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In vitro antioxidant activity of different extracts of bulb of *Allium Sativum* Linn.

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ABSTRACT

Oxidative stress is one of the most popular terms in biomedicine. Oxidative Stress is a general term used to describe the steady state level of oxidative damage in a cell, tissue, or organ, results from an imbalance between radical-generating and radical scavenging systems, i.e. increased free radical production or reduced activity of antioxidant defenses or both. A great number of aromatic, medicinal, spice and other plants contain chemical compounds exhibiting antioxidant properties. In the present study the antioxidant activity and radical scavenging activity of aqueous and alcoholic extracts of *Allium sativum* Linn. traditionally used by Indian population as folk remedies was evaluated.

Introduction

Oxidative stress is one of major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, diabetes mellitus, cancer, Parkinson's disease, Alzheimer's disease, chronic renal failure, immune dysfunction, chronic inflammatory diseases and is involved in aging.[1-5] Harman first proposed the `free radical theory of ageing', which stated that free radicals produced during metabolism and other biological reactions cause cumulative cell damage leading to ageing and eventual death.[6]

The most effective way to eliminate free radicals which cause the oxidative stress is with the help of antioxidants. Antioxidants are those substances which possess free radical chain reaction breaking properties. Antioxidants, either exogenous or endogenous, whether synthetic or natural, can be effective in preventing free radical formation by scavenging them or promoting their decomposition and suppressing such disorders[7]. The human body has a number of antioxidant defense mechanisms and repair systems to protect against the damage and reduce the adverse effects of free radicals and other reactive oxygen species.

Plants are potent sources of biochemical constituents and have been components of phytomedicine since very long times. Among the numerous naturally occurring antioxidants; ascorbic acid, carotenoids and phenolic compounds are more effective. They are known to inhibit lipid peroxidation (by inactivating lipoxygenase), to scavenge free radicals and active oxygen species by propagating a reaction cycle and to chelate heavy metal ions.

The present study evaluated the potential antioxidant activity and radical scavenging activity of of different extracts of Allium sativum Linn.

Experimental:

Collection of the plant material:

Six months old (after harvest) garlic (Allium sativum Linn.) bulbs were collected from the local market from the rural areas of Gorakhpur district of eastern Uttar Pradesh and authenticated by Dr. M.B. Singh, Programme Coordinator, KVK Sultanpur, U.P.

Extraction:

Garlic bulbs were separated, peeled and washed with distilled water. After drying in shed, about 500 g of clean garlic bulbs were crushed with the help of electronic grinder. Aerial parts of the plants were allowed to dry in shed and then cut into small pieces. The bark of the plants was cut into small pieces. About 500 g of these dried small pieces of the plants were powdered with the help of electronic grinder.

Powdered plant material was charged in a ‘thimble’ made of cellulose it was placed in a central compartment of Soxhlet assembly. By using Soxhlet extractor exhaustive extraction with a series of solvents of increasing polarity was done. Solvents used with increasing polarity: ethanol and finally water.

Extraction with alcohol

Defined quantities of plant material were collected, shade dried at room temperature, pulverized and extracted with 95% ethanol in a Soxhlet extractor. The extract was concentrated and dried using rotary flash evaporator. It was kept in dessicator until further used.

Aqueous Extraction by Maceration:

Defined quantities of plant material were collected were collected; shade dried at room temperature, pulverized and was macerated with 3% chloroform water for seven days with occasional shaking to get aqueous extract. The aqueous extract was concentrated and dried using rotary flash evaporator. It was kept in dessicator until further used.

Phytochemical analysis of different extract:

Different chemical tests were carried out for both extracts of Allium sativum Linn to identify the presence of various chemical constituents like alkaloids, phytosterols, carbohydrates, terpenoids, saponins, flavanoid, phenolic compounds etc.

Determination of Antioxidant activity

DPPH Assay[8-10]

Free radical scavenging activity of different extracts was tested against a methanolic solution of 1, 1-diphenyl-2-picryl

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hydrazyl (DPPH). Antioxidants react with DPPH and convert it to 1-1-diphenyl -2-picryl hydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant extract. The change in the absorbance produced at 517nm has been used as a measure of antioxidant activity.

