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Extraction of phenolic compounds and evaluation of the antioxidant and phenolic compounds of onion (*Allium cepa* L.)

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Abstract
Onion is a versatile food which is used as traditional Indian spice. It is an essential part of food habit and has immense health benefits therefore consumed for its putative nutritional and health benefits for centuries. Onion (*Allium cepa* L.) belongs to Family: Liliaceae (lilies) and easily digestible aromatic vegetable used globally. Onions contains phenolics and flavonoids that have potential anti-inflammatory, anticholesterol, anticancer, and antioxidant properties. Moreover, onion is also prescribed to relieve headaches, coughs, snakebite, to facilitate bowel movements, erections and hair loss. A study found that on consumption of large amounts of Allium vegetables reduces the risk for gastric and prostate cancer. A Quercetin-3'-O-beta-D-glucoside isolated from *A. cepa* has antioxidant activity. Moreover, onion flesh and onion peel were also found to enhance antioxidant status in aged rats. Such an outcome has been attributed to the antioxidant, xanthine oxidase inhibitory, and superoxide radical scavenging activities of onion peel. Some epidemiological studies relate onion's antioxidant activity to the presence of organosulfur-compounds and flavonoids.

Keywords: *Allium Cepa*, xantine oxidase, flavonoids, antioxidant

Introduction
Depression is also acknowledged as depressive condition and considered as multi-factorial disease associated with psychological and emotional imbalance. Depression is a chronic and life-threatening disorder affecting with a prevalence of approximately 5% in the general population. Moreover, depression is also responsible for huge burden on individual as well as families (P. Sobocki., 2006; N. Abe-Higuchi, 2016) [1, 2]. Since last several decades, the age for the onset of major depression has been decreased leading to a significant increase in overall incidences of depression (Blazer, 2000) [3]. Moreover, the enhanced numbers of incidences are not the result of changed approach of health experts or society, investigative criterion, reporting predisposition, and other parameters (Klerman and Weissman, 1989; Klerman, 1988) [4, 5]. Generally, any type of stressful event in life is considered as an initial sign of depression, therefore relating depression to the stressful events. Moreover, one more factor called as oxidative stress is found to have a significant role in the pathogenesis of depression (Nielsen *et al*., 2004) [6]. Onion is a versatile food which is used as traditional Indian spices. It is an essential part of food habit and has immense health benefits therefore consumed for its putative nutritional and health benefits for centuries (Krishnaswamy, 2008) [7]. Onion (*Allium cepa* L.) belongs to Family: Liliaceae (lilies) is easily digestible aromatic vegetable used globally. Onions contain phenolics and flavonoids that have potential anti-inflammatory, anticholesterol, anticancer, and antioxidant properties. Moreover, onion is also prescribed to relieve headaches, coughs, snakebite, to facilitate bowel movements, erections and hair loss (Park *et al*., 2007) [8]. A Quercetin-3'-O-beta-D-glucoside isolated from *A. cepa* has antioxidant activities (Yang *et al*., 2004) [9]. Moreover, onion flesh and onion pee were also found to enhance antioxidant status in aged rats (Di Renzo *et al*., 2007) [10]. Considering the presence of plethora of chemical constituents and numerous activities of *A. Cepa*, present study was designed to investigate the antidepressant activity of *Allium cepa* in laboratory animals.

Material and Methods
Swiss albino male mice (22-26 g) were used for the study. The animals were obtained from the animal house of M. M. College of Pharmacy, Mullana, Ambala. All the mice were kept in plastic rat cages with stainless steel coverlids and rice straw was used as bedding material. The animals were facilitated with environmental conditions of photoperiod (12:12 h dark: light cycle) and temperature (25 ± 2 °C). Animals were kept on commercial diet and water.

ad libitum. The animals were kept fasted 2hr before and 2hr after the administration of the drugs. The experiments were performed between 8.00 am to 1.00 pm. The study protocols were approved by Institutional Animal Ethics Committee (I.A.E.C) and animal care was taken as per the guidelines of committee for the purpose of control and supervision of experiment on animals (CPCSEA) Govt of India.

Collection and preparation of samples

The whole fruit of *Allium cepa* was collected from the local market. The whole fruit of *Allium cepa* was collected & shade dried. Coarse powder is made from whole plant of *Allium cepa* and subjected to extraction in increasing polarity. Various extracts are prepared by using suitable solvents like petroleum ether, chloroform, and hydroalcohol solvent.

