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IN VITRO ANTIOXIDANT, TOTAL PHENOLIC AND FLAVONOID CONTENTS OF SIX *ALLIUM* SPECIES GROWING IN EGYPT

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ABSTRACT

This study was designated to determine the total phenolic and flavonoid contents as well as evaluation the *in vitro* antioxidant activity of the defatted methanolic extracts of six *Allium* species growing in Egypt. Three of them are subspecies of *Allium cepa* L. (ssp. red onion, ssp. white onion and ssp. green onion), the other three species are *Allium sativum* L. (garlic), *Allium porrum* L. (leek) and *Allium kurrat* L. (kurrat baladi). The results exhibited that *A. cepa* (ssp. red onion) and *A. porrum* have the highest phenolic contents. On the other hand, *in vitro* antioxidant activity using three methods, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical, phosphomolybdate and reducing power assays revealed that *A. cepa* (ssp. red onion) and *A. porrum* have high antioxidant activities. Moreover, there was positive correlation between the antioxidant activity and total phenolic contents of the tested *Allium* species. Therefore, the two plant species *A. cepa* (ssp. red onion) and *A. porrum* were submitted to fractionation process using chloroform, ethyl acetate and n-butanol. The results showed that the ethyl acetate fractions of the two plants have high phenolic and flavonoid contents as well as have high antioxidant activities. Also, the preliminary phytochemical screening of the tested *Allium* species showed that *A. cepa* (ssp. red onion) and *A. porrum* have high quantities of flavonoids, steroids, terpenoids and saponins.

Keywords: *Allium* species, plant extract, antioxidants, phenolics, flavonoids

INTRODUCTION

Phenolic compounds exhibiting antioxidant properties. Therefore, they play an important role in protecting cells and organs from oxidative damage **(Osawa, 1999).** These phenolic compounds have one or more phenolic groups for hydrogen proton donors and neutralize free radicals **(Osamuyimen *et al.*, 2011).** Antioxidants protect the human body from free radicals and reactive oxygen species (ROS) effects **(Gülcin, 2006).** Reactive oxygen species (ROS) such as superoxide anion ($O_2^{-\bullet}$) ,hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$) and hydrogen peroxide (H_2O_2) may attack the biological macromolecules which leads to protein, lipid or DNA damage and causes many diseases such as aging, cancer, coronary heart diseases and Alzheimer's disease (**Reşat *et al.*, 2013**).
The *Allium (*family *Alliaceae*) has over 700 members each of them has special tastes, forms and colors. They have several pharmacological and biological properties such as anti-bacterial, anti-fungal and anti-inflammatory activities. During the last 20 years, *Allium* spices have been among the most studied vegetables and aroused great interest **(Benkeblia, 2005; Siti *et al.*, 2011).** Many studies showed that *Alliums* have great importance for they use in prevention and treatment of different diseases and contribute to their therapeutic effects **(Stajner and Szöllôsin, 2003).** Now increasing attention has been paid in Europe to the medical use of garlic and onion. Therefore, the present study aimed to evaluate the antioxidant properties of the defatted methanolic extracts of six *Allium* species; *A. cepa* L. (ssp. red onion, ssp. white onion and ssp. green onion), *A. sativum* L., *A. porrum* L. and *A. kurrat* L. as well as to determinate the total phenolic and flavonoid contents in these extracts. Also, preliminary photochemical screening of the tested *Allium* species was carried out.

MATERIAL AND METHODS

Plant materials

The leaves and bulbs of *Allium* species in this study were collected from different localities in Egypt especially from El- Sharkia governorate and Kalubia governorate - Egypt in March 2012. The collected plants were kindly identified by Prof. Dr. Waffa Amer, Professor of plant taxonomy, Faculty of Science, Cairo University. The fresh bulbs of *A. cepa* L. (ssp. red onion and ssp. white onion) and *A. sativum* L., fresh leaves of *A. kurrat* L. and fresh leaves and bulbs of *A. cepa* L. (ssp. green onion) and *A. porrum* L. were cut to small pieces and submitted to extraction process.

Extraction and fractionation

Five hundred grams of *Allium* species were separately extracted four successive times with 750 ml of pure methanol. The methanolic extract of each plant was evaporated under vacuum to dryness using rotatory evaporator. The methanolic extract was defatted with petroleum ether. The defatted methanolic extracts of *A. cepa* (ssp. red onion) and *A. porrum* was successively fractionated with different organic solvents such as chloroform, ethyl acetate (EtOAc) and n-butanol (n-BuOH) then the different fractions was evaporated under reduced pressure to dryness. The dried extracts were kept away from any moisture in well plastic vials for determination of phenolic and flavonoid contents as well as antioxidant studies.