The samples of different extracts were prepared in various concentrations viz. 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 µg/ml in AR grade methanol. 1 ml samples of above concentrations were mixed with equal volume of 0.1mM methanolic solution of DPPH (0.39mg in 10 ml methanol). An equal amount of methanol and DPPH was added and used as a control. Ascorbic acid solution of various concentrations viz. 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 µg/ml in distilled water were used as standard. After incubation for 20 minutes in dark, absorbance was recorded at 517 nm. Experiment was performed in triplicates. % scavenging was calculated by using the following formula:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

A graph was plotted with concentration (µg/ml) on X axis and % scavenging on y axis and IC50 values were calculated, which represents the concentration of the scavenging compound that caused 50% neutralization.

Nitric oxide radical scavenging (NO) assay[11-12]

This procedure is based on the principle that sodium nitropruside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions, which can be measured using a Griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO.

The samples of different extracts were prepared in various concentrations viz. 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml in AR grade methanol. 2 ml samples of above concentrations were mixed with 3 ml of solution of 10 mM sodium nitroprusside. The same reaction mixture without the methanolic extract of sample but with equivalent amount of methanol served as control. The reaction mixture was allowed to incubate at room temperature for 180 minutes. Ascorbic acid solution of various concentrations viz. 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml in distilled water were used as standard for comparison. After incubation the samples were reacted with Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 2% phosphoric acid). The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dichloride was read at 546 nm and referred to the absorbance of ascorbic Acid, used as a positive control treated in the same way with Griess reagent. Experiment was performed in triplicates. % scavenging was calculated by using the following formula:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

A graph was plotted with concentration (µg/ml) on X axis and % scavenging on y axis and IC50 values was calculated.

Determination of Reducing Power[13-14]

The reducing power (or ability) describes how easily one substance can give electrons to another. A powerful reducing agent is keen to donate electrons. This method measures the ability of antioxidants to reduce ferric ion. Reducing power was investigated using the method developed by Oyaizu.

The samples of different extracts were prepared in various concentrations viz. 250, 500 and 1000 µg/ml in distilled water. 1.25 mL of sample aliquots was mixed with 1.25 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixtures were incubated at 50 ºC for 20 minutes. The resulting solution was cooled rapidly, mixed with 1.25 mL of 10% trichloroacetic acid and centrifuged at 3,000rpm for 10min. The supernatant (2.5 mL) was taken out and immediately mixed with 2.5 mL of distilled water and 500 µL of 1.0 % ferric chloride ($FeCl_3$) was then added. After incubation for 10 min, the absorbance (abs) against blank was determined at 700 nm. All samples were assayed in triplicate. Ascorbic acid standard was utilized for comparison.

Folin-Ciocalteau Total Phenolic Assay [15-16]

This assay measures the change in colour metal oxides are reduced by polyphenolic antioxidants such as gallic acid and catechin, resulting in a blue solution with maximal absorption at 765 nm. The standard curve is prepared using gallic acid, and results are reported as gallic acid equivalents. Total phenols were determined by Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. However, this reagent does not only measure total phenols and will react with any reducing substance. The reagent therefore measures the total reducing capacity of a sample, not just the level of phenolic compounds.

A dilute sample of diffrent extract (0.5 ml of 1:10 g/ml) or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250, 300 mg /L solutions of gallic acid in methanol: water (50:50, v/v). The total phenolic content was expressed as mg/g equivalents of gallic acid using the following equation based on the calibration curve: y= mx + c (y= absorbance, m = slope, x = concentration, c = intercept), which is a common reference compound.

Results and Discussion:

The average value of extractive of aqueous and ethanolic extract of Allium sativum Linn was found to be 9.55% and 5.40 respectively.

The obtained extracts were subjected to phytochemical screening for its constituents by standard methods and the results were tabulated in table 1.

Table 1:- Phytochemical screening of bulbs of Allium sativum Linn.

