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In vitro Antioxidant Activity of Aged Extracts of some Italian *Allium* Species

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Abstract Antioxidant activity of fresh *Allium sativum* L. (garlic) is well known and is mainly due to unstable and irritating organosulphur compounds. Fresh garlic extracted over a prolonged period (up to 20 months) produces odourless aged garlic extract (AGE) containing stable and water soluble organosulphur compounds that prevent oxidative damage by scavenging free radicals. The aim of this study was to investigate the *in vitro* antioxidant activity of aged (up to 20 months) 15% hydroethanolic extracts of different parts (bulbs, bulblets, flower bulblets, flowers, and leaves) of three *Allium* spontaneous species which are endemic for Italian flora: *Allium neapolitanum* Cyr., *Allium subhirsutum* L., *Allium roseum* L. and to compare it with the *in vitro* antioxidant activity of aged 15% hydroethanolic extracts of bulbs and leaves of garlic. The antioxidant potential of aged extracts of all species has been evaluated using two different spectrophotometric assays: 2,2-diphenylpicrylhydrazyl (DPPH) test and the ferric reducing/antioxidant power (FRAP) assay. Furthermore the polyphenol content was determined. The aged extracts obtained from the leaves showed the best antioxidant activity, followed by flowers and then by bulbs in both used tests, while flower bulblets and bulblets exhibited lower results or no activity. The polyphenol content was generally directly correlated with antioxidant/antiradical activity. This study confirms the data obtained in previous researches, the wild-type species of *Allium* and in particular organs other than bulbs are more active and effective than garlic bulb. Surely leaves of these *Allium* spp. deserve special attention.

Keywords Garlic · *Allium* species · Wild-type · Antioxidant activity · Scavenger · Polyphenol content

Abbreviations

AGE	aged garlic extract
DPPH	2,2-diphenylpicrylhydrazyl
FRAP	ferric reducing/antioxidant power
GAE	gallic acid equivalent
I%	percentage of inhibition
IC_{50}	concentration inhibiting the 50% values of DPPH radical
SE	standard error
R^2	coefficient of determination
TPTZ	2,4,6-tris(2-pyridyl)-s-triazine

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Introduction

Garlic is used as flavour compound in cooking and it contains sulphurs, arginine, oligosaccharides, flavonoids, and selenium, all substances of which may be beneficial to health.

The medicinal effects of garlic (*Allium sativum* L.) in treating a wide variety of human diseases have been known for centuries. Garlic enhances immune functions and shows antibacterial, antifungal, antivirus and antioxidant activities;

it is used in preventing age-related diseases and cardiovascular diseases [1–3]. However, it is not practical to use fresh garlic because it is irritating and generates gastric side effects [4, 5]. Among the many preparations of garlic the aged garlic extract (AGE), obtained from fresh garlic bulb by extraction over a prolonged period (up to 20 months), has been extensively studied and it is known that is less irritating and toxic than fresh garlic [5, 6]. A large number of pharmacological studies have demonstrated that AGE possesses antioxidative [7–11], immunomodulating [12], cardiovascular- [13, 14] and hepato-protective properties [15, 16]. Moreover, AGE has been shown to be superior to raw garlic in terms of its antioxidant properties [9, 17]. Prolonged extraction of fresh garlic with an hydroethanolic solution at room temperature produces an odourless extract modifying unstable molecules responsible of its characteristic flavour and odor. AGE contains stable and water soluble organosulphur compounds, such as S-allylcysteine and S-allylmercaptocysteine, having potent antioxidant properties [8, 9, 18]. In a previous study Nencini et al. yet showed the *in vitro* antioxidant activity of aqueous extracts of different fresh parts of garlic and of some *Allium* spontaneous species, endemic for Italian flora: the best antioxidant power was found in the extracts of spontaneous species [19]. These results suggest further researches on spontaneous *Allium* species, in the past used as food or vegetables [20] and actually not much investigated. In effect these spontaneous *Allium* species resulted more active than *A. sativum* also in two successive studies by Nencini et al. [21, 22]. In the present study, we investigated the *in vitro* antioxidant activity of aged (up to 20 months) 15% hydroethanolic extracts of bulbs, leaves, flowers, bulblets and flower bulblets (these latter obviously only when present) of three *Allium* spontaneous species, previously studied on fresh material [19]: *Allium neapolitanum* Cyr., *Allium roseum* L., *Allium subhirsutum* L., and the *in vitro* antioxidant activity of aged 15% hydroethanolic extracts of bulbs and leaves of garlic (*Allium sativum* L.). The antioxidant potential of aged extracts of all species was evaluated using two different spectrophotometric assays: 2,2-diphenylpicrylhydrazyl (DPPH) test [23], and the ferric reducing/antioxidant power (FRAP) assay [24]. Besides, the total phenolic content was measured in all aged extracts.