Chemicals

The chemicals used in this study include 1, 1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, ascorbic acid, vitamin E, ferric chloride, trichloracetic acid (TCA) and Folin-Ciocalteu reagent purchased from Sigma-Aldrich, (Steinheim, Germany). Phosphate buffer purchased from BDH Chemicals (Poole, England). Sodium carbonate, aluminum chloride, ammonium molybdate and sodium phosphate were purchased from Merck (Darmstadt, Germany). Gallic acid and rutin purchased from Sigma-Aldrich (St.Louis, USA).

Total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent, according to method reported by **Miguel *et al.*, (2010)** with little modification. Briefly, (50 µl) of each dried extract dissolved in methanol (500µg/ml) was combined with 250µl of the Folin–Ciocalteu reagent and 0.750 ml of sodium carbonate (20%). The mixture was shaken, made up to 5 ml using distilled water and allowed to stand for 2 h. Then the absorbance was measured at 765 nm against a blank with distilled water and using gallic acid as standard. The results were expressed as mg gallic acid equivalent per gram dry weight extract (mg GAE/g extract). The results were the averages of triplicate analyses.

Total flavonoid content

The total flavonoid content of each extract was determined according to the procedures described by **Kumaran and Karunakaran (2007**) using rutin as a standard. Plant extract (100 µl) in methanol (10 mg/ml) was mixed with 100µl of aluminum trichloride in methanol (20%) then add one drop of concentrated acetic acid and complete the total volume to 5 ml with methanol. The absorption at 415 nm was read after 40 min against the blank. All determinations were carried out in triplicate. The total flavonoid in each plant extract was determined as mg rutin equivalents per gram extract (mg RE /g extract).

DPPH radical scavenging activity

The DPPH radical scavenging activity of plant extracts was measured according to the procedures described by **Liu *et al.*, (2008).** In this method, the various concentrations of the tested extract in methanol were added to 2 mL of a solution of DPPH in methanol (0.1 m mol L^{-1}). After 30 min of incubation at 37 °C in the dark and the absorbance was recorded at 517 nm**.** Ascorbic acid and vitamin E were used as standards and all experiments were carried out in triplicate**.** The decrease of the DPPH solution absorbance indicates an increase of the DPPH radical scavenging activity. The DPPH scavenging activity of the extracts was calculated and SC_{50} (Concentration of sample required to scavenge 50 % of DPPH radicals) value was determined from this equation:

Scavenging activity % = $[(A_{control} - A_{sample})/A_{control}] \times 100$

Where A_{sample} is the absorbance of a sample solution, and $A_{control}$ is the absorbance of the control solution (containing all of the reagents except the test sample).

Total antioxidant capacity (Phosphomolybdate assay)

The total antioxidant capacity of the tested extracts was determined by phosphomolybdate method using ascorbic acid as a standard **(Prieto *et al.*, 1999).** An aliquot of 0.5 ml of extract (100µg /ml) solution was mixed with 5 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. Then, the samples had cooled to room temperature and the absorbance of the mixture was measured at 695 nm against a blank. A typical blank contained 5 ml of the reagent solution and the appropriate volume of the solvent and incubated under the same conditions. The antioxidant activity of the extracts was expressed as the number of mg equivalents to ascorbic acid (AAE) / g extract. All experiments were carried out in triplicate.

Reducing power assay

Reducing power of extracts was evaluated according to the method of **Oyaizu, (1986).** Each sample (200µg /ml) 1 ml in methanol was mixed with 2.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$. This mixture was incubated at 50 °C for 20 min. Then adding 2.5 ml of trichloro acetic acid (TCA) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant solution (2.5 ml) had taken out and immediately mixed with 2.5 ml of methanol and 0.5 ml of 0.1% ferric chloride. After incubation for 10 min at room temperature, the absorbance against blank was measured at 700 nm. Increase in absorbance of the reaction mixture indicates increased reducing power. All the tests were performed in triplicates. Ascorbic acid and vitamin E standards were used for comparison.