Test	Ethanolic Extract	Aqueous Extract
Alkaloids	-	-
Carbohydrates	+	+
Phytosterols	+	+
Terpenoids	+	+
Saponins	+	+
Phenolic compounds	+	+
Flavonoids	-	-
Tannins	-	-

(+ = Present, - = Absent)

In vitro tests of alcoholic and aqueous extract of bulb of Allium sativum Linn. evaluated for its antioxidant property

revealed DPPH, Nitric oxide free radical, reducing power and total phenolic content effect.

The antioxidant reacts with stable free radical, DPPH and converts it to 1,1-diphenyl–2-picryl hydrazine. The ability to scavenge the stable free radical DPPH was measured by decrease in the absorbance at 517 nm[10]. The ethanol and water extracts of Allium sativum Linn. exhibited a significant dose dependent inhibition of DPPH activity (Table 2). A concentration dependent assay was carried out with these extracts and the results are presented in Fig.1. The amount of extract needed for 50% inhibition of DPPH free radical is known as IC50 value of the extract. Lower the IC50 value shows better scavenging ability of the sample. The IC50 value of aqueous and alcoholic extract was found to be 867.49 and 413.54 µg/ml respectively. Hence, the aqueous extract has more prominent scavenging activity rather than alcoholic extract.

Table 2:- DPPH radical scavenging of bulbs of Allium sativum Linn.

S. No.	Conc. (µg/ml)	% Scavenging		
		Aqueous extract	EtOH extract	Ascorbic acid
1	2	0.29±0.36	2.21±0.54	9.56±0.47
2	4	1.21±0.47	3.22±0.65	28.24±0.28
3	8	3.64±0.18	8.54±0.47	52.08±0.37
4	16	8.54±0.27	16.42±0.52	89.76±0.43
5	32	13.33±0.36	22.58±0.74	93.27±0.72
6	64	19.45±0.87	32.49±0.38	95.73±0.37
7	128	26.42±0.86	37.51±0.67	95.92±0.81
8	256	31.25±0.82	41.26±0.51	95.81±0.49
9	512	37.43±0.71	51.24±0.67	87.17±0.43
10	1024	51.22±0.48	64.27±0.74	83.58±0.28

All values in this table represent the mean ±SD (n=3).

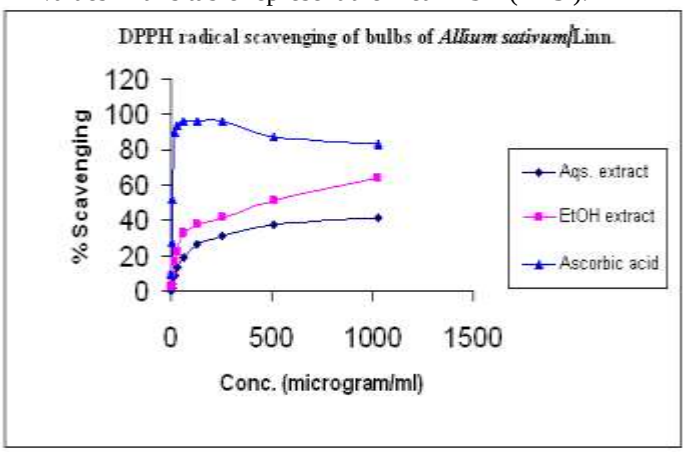

Fig. 1:- DPPH radical scavenging of bulbs of Allium sativum Linn.

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities[12]. Figure 2 & table 3 illustrates the percentage inhibition of nitric oxide generation by Allium sativum Linn. The IC_{50} value of aqueous and alcoholic extract was found to be 89.93 and 78.34 µg/ml respectively. Hence, the alcoholic extract has more prominent scavenging activity of NO free radicals rather than aqueous extract.

Table 3:- Nitric oxide scavenging activity of bulbs of Allium sativum Linn.

S. No.	Conc. (µg/ml)	% Scavenging		
		Aqueous extract	EtOH extract	Ascorbic acid
1	20	8.76±0.65	11.64±0.39	18.64±0.62
2	30	11.54±0.26	14.74±0.78	28.24±0.52
3	40	17.65±0.38	19.43±0.64	57.08±0.31
4	50	23.86±0.95	28.41±0.29	86.76±0.94
5	60	25.82±0.64	32.67±0.86	93.27±0.27
6	70	31.76±0.66	38.23±0.94	95.73±0.26
7	80	46.12±0.28	52.35±0.66	95.92±0.19
8	90	54.87±0.94	58.90±0.42	95.89±0.54
9	100	59.62±0.86	61.05±0.34	96.06±0.67

All values in this table represent the mean ±SD (n=3).