Materials and Methods

Plant Collection - *Allium neapolitanum* Cyr. and *Allium roseum* L. were collected nearby Siena (Tuscany, Italy) during their blooming time (late spring), respectively in April and at the end of May; *Allium subhirsutum* L. was collected in May nearby Grosseto (Tuscany) not far from the sea. *Allium sativum* was cultivated in Poggibonsi (Siena). Spontaneous *Allium* species were identified by Prof. G.G. Franchi, University of Siena.

Preparation of Aged Extracts - Peeled bulbs, bulblets, flower bulblets, leaves and flowers were washed, sliced and soaked in 15% aqueous ethanol (500 mg/ml) for up to 20 months at room temperature in dark. The extracts were filtered and evaporated to dryness under nitrogen to obtain aged extracts.

Antioxidant Assays

DPPH Assay - The scavenging activity of the DPPH (2,2-diphenylpicrylhydrazyl) radical was measured as described by Ramadan et al. [23].
Aged extracts were weighed and diluted with 15% aqueous ethanol in a range of concentration 1 to 250 mg/ml. 950 μl of 10^{-4} M DPPH methanol solution was added to 50 μl of aged diluted extracts. Each mixture was then shaken and kept for 30 min in dark, at room temperature. The decrease in absorbance of DPPH solution was evaluated at 515 nm using a spectrophotometer Beckman DU 650. The test was carried out in triplicate. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging effect (I\%)} = [(A_0 - A)/A_0] \times 100$$

where A_0 was the absorbance of DPPH without sample and A was the absorbance of sample with DPPH.

FRAP Test - Total antioxidant power was measured according to the FRAP test [24], which was initially developed to assay plasma antioxidant capacity but can also be used on other fluids. In the FRAP test, reductants (antioxidants) in the sample reduces ferric-tripyridyltriazine complex (Fe^{3+}-TPTZ), in stoichiometric excess, to a blue colored ferrous form (Fe^{2+}), with an increase in absorbance at 593 nm. The working FRAP reagent was prepared *ex tempore* by mixing 10 volumes of 300 mmol/l acetate buffer, pH 3.6, with 1 volume of 10 mmol/l TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) in 40 mmol/l HCl and with 1 volume of 20 mmol/l $FeCl_3 \cdot 6 H_2O$. Then, the working FRAP reagent was warmed to 37 °C and its absorbance against water was read at 593 nm (reagent blank) using a spectrophotometer Beckmann DU 650. Subsequently 30 μl of diluted aged extracts (250 mg/ml in 15% aqueous ethanol) were added to 970 μl of FRAP reagent and the absorbance was monitored for 4 min. Each sample was carried out in triplicate. The ΔA (the absorbance of the samples at the 4th minute minus the absorbance of the blank) is proportional to the combined ferric reducing/antioxidant power (FRAP value) of the antioxidant in the sample. The

results were expressed as μmol of ferric reducing/antioxidant power (the FRAP value)/g of aged extract and were compared with the standard curve prepared using $FeSO_4 \cdot 7\ H_2O$ in a range of concentration from 0.01 to 5 μmol/ml.

Determination of Polyphenol Content - The amount of the total phenolics in the *Allium* aged extracts was determined with Folin-Ciocalteu reagent according to Koski et al. [25] using gallic acid as a standard. 200 μl of aged diluted extracts (250 mg/ml in 15% aqueous ethanol) and 1 ml of Folin-Ciocalteu reagent diluted 30 times with water were placed into tubes. After 3 min, 800 μl of Na_2CO_3 aqueous solution (7.5%) were added and incubated for 30 minutes at room temperature. Each sample was performed in triplicate. The absorbance of 1 ml of each sample was measured at 765 nm using a spectrophotometer Beckmann DU 650.
Quantification was obtained by reporting the absorbance in the calibration curve of gallic acid used as standard phenol (concentration range 0.4-0.025 mg/ml). The results were expressed in milligrams of gallic acid equivalent for gram of aged extract (mg GAE/g of aged extract).

Statistical Analysis - Data are presented as mean ± S.E. of triplicate determinations. Statistical analysis was performed with SPSS (SPSS Inc., Chicago, IL). The Levene's test was used to assess the homogeneity of the variance of the groups. One-way analysis of variance (ANOVA) was utilized to evaluate differences among the groups.
It was mainly used for multiple comparisons the Tukey's test when variances of the groups were homogeneous and the Tamhane's test when they were not. The values of $P<0.05$ were considered significant. Dunnett's test was also used for multiple comparisons of all groups against one, the "*A. sativum* bulbs aged extract".

