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Evaluation of antioxidant defense system of Wild *Allium neapolitanum* Cyr. From Italy

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Abstract
This study examines the antioxidant status of fresh organs of *Allium neapolitanum* Cyr. And compare the results with those obtained from *Allium sativum* L.
The antioxidant enzymes activities of catalase, glutathione peroxidase and glutathione reductase and the levels of glutathione, the content of thiosulphinates and ascorbic acid were determined in bulbs, bulblets, leaves and flowers.
Allium neapolitanum exhibits antioxidant ability in all organs investigated: the bulb presents the highest levels of ascorbic acid (P<0.001 *versus* leaves, flowers and bulblets), the leaves have the highest activity of catalase and glutathione peroxidase (P<0.001 *versus* flowers and bulblets), while the reduced glutathione levels and glutathione reductase activity are greater in the flowers (P<0.001 *versus* bulbs). An important result is the high activity of glutathione peroxidase in the leaves of *Allium neapolitanum* greater than the bulb of *Allium sativum* (*P*<0.05). *Allium neapolitanum* possesses properties similar to garlic indicating its possible nutritional and medicinal value.

Keywords*:* antioxidant capacity; ascorbic acid; catalase; glutathione peroxidase; leaves*;* garlic.

1. Introduction
In recent years, numerous studies have reported that several natural foods could prevent the development of several diseases [1]. *Allium* species (Alliaceae) have been used as food plants or as medicinal plants since ancient times; several investigations have previously demonstrated that different *Allium* species may be useful for the prevention of pathological conditions such as atherosclerosis, carcinogenesis, pulmonary damages, liver necrosis, coronary heart disease, obesity, hypercholesterolemia, diabetes type 2, hypertension, cataract, depression and disturbances of the gastrointestinal tract [2-8].

The genus *Allium* is an important dietary sources of antioxidant phytochemical products which are capable of scavenging free radicals: organosulphur compounds, flavonoids, phytoalexin (such as allixin), trace of elements such as germanium Ca, Fe, Zn, Se and volatile oil containing sulphur constituents [9].
Furthermore, biochemical investigations showed that *Allium* spp. contain amino acids, proteins, fat, carbohydrates, vitamins and also different antioxidants and enzymes which contribute to their nutritional and therapeutic characteristics [10].
The beneficial effect of garlic (*Allium sativum* L.) may be explained at least in part by its ability to enhance or maintain the total antioxidant capacity of individuals who include these bulbs in their diet.
Previously, we have demonstrated that some *Allium* species (*Allium neapolitanum* Cyr., *Allium subhirsutum* L., *Allium roseum* L.), endemic of Italian flora, have an antioxidant activity, *in vitro*, similar or better than *A. sativum* [11, 12]. Moreover, we have showed the protective effect of *Allium neapolitanum* Cyr. on liver injury induced by ethanol in rats [13].
The aim of this study was to measure the antioxidant status of fresh organs (bulbs, bulblets, leaves and flowers) of wild *Allium neapolitanum*, previously investigated, and to compare the data with those obtained by bulb and leaves of the extensively studied garlic.
The activities of antioxidant enzymes (catalase, glutathione peroxidase and glutathione reductase), the levels of reduced and oxidized glutathione and also the content of thiosulphinates, of ascorbic acid and soluble proteins were determined. In addition, the polyphenols content was measured again in the organs of both *Allium* species, to confirm the results previously described in Nencini *et al.* [11].

2. Materials and Methods

All reagents were of analytical grade. HPLC solvents were purchased from Merck (Darmstadt, Germany). Standard molecules and chemicals were purchased from Sigma- Aldrich (St. Louis, Missouri). Milli-Q (Millipore-Lab, Bedford, Massachusetts) purified water was used for all analyses.

2.1 *Plant collection*

Two Allium species were investigated: *Allium sativum* L. cultivated in rural farms of Poggibonsi (Siena) and wild *Allium neapolitanum* Cyr. Collected nearby Siena (Tuscany, Italy). The plants were collected respectively in March 2011 and during their blooming time in April 2011.

The identification of a sample of *Allium neapolitanum* was performed by Dr. Ilaria Bonini and a voucher specimen (SIENA-N°7745) was deposited in the Herbarium Universitatis Sinensis [SIENA], Department of Life Sciences, University of Siena.