Phytochemical screening

Phytochemical screening of the extract was carried out to identify the secondary metabolites such as flavonoids (Shinoda test), terpenoids (Salkowski test), tannins (Ferric chloride test), saponins (Frothing test), and anthraquinones (Borntrager's test) according to standard phytochemical methods as described by **Harborne, (1973); Trease and Evans, (1978); Sofowora, (1993).**

Statistical analysis

All experimental were carried out in triplicate, and statistical analysis were performed using SPSS (13) software and Microsoft Excel program. The results were given as means ± standard deviation.

RESULTS AND DISCUSSIONS

Total phenolic content

Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidants in terms of their ability to act as efficient radical scavengers **(Narendhirakannan and Rajeswari, 2010).** Total phenolic content of the plant extracts was examined, using Folin-Ciocalteu method and was expressed as gallic acid equivalents. The results in Table 1 exhibited that, the methanol extract of *A. cepa* (ssp. red onion) and *A. porrum* have the highest phenolic contents (123.69 ± 2.68 and 108.78 ± 5.07 mg gallic acid equivalent per gram plant extract) respectively. The phenolic content of other *Allium* sp. decreased in order *A. kurrat* > *A. cepa* (ssp. green onion) >*A. sativum* > *A. cepa* (ssp. white onion). From the results in Table 2, it was established that the ethyl acetate fractions derived from the methanolic extract of *A. cepa* (ssp. red onion) and *A. porrum* contained high phenolic contents (171.13 ± 5.98 and 162.92 ± 8.70 mg gallic acid equivalent /g plant extract) than the other fractions. The phenolic content of the butanolic fraction of each tested plant was higher than the chloroform fraction of the two plants. Previous studies showed that phenolic compounds of the plant extract have been shown to be responsible for their antioxidant activity **(Rice-Evans *et al.*, 1996).**

Total flavonoid content

The total flavonoids content was determined spectrophotometrically using aluminum chloride. The content of flavonoids was expressed in terms of rutin equivalents. The results in Table 1 showed that, the total flavonoid contents of *A. cepa* (ssp. red onion) and *A. porrum* were 16.79 ± 0.28 and 19.48 ± 1.40 mg rutin equivalent /g plant extract respectively. Although, *A. cepa* (ssp. red onion) was reported to have the highest phenolic compounds, the low total flavonoid content was found in its defatted methanol extract with 16.79 ± 0.28 mg rutin equivalent /g plant extract, which suggest that the total flavonoids and their derivatives are not the main contributor to the high phenolics content because there are other phenolics in *A. cepa* (ssp. red onion) such as phenolic acids and tannins **(Yesilyurt *et al.*, 2008).** On the other hand, the results in Table 2 revealed that the total flavonoid content of the ethyl acetate derived from the methanolic extracts of *A. cepa* (ssp. red onion) and *A. porrum* were 144 ± 5.63 and 34.49 ± 1.22 mg rutin equivalent /g plant extract, respectively.

Table 1 Total phenolic and flavonoid contents of the defatted methanolic extracts of *Allium* species

Plant	Total phenols (mg gallic acid equivalent/g plant extract)	Total flavonoids (mg rutin equivalent/g plant extract)
A. cepa (ssp. red onion)	123.69 ± 2.68	16.79 ± 0.28
A. cepa (ssp. white onion)	73.01 ± 3.09	7.98 ± 0.29
A. cepa (ssp. green onion)	98.75 ± 4.33	15.75 ± 0.63
A. porrum	108.78 ± 5.07	19.48 ± 1.40
A. kurrat	102.83 ± 3.65	24.89 ± 0.63
A. sativum	89.66 ± 0.01	6.90 ± 0.16

Values are expressed as mean of triplicate determinations ± standard deviation

Table 2 Total phenolic and flavonoid contents from different fractions derived from the defatted methanol extracts of *Allium cepa* (ssp. red onion) and *Allium porrum*

Fraction	Total phenols (mg gallic acid equivalent/g plant extract)	Total flavonoids (mg rutin equivalent/g plant extract)
A. cepa (ssp. red onion) EtOAc ext.	171.13 ± 5.98	144.34 ± 5.63
A. cepa (ssp. red onion) n-BuOH ext.	168.72 ± 5.65	24.91 ± 0.48
A. cepa (ssp. red onion) $CHCl_3$ ext.	102.45 ± 4.51	28.03 ± 0.21
A. porrum EtOAc ext.	162.92 ± 8.70	34.49 ± 1.22
A. porrum n-BuOH ext.	140.34 ± 4.45	33.10 ± 1.08
A. porrum $CHCl_3$ ext.	95.26 ± 3.50	20.57 ± 0.60

Values are expressed as mean of triplicate determinations ± standard deviation**.**