Results and Discussion

The free radical scavenging activity obtained with DPPH test and expressed as percentage of inhibition (I%) at 12.5 mg/ml, the higher concentration tested, is reported in Table 1. Also, in the same table, we reported the IC_{50} values (concentration inhibiting the 50% of DPPH radical) calculated only for the extracts reaching a percentage of radical inhibition higher than 50 at the tested concentrations. The antioxidant capacity expressed as FRAP value and the polyphenol content in various parts of *Allium* spp. are reported in Table 2. In general, among all the assayed species, the aged extracts obtained from leaves showed the best antioxidant activity, followed by aged extracts of flowers and then by aged extracts of bulbs; on the other hand, the aged extracts of flower bulblets and of bulblets exhibited lower results or no activity at all. In particular, the leaves of *A. roseum* and *A. sativum* displayed the strongest radical scavenging activity (IC_{50}: 6.59±0.26 mg/ml and 7.21±0.39 mg/ml, respectively) and antioxidant activity (FRAP values are 7.05 μmol/g and 7.99 μmol/g respectively). In fact, the statistical analyses carried out inside the species showed for *A. roseum* that leaf aged extract had greater antioxidant activity than flower aged extract in

Table 1 Percentage of inhibition (I%) values (mean ± standard error) at 12.5 mg/ml and IC_{50} values of *Allium* spp. aged extracts obtained with DPPH test

Allium spp. aged extracts		I%	IC_{50} (mg/ml)
A. neapolitanum Cyr.			
	bulbs	n.a.	
	bulblets	n.a.	
	leaves	45.96±1.85	
	flowers	44.17±4.21	
A. roseum L.			
	bulbs	n.a.	
	bulblets	n.a.	
	flower bulblets	n.a.	
	leaves	70.84±5.81	6.59±0.26[d]
	flowers	43.79±3.88[a]	
A. subhirsutum L.			
	bulbs	5.07±0.15[b]	
	leaves	15.57±1.35[b]	
	flowers	56.46±1.19	10.44±0.29
A. sativum L.			
	bulbs	11.36±1.13[c]	
	leaves	66.48±1.85	7.21±0.39[d]

n.a. no active
[a] *vs A. roseum* leaves with $P<0.02$
[b] *vs A. subhirsutum* flowers with $P<0.001$
[c] *vs A. sativum* leaves with $P<0.001$
[d] *vs A. subhirsutum* flowers with $P<0.001$

Table 2 FRAP values of *Allium* spp. aged extracts obtained with FRAP test and polyphenol values of *Allium* spp. aged extracts

Allium spp. aged extracts		FRAP value (μmol/g) mean ± s.e.	Polyphenol levels (mg GAE/g) mean ± s.e.
A. neapolitanum Cyr.			
	bulbs	0.20±0.04[aa]	0.25±0.01[a]
	bulblets	0.62±0.04[aa]	0.35±0.02[a]
	leaves	6.37±1.27	1.36±0.04
	flowers	1.97±0.29[a]	1.13±0.02[a]
A. roseum L.			
	bulbs	0.87±0.32 [bb]	0.22±0.01[bbb]
	bulblets	0.35±0.01[b]	0.62±0.01[bbb]
	flower bulblets	0.13±0.02[b]	0.27±0.01[bbb]
	leaves	7.05±0.39	1.31±0.02
	flowers	1.91±0.12[b]	1.07±0.01[bbb]
A. subhirsutum L.			
	bulbs	0.49±0.09[c]	0.39±0.01[cc]
	leaves	1.94±0.18	1.22±0.01
	flowers	1.25±0.29	1.09±0.02[cc]
A. sativum L.			
	bulbs	0.38±0.27[d]	0.73±0.01[d]
	leaves	7.99±0.54	1.23±0.02

[a] *vs A. neapolitanum* leaves with $P<0.01$; [aa] *vs A. neapolitanum* leaves with $P<0.001$; [b] *vs A. roseum* leaves with $P<0.05$; [bb] *vs A. roseum* leaves with $P<0.01$; [bbb] *vs A. roseum* leaves with $P<0.001$; [c] *vs A. subhirsutum* leaves with $P<0.02$; [cc] *vs A. subhirsutum* leaves with $P<0.001$; [d] *vs A. sativum* leaves with $P<0.001$

DPPH and FRAP tests with $P<0.02$ and $P<0.05$, respectively, and for *A. sativum* that aged extract of leaves had an antioxidant power greater than aged extract of bulbs ($P<0.001$) in both tests used. These data were also confirmed by a statistical analysis performed independently from the species, where the highest antioxidant activity in FRAP test is shown by leaf aged extracts, with $P<0.001$ in respect to all the other aged extracts.

The polyphenol content in aged extracts of *Allium* spp. ranged from 0.22 to 1.36 mg of GAE/g and it was higher in the leaves ($P<0.001$) in respect to flowers and bulbs.

