British Journal of Pharmaceutical Research
4(23): 2662-2669, 2014
ISSN: 2231-2919

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Correlation of Total Secondary Sulfur Compounds, Total Phenols and Antioxidant Capacity in the Ramsons and Garlic

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OM and LK designed the study. Authors IT, AC, MM and OM carried out the analysis. Authors SI and OM wrote the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2014/13977
Editor(s):
(1) Syed A. A. Rizvi, Department of Pharmaceutical Sciences, College of Pharmacy, Nova Southeastern University, USA.
Reviewers:
(1) Anonymous, National Research Center, Egypt.
(2) Anonymous, Khon Kaen University, Thailand.
(3) Fernanda Carlini Cunha dos Santos, Faculdade de Veterinária, Universidade Federal de Pelotas (UFPel), Pelotas, Rio Grande do Sul, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=858&id=14&aid=7021

Short Research Article

Received 12th September 2014
Accepted 4th November 2014
Published 19th November 2014

ABSTRACT

Aims: This study explores the total quantity of sulfur secondary metabolites in the plant organs of garlic and ramsons, the content of total phenol and their correlation to the antioxidant capacity (AOC). There are different reports about correlation of secondary

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sulfur compounds and phenols from garlic to AOC. The aims of this research was to investigate this correlation of total secondary compounds of sulfur as whole, not some isolated molecules to the AOC, and to reveal the medicinal valuation of ramsons leaf at late spring period.

Methodology: Samples of garlic and ramsons were collected from rural locations in the surroundings of Sarajevo (Bosnia and Herzegovina), in the late May period. The AOC was carried out through the ORAC (Oxygen Radical Absorbance Capacity) method using a generated peroxyl radical ($ROO^{\bullet}$). The quantity of total sulfur was being determinate using the Ion chromatography method (HPIC), in the form of a sulfate ion. The samples were treated in a strong oxidizing media. The total phenols content has been measured by Folin-Ciocalteu method.

Results: AOC is higher in the leaves than in the bulbs during most of the vegetative period. Content of phenol compounds is multiple times higher in the leaves than in the underground parts of the plants. During the first period of vegetation, concentration of sulfur compounds is higher in leaves than in the bulbs.

Conclusion: Secondary sulfur compounds have no influence on the AOC of garlic. Moreover, there was no significant influence of phenolic compounds on the antioxidant capacity. AOC show dependence on the age of plant. Optimal time for consumption of ramsons is the early spring period.

Keywords: Antioxidant capacity; allicin; leaf; bulb.

1. INTRODUCTION

Sulfur compounds dominate in the chemical composition of secondary metabolites of garlic (*Allium sativum* L.) and wild garlic (*Allium ursinum* L.) or ramsons. These compounds are responsible for the aromatic character of these herbs and they have a significant pharmacological effect (Table 1), mainly in prevention of cardiovascular diseases [1-3]. Secondary sulfur compounds, cysteine sulfoxides (alliin, isoalliin, methiin) and γ-glutamil cysteines are most abundant sulfur ingredients of garlic and ramson [4,5]. The well determined ratio of these compounds in the total sulfur content, provides the possibility to use the total sulfur content as a valid parameter of aromatic and medicinal effect of these plants [4].

Garlic is an annual plant. The use of garlic is related to its bulb and can last through nearly the entire year. In temperate climates of the Northern hemisphere two kinds of garlic can be found, fall garlic and spring garlic, which are planted in the aforementioned seasons, and picked in July (fall species) and August (spring species). Proper storing makes it possible to conserve the properties of garlic bulbs and it extends the period of consumption through spring of the following year. Regardless of the storing conditions, the properties fade away with time and they are minimal at the onset of spring. An excellent substitute for garlic in the period of spring can be found in the leaves of ramsons [5]. Ramsons or wild garlic is a perennial plant in the *Alliaceae* family which grows in Europe and Asia, and is similar to garlic in chemical composition and aroma. Its use is related to the fresh leaves and it lasts through the spring season.