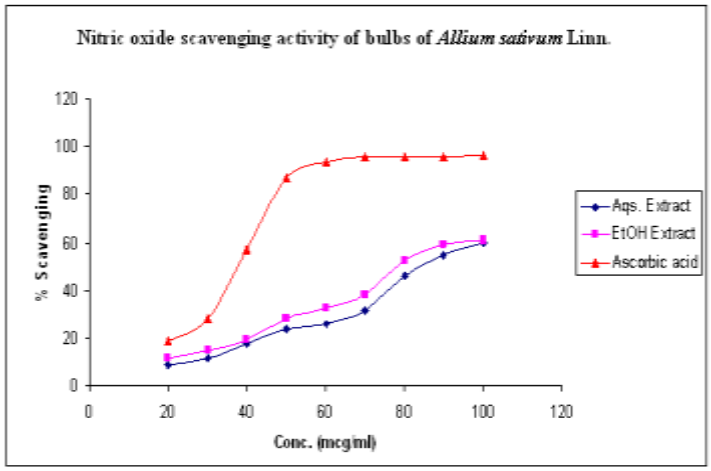

Fig. 2:- Nitric oxide scavenging activity of bulbs of Allium sativum Linn.

The reducing power of the plant extracts involved the transformation of Fe^{3+} to Fe^{2+}. The reducing ability of a molecule may serve as an indicator of its potential antioxidant ability[13]. The reducing capacity of ethanolic extract of bulbs of Allium sativum was close to ascorbic acid, but aqueous extract has some less reducing capacity than standard (table 4 & fig. 3). Better reducing capability at higher concentrations was found.

Table 4:- Reducing power ability of bulbs of Allium sativum Linn.

S. No.	Conc. (mg/ml)	Absorbance		
		Aqueous extract	EtOH extract	Ascorbic acid
1	0.25	0.2546±0.39	0.3342±0.23	0.4122±0.94
2	0.50	0.5168±0.65	0.6652±0.51	0.8172±0.35
3	1.0	1.0352±0.24	1.3411±0.64	1.6545±0.49

All values in this table represent the mean ±SD (n=3).

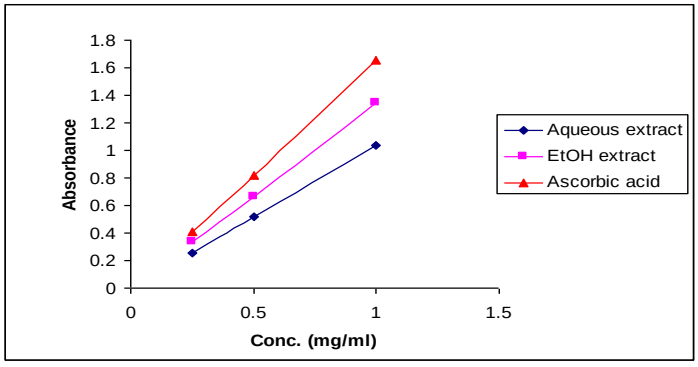

Fig. 3:- Reducing power ability of bulbs of Allium sativum Linn.

A strong relationship between the total phenolic content and antioxidant activity in fruits, vegetables, grain products, and plant subjects of ethnopharmacological treatments has also been reported. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups[15].

The content of phenolic compounds (mg/g GAE) in ethanolic and water extract was determined from regression equation of calibration curve ($y = 0.0029x + 0.0903$, $R^2 = 0.9725$) and expressed in gallic acid equivalents (GAE). The aqueous and ethanolic extracts of Allium sativum Linn have 0.484 and 0.757 mg/g GAE respectively; hence it may be a moderate antioxidant.

Conclusion:

The results of all methods have proven the effectiveness of the plant extracts compared to the reference standard antioxidant ascorbic acid. Further investigation on the isolation and identification of antioxidant component(s) in theplant may lead to chemical entities with potential for clinical use.

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