2.2 *Tested material*

Fresh organs (bulbs, bulblets, leaves and flowers) were cleaned to remove impurities; after cut and homogenized (500 mg/ml) in MilliQ purified water or different buffer (as detailed below for each parameter) using a homogenizator (T25 Basic Ultra-Turrax, Janke & Kunlel, IKA- Laboratechnik, Staufen, Germany).

The obtained homogenates were treated with opportune reagents and centrifuged to different gravities according to the several methods described below. Subsequently, all supernatants were stored at -80°C before starting their analytical procedures.

3. Analytical procedures

3.1 *Thiosulphinates*

The organs of plants were homogenized in ice-cold phosphate buffer (0.125 M, pH 7.4) and were centrifuged at low speed (2,000 g) for 10 min at 0 °C. Total thiosulphinates (Thio) were determined in supernatants with a spectrophotometric procedure [14].

One molecule of allicin reacts rapidly with two molecules of cysteine to form two molecules of S-allil mercapto-cysteine. The decrease in cysteine concentrations is measured by reaction with DTNB 5,5′-dithiobis(2-nitrobenzoic acid) at 412 nm. The results are expressed as mM/g of fresh weight.

3.2 *Glutathione oxidized and reduced*

The organs of plants were homogenized in ice-cold phosphate buffer (0.125 M, pH 7.4) containing 1 mM EDTA and then were added to an equal volume of 10% metaphosphoric acid. Samples were centrifuged at low speed (2,000 g) for 10 min at 0 °C. Total glutathione (GSH) and oxidized glutathione (GSSG) were quantified in supernatant using a microassay procedure [15]. Results were expressed in nmol of GSH or GSSG / mg protein.

3.3 *Ascorbic acid*

The organs of plants were homogenized (1:3; w/v) in phosphate buffer (pH 7.4) at 0 °C. The samples were added to an equal volume of 10% metaphosphoric acid and immediately centrifuged at 2,000 g and 0°C for 10 min. The ascorbic acid (AA) was measured by HPLC method. The supernatants were filtered (Anotop 0.2 Am, Merck, Darmstadt, Germany), and 20 µl aliquots were injected into the high-performance liquid chromatography column (Reverse phase C18) with UV detector at 262 nm [16]. Results were expressed as nmoles/g of fresh weight.

3.4 *Glutathione reductase activity*

The organs of plant were homogenized in cold 0.25M sucrose in 0.1 M phosphate buffer, pH 7.4. The homogenates were centrifuged at 40,000 g for 20 min at 4 °C, and the supernatants were used for the glutathione reductase (GR) assay [17]. The method is based on the increase in absorbance at 415 nm when 5,5'-dithiobis(2-nitrobenzoic acid) is reduced by GSH generated from an excess of GSSG. Samples were prepared in 96-well plates, and absorbance was measured every 30 seconds for 3 minutes with a programmable microplate reader. The rate of increase in absorbance is directly proportional to the amount of GR in the sample. The results were expressed as U/mg protein.

3.5 *Glutathione peroxidase assay*:

The organs of plants were homogenized using the same procedure as described for glutathione reductase. The glutathione peroxidase activity (GPx) is quantitated by measuring the change in absorbance at 340 nm caused by the oxidation of NADPH [18]. One unit of GPx activity is defined as the amount of enzyme that oxidizes 1µmol of NADPH at 37 °C per minute. Enzyme activity was expressed in units of GPx per mg of protein.

3.6 *Catalase activity*

The organs of plants were homogenized in ice-cold phosphate buffer (0.125 M, pH 7.4) containing 1mM EDTA and then centrifuged at 4,000 g for 15 min at 4 °C. To determine catalase (CAT) a microassay procedure was used [19].

This method is based on the reaction of the CAT with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde production was measured spectrophotometrically at 540 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as a chromogen. One unit of catalase activity is defined as the amount of enzyme that will cause the formation of 1 nmol of formaldehyde per minute at 25°C. Results were expressed as U/mg of protein.

3.7 *Proteins assay:* protein concentrations were determined by the method of Lowry *et al*. and the calibration curves were prepared with dry bovine serum albumin [20].