Determination of phenolic compound

Total soluble phenolics in the extracts were determined with Folin–Ciocalteau reagent according to the method using gallic acid as a standard phenolic compound (Slinkard and Singleton, 1977) [11]; 1.0 ml of extract solution containing 1.0 g extract in a volumetric flask was diluted with 45 ml of methanol. 1.0 ml of Folin–Ciocalteau reagent was added and mixed thoroughly. Three minutes later 3.0 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 3 h with intermittent shaking. The absorbance of the blue color that developed was read at 760 nm. The concentration of total phenols was expressed as mg/g of dry extract (Kim *et al*., 2003) [8, 12]. All determinations were performed in triplicate. Total content of phenolic compounds in plant extract was determined as µg of gallic acid equivalents (GAE)

Reducing power assay

The reducing power of the extract was determined using ascorbic acid as a standard. 1 ml of different concentrations of extract or ascorbic acid i.e. 10 - 160 µg/ml were taken in different test tubes and the volumes was made up to 2 ml using distilled water and were mixed in to the mixture of 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50^0 C for 20 min. Following incubation, 2.5 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated the increased reducing power.

Reducing power ability (%) = [(Absorbance of Control - Absorbance of test Sample)/(Absorbance of Control)] x 100

Hydrogen peroxide-scavenging activity

The Hydrogen peroxide-scavenging activity of extract was determined by the method of (Ruch *et al*., 1989) [13]. The extract (20-320µl/ml) was dissolved in 3.4 mL of 0.1M phosphate buffer (pH 7.4) and mixed with 600 µL of 43 Mm solution of hydrogen peroxide. The absorbance value (at 230 nm) of the reaction mixture was recorded at 10 min intervals between zero and 40 min. for each concentration, a separate blank sample was used for background subtraction. The percentage of hydrogen peroxide scavenging effect is calculated using following formula.

Percentage H_2O_2scavenging effect = $[A_0 – A_1 / A_0]$ X 100

Where, A_0 absorbance of blank, A_1 absorbance standard or extract.

Determination of DPPH (1-1-diphenyl 2-picryl hydrazyl) radical-scavenging activity

The free radical-scavenging activity of the NA hydro-alcoholic extract was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH. 0.1 mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in water at different concentrations (10 - 320µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Ascorbic acid was used as the reference compound. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. Radical-scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ inhibition} = (A_0 - A_t)/A_0 x\ 100;$$

Where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

Total phenolic contents

The total phenolic content in Hydroalcoholic (HA) and Choloroform (CH) extract of *Allium cepa* was determined with the Folin-Ciocalteu reagent (according to the method of Slinkard and Singleton) using gallic acid as a standard and absorbance was measured at 765 nm. The total phenolic content in HA and CH extract was found 19.42 mg/g and 28.60 mg/g.

Ferric reducing antioxidant power

The reducing power of hydroalcoholic and chloroform extract of *Allium Cepa* increased with concentration. The maximum reducing power was obtained at 320µg/ml. EC_{50} (effective concentration at which the absorbance is 0.5) was calculated from the calibration curve and was found to be 30µg/ml for hydroalcoholic extract, 43µg/ml for petroleum ether extract.

Reducing power assay

Reducing power is the measure of reductive ability of antioxidant, and estimated by transformation of Fe (III) to Fe (II) in the presence of the sample extracts. The presence of antioxidants results in the conversion of ferric ions/ferricyanide complex to ferrous form. The absorbance is measured at 700 nm. Higher absorbance indicates higher reducing power.

The reducing power of hydroalcoholic and chloroform extract of *Allium Cepa* increased with concentration. The maximum reducing power was obtained at 320µg/ml. EC_{50} (effective concentration at which the absorbance is 0.5) was calculated from the calibration curve and was found to be 30µg/ml for hydroalcoholic extract, 43µg/ml for petroleum ether extract.

Hydrogen peroxide-scavenging activity

Hydrogen peroxide produces hydroxyl radicals in cells. Scavenging of these radicals by the test compound is used as a test for antioxidant activity. The reduction of these radicals is observed by the decreased absorbance at 230nm with increasing concentration of the test drug. In hydrogen peroxide scavenging assay, the percentage inhibition of hydroalcoholic extract on H_2O_2 at 160 µg/ml was found to be

52.18% whereas for the chloroform extract was found to be 53.24% while ascorbic acid shows 64.09% inhibition. IC_{50} value of hydroalcoholic extract of *Allium cepa* was found to be 120µg/ml whereas for the chloroform extract IC_{50} value was found to be 90µg/ml. The IC_{50} of ascorbic acid was found to be 42µg/ml.

