***In Vitro* Antioxidant Properties of Three Varieties of *Allium Sativum* L. Extracts**

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Abstract: Many herbs possess antioxidant ingredients that provide efficacy by additive or synergistic activities. *Allium sativum* L. is a strong astringent, used for the treatment of liver and spleen diseases, rheumatism and tumors. The antioxidant activities of different concentrations of ethanolic extracts of garlic bulb of three varieties were determined by the four assays *i.e.* DPPH radical scavenging assay, reducing power ability, hydrogen peroxide scavenging assay and total antioxidant capacity. Due to its natural origin and potent free-radical scavenging ability, *Allium sativum* L. could be used as a potential preventive intervention for free radical-mediated diseases.

Keywords: *In vitro* antioxidant, *Allium sativum* L., DPPH, Total antioxidant, free radical, H₂O₂, Garlic.

Introduction

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effects. The origin of diseases of multifactorial nature is being understood now due to the vitiation in basic homeostatic balance phenomenon in the body. The cause of a majority of disease conditions like atherosclerosis, hypertension, ischaemic diseases, Alzheimer's disease, parkinsonism, cancer, diabetes mellitus and inflammatory conditions are being considered to be primarily due to the imbalance between prooxidant and antioxidant homeostasis. Antioxidant principles from natural resources possess multifacetedness in their multitude and magnitude of activities and provide enormous scope in correcting the imbalance. Hence, there is no doubt that phytochemicals deserve a proper position in the therapeutic armamentarium¹.

Hence, the present study aims to investigate the *in vitro* antioxidant potential of *Allium sativum* L. Garlic also has a long history of medicinal use for a wide variety of conditions and was once known as poor-man's treacle (or cure-all). In folk medicine, garlic has been used to treat bronchitis and respiratory problems, gastrointestinal problems, flatulence, leprosy, menstrual cramps, high blood pressure, diabetes and has been used externally for warts, corns, arthritis, muscle pain, neuralgia and sciatica. Recently, science has begun to confirm some of garlic's long-standing medicinal uses. Garlic has been shown to lower blood cholesterol, blood pressure and blood sugar in studies and clinical trials and has also demonstrated anti-cancer, antibacterial, anti-fungal and anti-oxidant effects. Although garlic is best known for its culinary and medicinal uses it can also be used in homemade cosmetics and crafts like garlic braids and wreaths. Diallyl disulfide and diallyl trisulfide, two compounds in garlic oil, are insecticidal and make garlic a good ingredient for insect repellents². In this present study garlic can be chosen from various areas can be compared in their *in vitro* antioxidant activity.

Experimental

Allium sativum L. bulbs were collected from Ukkadam market Coimbatore, Namakkal and Kodaikanal farmers of Tamilnadu. The samples were labeled as UM, NM & KM respectively.

Chemicals

DPPH (1,1-diphenyl-2-picryl-hydrazil) free radical scavenging activity, trichloroacetic acid (TCA) were obtained from Sigma Aldrich, US. All other chemicals used were analytical grade and were obtained from Merck, US.

Extraction

Samples (50 g of tissues) were homogenized in 100 mL of ethanol using a Waring blender at high speed for 1 min at 4 °C. The extract was stirred 10 min at 4 °C and filtered through four layers of cheesecloth and the residue was re-extracted under the same condition with 100 mL of ethanol. The combined filtrate was concentrated under vacuum at 65 °C to dryness and the dry residue was dissolved in 10 mL of ethanol. These ethanol extracts were used for the determination of total phenolics, radical scavenging activity, H₂O₂-scavenging and reducing capacity³.

Total phenolics determination

Appropriately diluted sample in 1 mL was mixed with 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 mL of 5% Na₂CO₃ was added. Thereafter, it was thoroughly mixed and placed in the dark for 1 h and the absorbance was measured at 765 nm using UV-Visible spectrophotometer. A gallic acid standard curve was obtained for concentrations (50, 100, 150, 200, 250 mg/mL) of ethanol for the calculation of polyphenolic content⁴.

Estimation of the total flavonoids contents

The known method⁵ downscaled to 1 mL was followed. The sample (100 mg) was added to 0.4 mL distilled water followed by NaNO₂ (0.03 mL, 5%). After 5 min at 25 °C, AlCl₃ .6H₂O (0.03 mL, 10%) was added, followed by aqueous NaOH (0.2 mL, 1 M) after 6 min. The mixture was diluted with water to 1 mL and the absorbance at 510 nm was read and the total flavonoid content was estimated.