4. Statistical analysis

Statistical analysis was performed with the SPSS version 17 software package (SPSS Inc, Chicago, IL, USA). Results are expressed as the mean ± standard error of triplicate determinations.

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 HSD test when variances of the groups were homogeneous and the Games-Howell test when they were not. The values of $P<0.05$ were considered significant.

The correlations among variables were established using Spearman's rho correlation coefficients.

5. Results

The activities of antioxidant enzymes (catalase, glutathione peroxidase and glutathione reductase), and the relative levels of reduced and oxidized glutathione, thiosulphinates, and ascorbic acid are reported in Table 1 and Table 2. The results are expressed as a percentage (±standard error) of values obtained in the garlic bulb.

Table 1: Antioxidant enzymes activity in different fresh organs (bulbs, bulblets, leaves and flowers) of *Allium sativum* and *Allium neapolitanum*. The values are expressed as a percentage (±standard error) compared to garlic's bulb value, assumed as 100%

Enzymes activity		CAT	GPx	GR
Allium sativum	**Bulbs**	100	100	100
	Leaves	11.02+0.39 aaa	532.99±103.10 a	278.77±12.63 aaa
Allium neapolitanum	**Bulbs**	14.93+0.06 a	156.58+8.29	16.30+0.28 a, bb
	Leaves	64.20±6.38 ccc	742.21±13.46 a, ccc	85.57±0.58 b, ccc
	Flowers	35.65 ±2.47 aa, cc, dd	9.10±0.62 ccc, ddd	95.32±1.44 b, ccc, ddd
	Bulblets	15.71±0.73 a, ddd, ee	231.44±9.56 ccc, ddd, ee	32.73±0.84 a, b, ccc, ddd, eee

[a] vs *A. sativum* bulbs with $p < 0.05$; [aa] vs *A. sativum* bulbs with $p < 0.01$; [aaa] vs *A. sativum* bulbs with $p < 0.001$
[b] vs *A. sativum* leaves with $p < 0.05$; [bb] vs *A. sativum* leaves with $p < 0.01$; [bbb] vs *A. sativum* leaves with $p < 0.001$
[c] vs A. *neapolitanum* bulbs with $p < 0.05$; [cc] vs A. *neapolitanum* bulbs with $p < 0.01$; [ccc] vs A. *neapolitanum* bulbs with $p < 0.001$
[d] vs A. *neapolitanum* leaves with $p < 0.05$; [dd] vs A. *neapolitanum* leaves with $p < 0.01$; [ddd] vs A. *neapolitanum* leaves with $p < 0.001$
[e] vs A. *neapolitanum* flowers with $p < 0.05$; [ee] vs A. *neapolitanum* flowers with $p < 0.01$; [eee] vs A. *neapolitanum* flowers with $p < 0.001$

Table 2: Non-enzymatic antioxidants in different fresh organs (bulbs, bulblets, leaves and flowers) of *Allium sativum* and *Allium neapolitanum*. The values are expressed as a percentage (± standard error) compared to garlic's bulb value, assumed as 100%

Antioxidant content		GSH	GSSG	AA	Thio
Allium sativum	**Bulbs**	100	100	100	100
	Leaves	15.66±0.60 aaa	34.21±0.98 aa	210.46±2.68 aaa	72.11±8.12
Allium neapolitanum	**Bulbs**	0.61±0.02 bb	2.0±0.04 bb	75.29±0.48 aaa, bb	279.48±104.12
	Leaves	2.23±0.24bb, c	22.59±8.03	56.43±0.27 aa, bb, ccc	21.96±8.20 a
	Flowers	4.58±0.44 bb, ccc, dd	6.18±0.71 bbb	69.83±0.53 aa, bb, ccc, ddd	247.47±49.10
	Bulblets	1.18±0.34 bb, eee	2.14±0.08 bb	31.43±0.48 aaa, bbb, ccc, ddd, eee	383.88±49.04 d