***In-vivo* antioxidant assay**

Estimation of lipid peroxidation

The quantitative measurement of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation in heart was performed according to method of Ohkawa *et al.,* (1979) [15]. 0.2 ml of supernatant of homogenate was pipetted out in a test tube, followed by addition of 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 30% acetic acid (pH 3.5), 1.5 ml of 0.8% of thiobarbituric acid and the volume was made up to 4 ml with distilled water. The test tubes were incubated for 1 h at 95^0C, then cooled and added 1 ml of distilled water followed by addition of 5 ml of n-butanol-pyridine mixture (15:1 v/v). The tubes were then centrifuged at 4000 g for 10 minutes. The absorbance of developed pink color was measured spectrophotometrically at 532 nm. A standard calibration curve was prepared using 1-10 nM of 1, 1, 3, 3-tetra methoxy propane. The TBARS values were expressed as nanomoles per mg of protein.

Estimation of reduced glutathione

The reduced glutathione (GSH) content in tissue was estimated using method of Beutler *et al.,* (1963) [16]. The supernatant of homogenate was mixed with trichloroacetic acid (10% w/v) in 1:1 ratio. The tubes were centrifuged at 1000rpm for 10 min at 4^0C. The supernatant obtained (0.5 ml) was mixed with 2 ml of 0.3 M disodium hydrogen phosphate. Then 0.25 ml of 0.001 M freshly prepared DTNB [5, 5`-dithiobis (2-nitrobenzoic acid) dissolved in 1% w/v citric acid] was added and absorbance was noted spectrophotometrically at 412 nm. A standard curve was plotted using 5-50 µM of reduced form of glutathione and results were expressed as micromoles of reduced glutathione per mg of protein.

Estimation of superoxide anion generation

The left ventricular superoxide anion generation was estimated in terms of measuring reduced nitroblue tetrazolium (NBT). Weighed amount of left ventricular tissue (25 mg) was taken in 5 ml phosphate buffered saline containing 100 µM of NBT and incubated at 37^0C for 1.5 h. The NBT reduction was stopped by adding 5 ml of 0.5 M HCl. Then, the left ventricular tissue was taken out and was minced and homogenized in a mixture of 0.1 M sodium hydroxide and 0.1% sodium dodecyl sulphate in water containing 40 mg/l diethylene triamine penta acetic acid. The mixture was centrifuged at 20,000rpm for 20 min and the resultant pellet was suspended in 1.5 ml of pyridine and kept at 80^0C for 1.5 h to extract formazan, an adduct formed after reaction of NBT with superoxide anions. The mixture was again centrifuged at 10,000rpm for 10 min and absorbance of formazan was determined spectrophotometerically at 540 nm. The amount of reduced NBT was calculated using the formula

$$\text{Amount of reduced NBT} = \frac{\mathbf{A \times V}}{\mathbf{T \times Wt \times \varepsilon \times 1}}$$

Where A is absorbance, V is volume of pyridine (1.5 ml), T is time for which the tissue was incubated with NBT (1.5 h), Wt is blotted wet weight of tissue (25 mg), ε is extinction coefficient (0.72 l/mmol/mm) and l is length of light path (1 cm). Results were expressed as reduced NBT picomole per min per mg of wet tissue.

Statistical analysis

The results were expressed as Mean ± S.E.M. The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's test. The *p*-value <0.05 was considered to be statistically significant.

Result and Discussion

Total phenolic compound

Phenolic compounds are very important plant constituents because of their scavenging ability and are also considered as secondary metabolites which are produced in the shikimic acid pathway of plants and pentose phosphate through phenylpropanoid metabolization. Generally phenolic compounds contain benzene rings along with one or more hydroxyl substituents, ranging from simple phenolic molecules to highly polymerized compounds.

The total phenolic content in HA and CH extract of *Allium cepa* was determined with the Folin-Ciocalteu reagent (according to the method of Slinkard and Singleton) using gallic acid as a standard and absorbance was measured at 765 nm. The total phenolic content in HA and CH extract was found 19.42 mg/g and 28.60 mg/g as Gallic acid equivalent (GAE)

Reducing power assay of hydroalcoholic and chloroform extract of *Allium cepa*

Reducing power is the measure of reductive ability of antioxidant, and estimated by transformation of Fe (III) to Fe (II) in the presence of the sample extracts. The presence of antioxidants results in the conversion of ferric ions/ferricyanide complex to ferrous form. The absorbance is measured at 700 nm. Higher absorbance indicates higher reducing power.