Garlic as an antioxidant has received much attention, especially allicin - a derivative of alliin and most important constituent of crushed raw garlic. However little is known about the dynamics and efficacy of allicin as an antioxidant [6]. There are many reports about the *in*

vitro antioxidant activity of allicin and others sulfur compounds present in garlic [7]. The research by Okada et al. [6] supports the thesis about strong antioxidant effect of sulfur compounds derived from garlic, but there are reports that deny antioxidant effect of these substances [8,9]. Strong antioxidant activity of herb phenol compounds is well known. Thus in the case of garlic and related species, several articles have reported high positive correlation between total phenols content (TPC) and antioxidant capacity (AOC) [10,11]. However, there are also reports which considered correlation between AOC and phenols in garlic as very poor and deny influence of phenol ingredients on the AOC of garlic [12]. These opposite reports, related to impact/correlations of two main groups of secondary metabolites of garlic (phenol and sulfur compounds) on the AOC of garlic, were a motive for this research. This study explores the total quantity of sulfur compounds in the fresh leaves and bulbs of ramsons and garlic (fall and spring species) in the late spring period, total phenolics content, the AOC and investigates their correlation.

Table 1. Main constituents of intact and crushed garlic and their pharmacological actions [3]

Active component	Antibiotic	Antifungal	Anticancer	Antiatherosclerosis
Alliin				+
Allicin	+	+	+	+
Ajoene	+	+	+	+
Diallyl disulfide	+		+	+

2. MATERIALS AND METHODS

2.1 Samples

Analyzed samples include bulbs and leaves of ramsons and two garlic species, spring- and fall- garlic. These plants were harvested from rural locations in the surroundings of Sarajevo (Bosnia and Herzegovina), in the late May period. Relative ages of plants, in the period of sampling, were different considering that ramsons disappears with the start of June, while the picking periods for garlic are: late July (fall species) and late August (spring species). Period of planting for fall garlic is autumn, and the beginning of vegetation is in early spring. This coincides with the start of ramsons vegetation. Spring garlic species is planted later and the vegetation begins in the first part of April. These facts indicate a different relative age of plants at the time of sampling. All plants were stored for 2 days at 4℃ prior to analysis.

2.2 Chemicals and Equipment

The following chemicals and instruments were used for determination of the total antioxidant capacity: Trolox® - liposolubile vitamin E analog 97% (Sigma), fluorescein (Fluka), 2,2'-azobis-(2-amidino-propan)-dihydrochloride – AAPH 97% (Sigma), ethanol 95% p.a. (Kemika), deionized water, spectrofluorimeter (LS 55 Luminiscence; Perkin Elmer), balance (AB 104, Metler Toledo), centrifuge (Mikro 22 R, Hettich), thermostat (KP 20-D, Lauda).

The total sulfur analysis was performed using the following chemicals and equipment: nitric acid Suprapur® (Merck), perchloric acid Suprapur® (Merck), magnesium oxide p.a. (Fluka), magnesium carbonate p.a. (Merck), sodium carbonate p.a. (Merck), deionized water, HPIC

system (CDD-10A, LC-10AD, SIL-10Ai, DGU-14A, CTO-10A, SCL-10A; Shimadzu), column (IC SI-90G; Shodex), balance (AB 104; Mettler Toledo).

2.3 Methods

The quantity of total sulfur was analyzed using the Ion chromatography method (HPIC), in the form of a sulfate ion. The samples were treated in a strong oxidizing media consisting of the perchloric and nitric acid mixture [13,14,15].

The AOC was determined by the ORAC (Oxygen Radical Absorbance Capacity) method using a spectrofluorimeter [16]. The ORAC method is usually used to measure the AOC of agricultural and natural products, human and animal serum, pharmaceutical products etc. Last year (2013) ORAC was approved by AOAC as the First Action Method for measuring antioxidants in food. Values are expressed as equivalents of a standard antioxidant, which is usually Trolox® - a vitamin E analog. The radical attack was performed by a peroxyl radical ($ROO^{\bullet}$) which was generated from 2,2'-azobis (2-propanamidine) dihydrochloride (AAPH). Fluorescein was used as a target for the free radical attack [17].

Total phenols content was determined by the Folin-Ciocalteu method, using gallic acid as a standard - gallic acid equivalent (GAE).

2.3.1 Procedure for the antioxidant capacity measurement

Sample preparation: Approximately 1 g of fresh sample was weighed at analytical precision. Sample was macerated and homogenized, and deionized water was added to a total volume of 10 mL (initial solution). A part of the initial solution (about 1 mL) was centrifuged for 10 minutes at 15000 rpm. Only 0.1mL of the supernatant was carefully transferred (micropipette) to a test tube, which was filled up with 0.9 mL of deionized water (working solution). Aliquots of the working solution (0.1 mL) were a part of the reaction mixture of the sample (total volume 2 mL), the preparation of which is explained further in the paragraph AOC quantification.