[a] vs *A. sativum* bulbs with $p < 0.05$; [aa] vs *A. sativum* bulbs with $p < 0.01$; [aaa] vs *A. sativum* bulbs with $p < 0.001$
[b] vs *A. sativum* leaves with $p < 0.05$; [bb] vs *A. sativum* leaves with $p < 0.01$; [bbb] vs *A. sativum* leaves with $p < 0.001$
[c] vs A. *neapolitanum* bulbs with $p < 0.05$; [cc] vs A. *neapolitanum* bulbs with $p < 0.01$; [ccc] vs A. *neapolitanum* bulbs with $p < 0.001$
[d] vs A. *neapolitanum* leaves with $p < 0.05$; [dd] vs A. *neapolitanum* leaves with $p < 0.01$; [ddd] vs A. *neapolitanum* leaves with $p < 0.001$
[e] vs A. *neapolitanum* flowers with $p < 0.05$; [ee] vs A. *neapolitanum* flowers with $p < 0.01$; [eee] vs A. *neapolitanum* flowers with $p < 0.001$

The data showed that the investigated wild species possess effective antioxidant properties similar to garlic, however the levels of the enzymatic and non-enzymatic parameters are different in the various organs of the plant.

The leaves of *Allium neapolitanum* showed a high enzymatic activity: the CAT activity in the leaves is greater than the flowers ($P< 0.01$), bulbs and bulblets ($P<0.001$); the GPx activity in the leaves is greater than the bulbs, flowers and bulblets ($P<0.001$); finally the GR activity in the leaves is greater than bulbs and bulblets ($P<0.001$), although for highest GR activity is expressed in flowers ($P<0.001$ *vs* leaves). The enzymatic activities of GPx and GR in leaves of *A. sativum* are higher than in bulbs ($P<0.05$ and $P<0.001$ respectively); on the contrary the CAT activity in leaves of *A. sativum* is lower than that in bulbs ($P< 0.001$).

The highest activity of catalase is observed in the bulb of garlic when to compared with bulb, flowers and bulblettes of *A. neapolitanum* ($P< 0.05$, $P< 0.01$; $P< 0.05$ respectively), however, no statistical significance is observed when garlic bulb was compared to leaves of *A. neapolitanum*.

It is remarkable that the high activity for glutathione peroxidase observed in the leaves of *A. neapolitanum* is greater than bulbs of *A. sativum* ($P<0.05$).

The bulb of *A. neapolitanum* showed the highest level of AA when compared to leaves, flowers and bulblets ($P<0.001$); while the highest GSH levels were observed in flowers when compared to the bulb and bulblets ($P<0.001$) and leaves ($P<0.01$). The Thio levels were high in the bulblets when compared to leaves ($P<0.05$). The garlic bulb had higher levels of glutathione reduced and oxidized when compared to leaves ($P<0.001$ and $P<0.01$ respectively). On the contrary, the AA levels was higher in leaves than in bulbs ($P<0.001$). When the *A. neapolitanum* is compared to garlic we observed that the bulbs of *A. sativum* present the highest levels of AA compared to various other organs of *A. neapolitanum* ($P<0.01$ *vs* bulbs, leaves and flowers and $P<0.001$ *vs* bulblets). Also the Thio levels were highest in garlic bulb compared to leaves of *A. neapolitanum* ($P<0.05$).

6. Discussion

In this study, we observed a high antioxidant status in the various organs, indeed the high antioxidant capacities play a fundamental role in properties of *Allium ssp*.

In literature, studies on the enzymatic and non enzymatic antioxidant defence system of fresh organs of *Allium neapolitanum* compared to the antioxidant status of *Allium sativum* are not found.

The high levels of AA and the elevate activity of GPx and GR in the garlic leaves, greater compared to the bulbs, provide new information for *A. sativum,* particularly for the leaves that are little studied and poorly used as food.

Rojas-Graü and collaborators reported that the ascorbate shows inhibitive effect on the polyphenol oxidase, which oxidizes diphenols to quinones [21]. We found high levels of ascorbic acid, which protect the polyphenols by reactions of oxidation. Therefore the high AA levels contribute to maintain

high levels of polyphenols in *Allium* ssp [11]. In addition ascorbate is an important growth regulator, is related with cell division in plants and it may act to regulate cellular process including cell wall metabolism [22].

The glutathione, predominantly in the reduced form, has many roles similar to those found in mammalian cells. The bulbs of *A. sativum* had the highest levels of GSH and of GSSG, confirming that the garlic is rich in sulfur compounds that are also the source of many of its health-promoting effects [23]. The high levels of Thio in the leaves of *A. neapolitanum* suggest also the possible use as a source of sulphur species. Indeed, the presence of many reactive sulphur species are related to interesting biological activity *in vivo*, as antibiotic, fungicidal, pesticidal or anticancer activity [6, 24].