DPPH radical scavenging assay

To ethanolic solution (2 mL) of DPPH was added to 200 mL of extract and the mixture was vortexed. The decrease in absorption at 515 nm was measured in 1 cm quartz cell during 300 min

using an UVmini-1240 recording spectrophotometer (Shimadzu, Kyoto, Japan). Radical scavenging assay toward DPPH was estimated from the following equation:

$$\% \text{inhibition} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100$$

Rapid radical scavenging screening

The method⁶ as modified by Burits *et al.*⁷ was followed in screening for the antioxidant property of the extracts. With the aid of capillary tube, stock solutions (1 mg/mL) of extracts were spotted on silica gel thin layer chromatographic (TLC) plate and developed with a solvent system of ethanol: methanol (90:10). After development, the chromatograms were dried and sprayed with a 0.3 mM solution of the stable radical DPPH. Purple spot formed were taken as positive results. The duration for the development of yellow colour indicated whether the antioxidant activity was strong or not.

Hydrogen peroxide scavenging Assay

The ability of garlic extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.*⁸. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4) and concentrations were determined spectrophotometrically at 230 nm. Extracts (25-50 µg/mL) in distilled water was added to a hydrogen peroxide solution (0.6 mL, 40 mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 19 min against a blank solution in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of extracts and standard compounds was calculated using the following equation:

$$\% \text{ scavenged } [\text{H}_2\text{O}_2] = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance of garlic extracts or standards.

Reducing power test

Different concentration of garlic extract solution (final concentration 100 - 500 mg/L) was mixed with 2.5 mL phosphate buffer (0.2M, pH 6.6) and 2.5 mL potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (10 g/L), then mixture was incubated at 50 °C for 20 minutes. 2.5 mL of trichloroacetic acid (100 g/L) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL FeCl_3 and absorbance measured at 700 nm in UV-Visible spectrophotometer. Ascorbic acid (AA) was used as standard and phosphate buffer used as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power⁹.

Total antioxidant capacity

For total antioxidant capacity assay, 0.1 mL of the extract (10 mg/mL) dissolved in water was combined in an Eppendorf tube with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid (AA) was used as the standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid¹⁰.

Statistical analysis

All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant

difference (LSD) test. *P* values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean $\pm$ S.D. for five experiments in each.

Results and Discussion

Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defences¹¹. Oxidative stress, in which large quantities of reactive oxygen species (ROS) like hydrogen peroxide, superoxide, hydrogen radical, singlet oxygen and nitrogen species are generated, one of the earliest responses to stress. These ROS have a role in disease and aging in animals¹². The anti-oxidative system protects the organism against ROS-induced oxidative damage. There are restrictions on the use of synthetic antioxidants such as BHT, as they are suspected to be carcinogenic¹³. Natural antioxidants therefore have gained importance, some of our previous studies reported that medicinal plant (*Cleome gynandra*) has potential antioxidant capacity^{14,15}.

Phytochemical screening

The total phenols and flavonoid contents in the ethanolic extract were found to be higher than in the other extracts (Petroleum ether, ethyl acetate and chloroform). The preliminary phytochemical analysis indicated the presence of steroids and triterpenoids in the petroleum ether extract. Alkaloids, flavonoids, phenols, saponins, tannins in the ethyl acetate extract. Alkaloids, flavonoids, phenols and triterpenoids in the chloroform extract and alkaloids, phenols, saponins and tannins in the chloroform extract in all the sample extracts. Total phenol and flavonoids shown in the Table 2 exhibits the phenol and flavonoid content of NM, UM and KM. The total phenol and flavonoid content in KM were found to be higher when compared to UM and NM. Rapid radical scavenging screening: The result of the rapid radical scavenging screening is shown in Table 3. KM showed an immediate reaction from purple to yellow and hence more active than NM and UM.

Table 1. Extraction, phytochemical analysis and *in vitro* antioxidant activity of *Allium sativum* L. from different places

Extracts/standard	Extraction	
	Characteristics	Phytochemical constituents
Petroleum ether extract	Pale white residue	Steroid, triterpenoid
Chloroform extract	Pale white residue	Alkaloid, flavonoids, phenols, Triterpenoids
Ethylacetate extract	Pale white residue	Flavonids, phenols, saponins, tannins
Ethanol extract	Pale white sticky residue	Alkanoids, flavonoids, phenols, tannins.