Preparation of standard: 10 mg of Trolox® was dissolved in 0.5 mL of ethanol and filled up with deionized water to a total volume of 40 mL (initial solution C_1 = 1 µmol/ mL), 20 µL of this solution was transferred to a test tube and supplemented with 980 µL of deionized water (working solution C_2 = 0.02 µmol/mL). An aliquot of the working solution (0.1 mL) constituted a part of the reaction mixture of the standard (total volume 2 mL), the preparation of which is explained further in the paragraph AOC quantification.

AOC quantification: The final mixtures of the standard and samples were prepared in cuvettes of spectrofluorimeter. Each mixture consisted of: aliquots of sample/standard final solution with a volume of 0.10 mL; 0.05 mL fluorescein solution (c = 0.32 µmol/L); 0.2 mL AAPH solution (c = 320 mmol/L); and 1.65 mL of deionized water. The blank was prepared in the same way, but without the standard or sample solution, and the water portion was 1.75 mL. The order of preparation of the reaction mixture: working solutions of sample/standard, fluorescein and water were mixed in cuvettes in the aforementioned amounts and thermostated for about 15 minutes at 37°C. After thermostating, a solution of AAPH (free radical source) was added and immediately the fluorescence intensity was measured. The measurement consisted of measuring points which are repeated every 10 minutes, while maintaining the mixture at 37°C. The measuring point of the fluorescence decline was an average value of fluorescence intensity within the measuring interval of 30

seconds. Reason for using the average value is the fluctuations of the fluorescence intensity. The excitation wavelength was at 485 nm while the emission wavelength was at 520 nm.

2.3.2 Procedure for the total sulfur determination

Sample preparation: Approximately 0.15 g of fresh sample was weighed at analytical precision. The sample was mixed with 2 mL of 65% nitric acid and 1 mL of 70% perchloric acid (nitric acid contained a magnesium oxide at the concentration of 3 g/L). The mixture was stored for 24h and then gently heated on the plate (avoiding boiling) until almost a dry residue, approximately about four hours (the heat power of the plate ensured the temperature of water between 50℃ and 60℃). Cooled residue was dissolved in de-ionized water up to the 50 mL mark. This solution was filtrated and was injected to the HPIC system. The blank was prepared on the same way, but without the sample.

Preparation of standard: Calibration curve was made by a series of standard solutions of magnesium sulfate in the following concentrations of sulfate anion: 2; 4; 8; 16; 48; 64; 80 mg/L.

Measurement: 990 µL of solution (sample/blank/standard) and 10 µL of carbonate buffer solution (0.17 mol/L of $NaHCO_3$ and 0.18 mol/L of Na_2CO_3) were mixed in a vial of HPIC and 10 µL of this solution was injected in a column of HPIC. Chromatographic parameters were as follows - mobile phase: carbonate buffer (0.0017 mol/L of $NaHCO_3$ and 0.0018 mol/L of Na_2CO_3); flow-rate: 1 mL/min; temperature: 40℃; time: 20 minutes; column: strong anion exchange (SAX). At these conditions the retention time for sulfate anion was 10.7 min.

2.4 Statistical Analysis

All results were expressed as mean value ± standard deviation (SD) of triple measurements. All measurements were statistically analyzed using ANOVA, followed by multiple comparisons by Tukey test (SPSS). P-values of less than 0.05 were considered significant.

3. RESULTS AND DISCUSSION

The results presented in Table 2 show that the sulfur level in both species of garlic was higher in leaves than in the bulbs at the period of sampling (late spring). The distinction is higher in spring garlic, where total sulfur content was about 45% higher in leaves than in bulbs. For fall garlic the distinction between bulb and leaf is not significant. Results for ramsons are opposite, bulbs were found to contain about 25% more total sulfur than leaves which is a consequence of the migration of organosulfur compounds to bulbs in the end of vegetative period [4].