Our data show that also the flowers are rich of antioxidants substances and thus their use in the popular medicine could be made as well as for the flowers of *A. leucanthum* C. Koch [25]. A recent study, investigate on the possibilities of culinary use of flowers of *A. neapolitanum* [26].

The antioxidant properties of leaves garlic's are confirmed by the activity of GPx higher than bulbs. A remarkable result is represented by high activity of GPx enzyme in leaves of *A. neapolitanum* compared to bulbs and leaves of *A. sativum*. On the other hand a high activity of GPx in leaves is reported by Stajner *et al.* for others *Allium* species [27].

In the garlic's leaves we found an activity of glutathione reductase higher than bulbs; this event has no effect on the glutathione content and on the GSH/GSSG ratio of the leaves [28]. The GR plays a key role in the response to oxidative stress by maintaining especially functions as an antioxidant that scavenges reactive oxygen species such as hydrogen peroxide and superoxide.

In addition, we have confirmed results of our previous study which concluded that all organs of these *Allium* species have a high content of polyphenols and a high radical-scavenging activity (evaluated using two different assays: the Ferric reducing-antioxidant power = FRAP method and the 1,1-diphenyl-2-picrylhydrazyl = DPPH test) [11].

Furthermore, in both *Allium* species, a significant correlation (Figure 1) between total polyphenols content and Thio levels was estimated (Spearman rho coefficient 0.603; $P<0.01$). Finally, a negative correlation between polyphenols content and oxidized glutathione and catalase activity was found (Spearman rho coefficient -0.554; $P < 0.02$ and Spearman rho coefficient -0.562; $P < 0.02$ respectively).

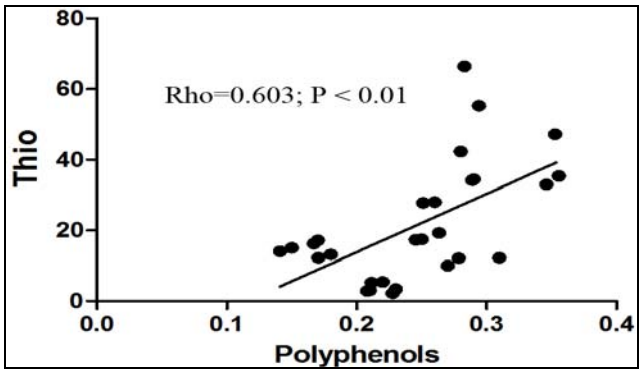

Fig 1: Relation between total thiosulphinates (Thio) and polyphenols content of various organs of two *Allium* species (Rho = Spearman correlation coefficient).

7. Conclusion

In *Allium* species investigated, the polyphenols provide antioxidant properties, but the antioxidant capacity is also related to the antioxidant status and to the thiosulfinates content.

The negative correlation between polyphenols content and GSSG levels or CAT activity shows that the organs of *Allium* species are protected against oxidative stress by the antioxidant constituents (as the high polyphenols content), certainly where the polyphenol content is higher, the CAT activity is lower and the GSSG levels to.

The epidemiological evidence that consumption of fruit and vegetables decreases the frequency of the most important diseases in the developed countries, had increased the importance on the identification of plant dietary compounds or spices as a source of natural antioxidants.

Allium neapolitanum exhibits antioxidant ability in all investigated plant organs, especially in the leaves, these are the organ with higher enzyme activity compared to the others organs of plant. Moreover, the content of phenolic compounds is an important factor in determining the antioxidant activity of this plant.

Our results indicate that increased consumption of the investigated plant species could provide health benefit or could prevent disease related to oxidative stress. In addition for their properties, they could be used as new ingredients to improve the diversity in modern diet or as a vegetable and also in the pharmaceutical and cosmetic industries for manufacturing products with antioxidant activity [29].

Recently, the interest for the wild *Allium* species is grown and these data provide new information for the *A. neapolitanum,* a wild-growing *Allium* species, less studied and the references to nutritional and medical applications are rarely given [30].

Further pharmacological investigations will aid the development of natural, healthy foods and anti-cancer possible agents that may prevent or combat several diseases.

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9. References

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