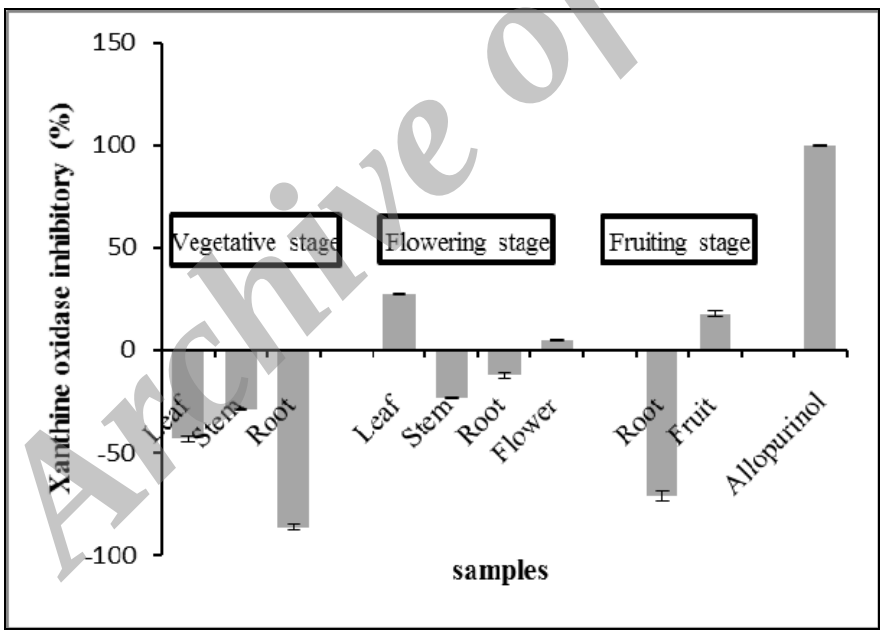

Fig. 1 Percent of xanthine oxidase inhibition by the plant extracts isolated from various stages of plant development.

Table 4 Correlation analysis between plant biochemical properties and percent of xanthine oxidase inhibition.

	Total Phenol content	Total Flavonoid content	Anthocyanin content	Carotenoid content	Soluble Sugar content	Antioxidant activity (FRAP)	Antioxidant activity (BCB)
(FRAP)	0.498**	0.154	0.469*	0.581**	0.744**		
(BCB)	0.179	0.264	0.419*	0.283	0.575**		
IFXO[a]	0.364	0.269	0.677**	0.628**	0.680**	0.806**	0.438*

** ($p < 0.01$).

* ($p < 0.05$).

[a]Inhibitory effect on xanthine oxidase

Conclusion

Here, we showed that *S. hortensis* extracts obtained from different stages of development contain various biochemical properties, hence, contrasting effects on the treatment of gout, via scavenging reactive oxygen species and inhibiting xanthine oxidase activity. The data show a positive correlation between xanthine oxidase inhibitory effects and the phenolic, carotenoid and soluble sugar contents. Extracts obtained from leaves of the flowering stage, which showed considerable *in vitro* inhibitory effects on xanthine oxidase activity (30% of Allopurinol), can be considered as a remedy for gout. Our data suggest that the consumption of various *S. hortensis* tissues, without careful consideration of their active compounds, may have adverse effects on gout treatment. Therefore, a differential screening of the compounds present in various tissues of the plant will probably reveal the nature of the plant's active compounds against gout and lead to new drug developments.

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