Results of antioxidant capacity (Table 2) show that the values, for leaves of spring and fall garlic, are significantly higher than in other samples ($p<0.05$). Antioxidants are compounds that reduce or stop oxidation processes. Fruits and vegetables have a high level of antioxidant capacity, which is mainly caused by the high concentration of phenol compounds [18]. Phenolic compounds are not dominant secondary metabolites in the chemical composition of the garlic family, but those are sulfur compounds. However, in forms of cysteine sulfoxides, more precisely its derivates - thiosulfinates (allicin etc.), sulfur compounds have low influences on the AOC, measured in vitro, as it has been shown in examples of ramsons and garlic. Ramsons and fall-garlic leaves had close values of total

sulfur but very different AOC ($p<0.05$), and for close levels of the total sulfur in fall-garlic organs, there was a great distinction of AOC ($p<0.05$). These *in vitro* obtained results indicate that the impact of garlic sulfur compounds on diseases could not be assigned to theirs antioxidant properties.

Table 2. Total sulfur content (TSC), total phenols content (TPC) and antioxidant capacity (AOC) of ramsons and garlic fresh samples and relative age of the plants in the time of collecting

Sample No.	Plant	Organ	TSC (mg/g)	TPC (mgGAE/g)	AOC (µmolTE/g)	Relative age
1	Ramsons	leaf	0.74±0.05	1.28±0.05	3.16±0.34	0.85
2	Ramsons	bulb	0.93±0.04	0.46±0.02	2.91±0.35	
3	Garlic (spring)	leaf	1.10±0.06	1.49±0.06	13.95±0.95	0.45
4	Garlic (spring)	bulb	0.70±0.04	0.28±0.02	2.22±0.50	
5	Garlic (fall)	leaf	0.66±0.05	1.97±0.05	12.61±0.52	0.60
6	Garlic (fall)	bulb	0.64±0.05	0.48±0.02	2.76±0.42	

The plants were collected in the same period, but their relative ages at the time of sampling were not same. This is due to the variation in their respective periods of vegetation. The results can be considered with respect to the relative age, on a scale from 0 to 1 (zero for the start of vegetation period, value one for the end of vegetation period; values are approximately, vegetative periods of plants are given in 2.1 section). Comparison of the AOC values, total sulfur content and relative age of plants, leads to the conclusion that the most important influence on the AOC value derives from some non-sulfur compounds. This is also concluded by Yin et al. [19], who investigated the correlation of allicin and antioxidant activity - it was not found to be a strong correlation, and antioxidant activity has been ascribed to the other compounds. Using this research, we can suppose that concentration of these compounds correlates with the age of plant and obviously decreases with increasing age of the plant (Table 2).

AOC values of garlic leaves are significantly higher than in the bulbs ($p<0.05$). Leaves of garlic during spring period have a close AOC as most of the fruits [20] and AOC decreases with ageing of plant. This is the result of photosynthetic activity in the leaf that produces more free radical molecules and causes the synthesis of antioxidant compounds as protective agents of plant tissue. During this process leaves are exposed to external factors (UV irradiation, the cold weather, parasites, physical damage etc.) and they need protective substances more than bulbs do. When the plant enters the late phase of vegetation, values of vital compounds begin to decline in the leaf, as they migrate to the bulb or disappear. This is shown in the ramsons example, which had close AOC values of bulb and leaf, at the same time AOC of bulb and leaf of fall- and spring-garlic shows significant differences ($p<0.05$).The strong correlation of the total phenols and AOC values in garlic has been reported in numerous articles [10,11], however there are some reports which denied this correlation [12]. From Table 2 it is evident that a relatively high content of phenols in ramsons leaf do not give expected AOC, also the quantity of phenol compounds found in leaves of fall-garlic was more than 30% higher than in spring-garlic leaf ($p<0.05$), while the values of AOC were reversed - more precisely without significant difference. These facts support the results of Park et al. [12] and extend the lack of significant correlation on the ORAC method, besides previously been reported DPPH method [12], but it does not deny certain impact of phenol compounds on the AOC of garlic.

4. CONCLUSION

The sulfur compounds of crushed garlic, primarily allicin, have a negligible influence on the AOC. The phenol substances could only have a partial influence on the AOC of garlic and ramsons. The key influence on AOC should be found in other groups of compounds or in their interaction. Optimal time for the consumption of ramsons is the early spring period. Leaves of garlic are not commonly used for consumption, but could serve as a good substitute for ramsons in the period of late spring.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=858&id=14&aid=7021