A positive correlation was observed between antioxidant activity, evaluated with DPPH or FRAP tests, and polyphenol content (Figs. 1 and 2, respectively). An exponential function was adopted as model of regression because, unlike the linear model, this function assigns positive values of antioxidant activity for all values of polyphenols. The coefficient of determination (R-squared) was used to evaluate the goodness of fit of exponential model ($R^2=0.468$ for DPPH test and $R^2=0.669$ for FRAP test). In particular, the best results are obtained fitting an exponential regression model (graphs not shown) for each *Allium*

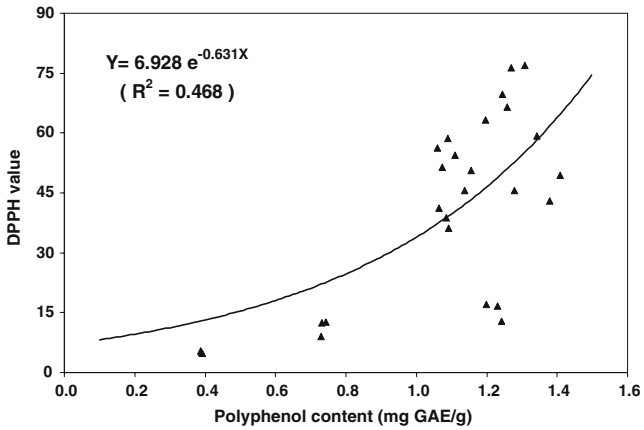

Fig. 1 Correlation between scavenger activity (DPPH) and polyphenol content

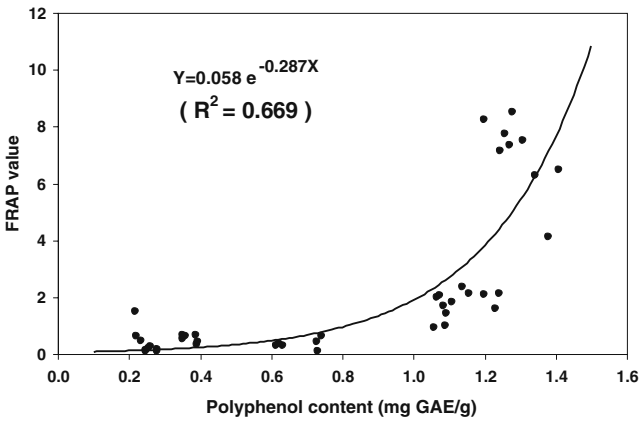

Fig. 2 Correlation between antioxidant activity (FRAP) and polyphenol content

species for FRAP test (with R^2=0.720 for *A. neapolitanum;* with R^2=0.908 for *A. roseum;* with R^2=0.938 for *A. sativum;* with R^2=0.794 for *A. subhirsutum*).

Finally, if we compare data relative to aged garlic extract of bulbs, versus all values obtained from aged extracts of the various parts of *Allium* spp., we observe that aged extracts of leaves and flowers of *A. neapolitanum* and *A. roseum*, and aged extracts of flowers of *A. subhirsutum* showed a greater antioxidant activity (P<0.001) in DPPH test; while in FRAP test aged extracts obtained from the leaves of *A. neapolitanum, A. roseum* and *A. sativum* displayed the highest activity (P<0.001). At last, as it regards the polyphenol content, aged extract of leaves and flowers from *Allium* spp. had the highest GAE content in respect to aged garlic bulb extract (P<0.001).

According to Park et al. [26], the total phenolic content of aged garlic was higher than in fresh *A. sativum* bulb extract. However, extracts of fresh bulbs, bulblets and flower bulblets of spontaneous species have a phenolic content higher than aged extracts [19]. The reduction of phenolic content, during the aging process, could be due to oxidative enzymes (polyphenoloxidases and peroxidases) contained in different amounts in the assayed species. We underline that perhaps not only organosulfur and polyphenolic compounds, but also other substances as alkaloids, formed during the aging process, may contribute to the antioxidant capacity, as reported for AGE by Ichikawa et al. [27]; probably the leaves have a more abundant content of these compounds.

This study confirms the results obtained in previous researches [19, 21, 22]; in fact, these studies yet showed that the wild-type species of *Allium*, and in particular leaves and flowers, are more active and efficient in respect to garlic bulbs.

However, the aged extract of garlic bulbs showed the highest antioxidant capacity in respect to aged extracts of bulbs of spontaneous species. Certainly, leaves of these *Allium* spp. deserve special attention because these organs have the best antioxidant activity and the highest polyphenol content.

In literature there are few studies on Italian wild *Allium* species and none on aged extract of such species or aged extracts of garlic leaves, therefore in this work we report new data on these *Allium* species and innovative and interesting information regarding leaves of garlic that need insights.

In conclusion, this study increases the knowledge on possible uses of garlic and of Italian *Allium* wild species, today almost neglected as food or as source of potential medicinal agents.

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