Table 2. Phenol and flavanoid content in UM, NM and KM

Total phenol, mg/g ^a		Total flavanoid, mg/g	
UM	40.8 $\pm$ 0.71	UM	128 $\pm$ 0.69
NM	38.3 $\pm$ 0.76	NM	90 $\pm$ 1.27
KM	44.58 $\pm$ 0.54	KM	145 $\pm$ 4.31

^a values are mean $\pm$ SD of five determinants

Table 3. Radical scavenging activities of the ethanolic extracts of UM, NM and KM using rapid DPPH TLC screening

Plant part	Reaction speed	Intensity of spots
NM	Intermediate	++
UM	Fast	+++
KM	Fast	+++

+ + + = Strong intensity (immediate reaction)

DPPH assay

Comparison of the antioxidant activity of the extracts and ascorbic acid by DPPH method is shown in Figure 1. The ethanol extract of NM, UM and KM exhibited significant dose dependent inhibition of DPPH activity. The scavenging activity of the extracts was very potent and the power of the extracts increased with increasing concentration.

Figure 1. Free radical scavenging activity of various amounts of ethanol extracts of NM, UM and KM (Values are mean $\pm$ SD of five determinations; AA=Ascorbic acid)

Among these, KM showed the highest potential to scavenge DPPH, followed by UM and NM. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to form a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. DPPH stable free radical method is an easy, rapid and sensitive way to evaluate the antioxidant activity of a specific compound or plant extracts^{16,17}. The significant decrease in the concentration of DPPH radical is due to the scavenging ability of garlic extracts¹⁸. The result of the rapid radical scavenging screening confirmed their high radical scavenging activity.

H₂O₂ assay

Ability of the investigated *Allium sativum* L. extracts to scavenge hydrogen peroxide is shown in Figure 2. The NM extract showed low scavenging activity, whereas other extracts of garlic showed higher activity. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H₂O₂ can probably react with Fe²⁺ and possibly Cu²⁺ ions to form hydroxyl radical and this may be the origin of many of its toxic effects¹⁸. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.

Figure 2. Hydrogen peroxide radical scavenging activity of NM, UM and KM (Values are mean $\pm$ SD of five determinations)

In our study, the decomposition of H_2O_2 by garlic extracts may at least partly result from its antioxidant and free radical scavenging activity. Assay of reducing power (Figure 3) reveals the reductive capabilities of the bulb extracts compared to ascorbic acid. The reducing power of bulb extracts was very potent and the power of the extract was increased with increasing concentration. The reducing power of the garlic extracts as a function of their concentration. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. Presence of reducers causes the conversion of the Fe^{3+} /ferricyanide complex used in this method to the ferrous form. By measuring the formation of Perl's Prussian blue at 700 nm, it is possible to determine the Fe^{2+} concentration. The reducing power of the garlic extracts increased with their concentrations or in par with the results of Deore *et al.*¹⁹.

Figure 3. Reducing power of NM, UM and KM compared to ascorbic acid
(Values are mean $\pm$ SD of five determinations; AA=Ascorbic acid)

Total antioxidant activity

The total antioxidant activity of different concentrations of NM, UM and KM were depicted in Figure 4. They exhibited effective antioxidant activity. The total antioxidant capacity of the extract was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at 695 nm. The higher total antioxidant capacity was found in KM and UM extracts than NM similar to AA.

Figure 4. Total antioxidant activity of ethanol extract of NM, UM and KM
(Values are mean $\pm$ SD of five determinations)

Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers²⁰. Therefore, in the present study, total phenolic content present in extract were estimated using modified Folin- ciocalteau method. The polyphenol and flavanoid are used for the prevention and cure of various diseases which

is mainly associated with free radicals²¹. The higher content of polyphenol and flavonoids may be attributed to the antioxidant potential of garlic.

Conclusion

The results obtained thus indicate that *Allium sativum* L. extract has potent antioxidant activity, achieved by scavenging abilities observed against DPPH, hydrogen peroxide reducing power assay mainly higher activity observed in Kodaikanal sample. The extracts has been reported to contain flavanoid and steroids which are known antioxidants, hence the antioxidant activity of the extract of the pulp also showed good antioxidant potential. Also the wide use of the extract in the Indian indigenous system of medicine as may be in part due to the antioxidant potential of the extracts. The bulb extracts merits further investigation in animal models to confirm its antioxidant properties. It can be used for minimizing or retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the shelf life of pharmaceuticals.

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