DPPH radical scavenging activity

1, 1-diphenyl-2-picrylhydrazyl (**DPPH**) is characterized as a stable free radical due to the delocalization of the free electron over the molecule as a whole. The delocalization of free electron also gives rise to the deep violet color and characterized by an absorption band in methanol solution centered at about 517 nm. When a solution of DPPH mixed with substrate which can donate a hydrogen atom, this gives rise to the reduced form with the loss of this violet color **(Nur *et al.*, 2013)**. Therefore the use of DPPH provides an easy and rapid way to evaluate antioxidant activity of any material. Results in Table 3 showed that the defatted methanolic extracts of *A. cepa* (ssp. red onion) and *A. porrum* showed the higher antioxidant activity compared to the other species (SC_{50}= 283.30 ± 4.46 and 347 ± 3.44 µg/ml, respectively). The activity of the other *Allium* species was decreased from *A. kurrat, A. cepa* (ssp. green onion), *A. sativum, A. cepa* (ssp.

white onion) with SC_{50} = 413.06 ± 6.81, 436.73 ± 6.06, 455.51 ± 2.22 and 832.43 ± 7.23, respectively. These results are in full agreement with previous studies on other *Allium* species (**Queiroz *et al.*, 2009**). Evaluation the antioxidant activity of different fractions of *A. cepa* (ssp. red onion) and *A. porrum* revealed that as shown in Table 4 the ethyl acetate fractions of *A. cepa* (spp. red onion) and *A. porrum* have antioxidant activity (SC_{50}= 31.73 ± 1.31 and 175.86 ± 0.93 µg/ml) respectively. The butanolic extract of *A. cepa* (ssp. red onion) and *A. porrum* have antioxidant activity (SC_{50} = 159.05 ± 1.93 and 229.32 ± 2.60 µg/ml) respectively. The chloroform fractions of two species have low activity (SC_{50} = 185.84 ± 3.21 and 308.14 ± 4.29 µg/ml) respectively.

Total antioxidant capacity (Phosphomolybdate assay)

The phosphomolybdate method is based on the reduction of Mo (VI) to Mo (V). The antioxidant sample which is detected by the formation of a green color phosphomolybdenum (V) **(Baig *et al.*, 2011).** The quantitative antioxidant activity is expressed as mg equivalent to ascorbic acid per g of extract (mg AAE/ g extract). The results in Table 3 showed that, the antioxidant capacity of the defatted methanolic extracts *A. cepa* (ssp. red onion) and *A. porrum* was higher than other *Allium* species (95.29 ± 6.25 and 87.25 ± 4.80 mg equivalent of ascorbic acid / g plant extract). The antioxidant capacity of the *Allium* species were arranged in order; *A. kurrat* > *A. cepa* (ssp. green onion) > *A. sativum* > *A. cepa* (ssp. white onion). Results in Table 4 exhibited that the antioxidant capacity of ethyl acetate fractions of *A. cepa* (ssp. red onion) and *A. porrum* were high than other species (270.25 ± 7.35 and 120.92 ± 6.86 mg equivalent of ascorbic acid /g plant extract, respectively). These results agreed with previous studies on other *Allium* species **(Mladenović e*t al.*, 2011; Chang *et al.*, 2013).**

Reducing power assay

Reducing power was measured by direct electron donation in the reduction of $[Fe^{3+}(CN)_6]^{3-}$ to $[Fe^{2+}(CN)_6]^{4-}$. The product was visualized by forming the intense Prussian blue color complex and then measured at λ700 nm **(Nishaa *et al.*, 2012).** The higher absorbance value indicates a stronger reducing power of the samples. As shown in Table 3 and 4 *A. cepa* (ssp. red onion), *A. porrum* and their ethyl acetate were more active than other *Allium* species (0.127 ± 1.55 and 0.113 ± 0.50 in case of methanol extracts and 0.156 ± 1.42 and 0.134 ± 3.06 in case of ethyl acetate fractions, respectively). Whereas the absorbance of ascorbic acid and vitamin E were 0.266 ± 2.77 and 0.206 ± 1.57, respectively at the same concentration.

Table 3 DPPH scavenging activity, total antioxidant capacity and reducing power assay of the defatted methanol extracts of *Allium* species

Plant	DPPH free radical scavenging activity SC_{50} [µg/ml]	Total antioxidant capacity [mg equivalent to ascorbic acid / g extract]	Reducing power assay (Absorbance at 700 nm)
A. cepa (ssp. red onion)	283.30 ± 4.46	95.29 ± 6.25	0.127 ± 1.55
A. cepa (ssp. white onion)	832.43 ± 7.23	32.01 ± 9.79	0.067 ± 2.08
A. cepa (ssp. green onion)	436.73 ± 6.06	62.17 ± 3.72	0.088 ± 3.51
A. porrum	347.17 ± 3.44	87.25 ± 4.80	0.113 ± 0.50
A. kurrat	413.06 ± 6.81	66.5 ± 7.92	0.095 ± 3.13
A. sativum	455.51 ± 2.22	54.13 ± 3.18	0.080 ± 2.56
Ascorbic acid	8.06 ± 0.70	--	0.266 ± 2.77
Vitamin E (α-tocopherol)	14.89 ± 0.26	--	0.206 ± 1.57

Values of SC_{50}, total antioxidant capacity and reducing power assay are expressed as mean of triplicate determinations ± standard deviation.

Table 4 DPPH scavenging activity, total antioxidant capacity and reducing power assay of fractions derived from the defatted methanolic extract of *Allium cepa* (ssp. red onion) and *Allium porrum*

Fraction	DPPH free radical scavenging activity SC_{50} [µg/ml]	Total antioxidant capacity [mg equivalent to ascorbic acid /g extract]	Reducing power assay (Absorbance at 700 nm)
A. cepa (ssp. red onion) EtOAc ext.	31.73 ± 1.31	270.25 ± 7.35	0.156 ± 1.42
A. cepa (ssp. red onion) n-BuOH ext.	159.05 ± 1.93	151.54 ± 5.74	0.143 ± 0.57
A. cepa (ssp. red onion) $CHCl_3$ ext.	185.84 ± 3.21	112.81 ± 3.54	0.115 ± 1.02
A. porrum EtOAc ext.	175.86 ± 0.93	120.92 ± 6.86	0.134 ± 3.06
A. porrum n-BuOH ext.	229.32 ± 2.60	97.13 ± 5.93	0.098 ± 2.08
A. porrum $CHCl_3$ ext.	308.14 ± 4.29	74.16 ± 1.25	0.076 ± 4.17
Ascorbic acid	8.06 ± 0.70	--	0.266 ± 2.77
Vitamin E (α-tocopherol)	14.89 ± 0.26	--	0.206 ± 1.57

Values of SC_{50}, total antioxidant capacity and reducing power assay are expressed as mean of triplicate determinations ± standard deviation.

Correlation between antioxidant activity and total phenolic content

Several studies reported that antioxidant properties of several plant extracts associated with their phenolic contents **(Maizura *et al.*, 2011).** In the present study there is a highly positive correlation between DPPH scavenging activity and total phenolic content (r^2= 0.850) as well as between total antioxidant capacity and total phenolic content (r^2= 0.957) of the tested defatted methanolic plant extracts were observed. The antioxidant power of the tested *Allium* species depend on their phenolic contents.

Qualitative phytochemical screening

The preliminary phytochemical investigation of different *Allium* sp. Table 5 showed the presence of many bioactive constituents such as tannins, saponins, flavonoids, terpenes, steroids. The presence of saponins, terpenoids and steroids in *Allium* species with high amounts means that these chemical constituents may be responsible for other biological and pharmacological activities of *Allium* species **(Hasanuzzaman *et al.*, 2013)**. The present study revealed that *A. cepa* (ssp. red onion) and *A. porrum* have lot of terpenoids and saponins.

Table 5 Qualitative phytochemical screening of the defatted methanol extracts of *Allium* species

Plant	Phytochemical Test					
	Anthraquinons	**Tannins**	**Flavonoids**	**Saponins**	**Steroids**	**Terpenoids**
A. cepa (ssp. red onion)	--	+	++	+++	+++	++
A. cepa (ssp. white onion)	--	+	--	+++	++	+
A. cepa (ssp. green onion)	--	--	+	++	++	++
A. porrum	--	+	+	+++	+++	++
A. kurrat	--	+	+	+++	++	++
A. sativum	--	--	--	++	++	+

- Absent + low ++ moderate +++ high

CONCLUSION

The present study showed that the defatted methanolic extracts of *Allium* species have high antioxidant activity and there is a positive correlation between the antioxidant activity and their phenolic content. *A. cepa* (ssp. red onion) and *A. porrum* have the highest antioxidant capacity, phenolic content, terpenoids and saponins. Therefore, the two plants will submit to chromatographic separation and structure elucidation of their chemical constituents.

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