The reducing power of hydroalcoholic and chloroform extract of *Allium Cepa* increased with concentration. The maximum reducing power was obtained at 320µg/ml. EC_{50} (effective concentration at which the absorbance is 0.5) was calculated from the calibration curve and was found to be 30µg/ml for hydroalcoholic extract, 43µg/ml for petroleum ether extract (Figure 1 and 2).

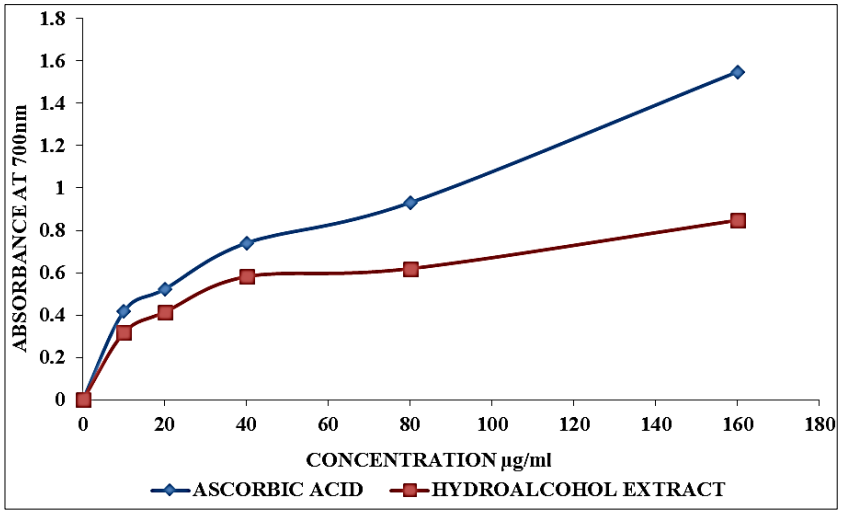

Fig 1: Effect of ascorbic acid and hydroalcoholicic extract of *Allium cepa* on reducing power assay

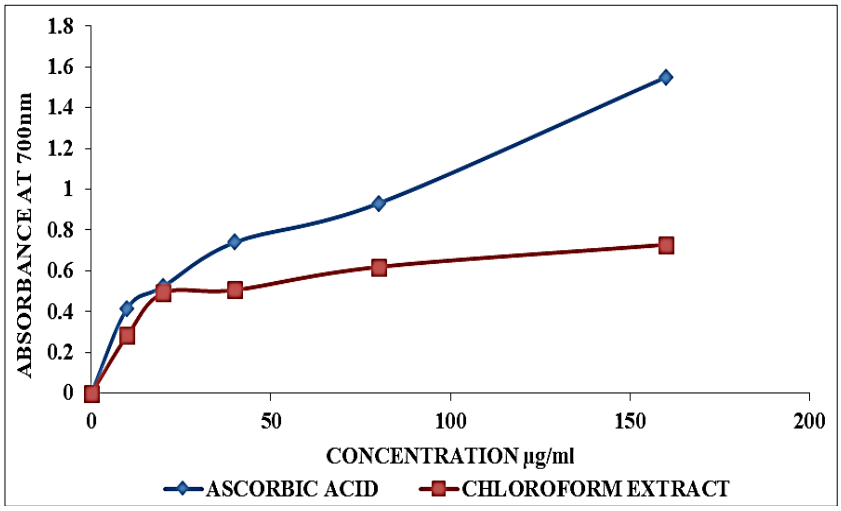

Fig 2: Effect of ascorbic acid and chloroform extract of *Allium cepa* on reducing power assay of *Allium cepa*

Effect of hydroalcoholicic extract extract of *Allium cepa* on hydrogen peroxide-scavenging activity

Hydrogen peroxide produces hydroxyl radicals in cells. Scavenging of these radicals by the test compound is used as a test for antioxidant activity. The reduction of these radicals is observed by the decreased absorbance at 230nm with increasing concentration of the test drug. In hydrogen peroxide scavenging assay, the percentage inhibition of hydroalcoholic extract on H_2O_2 at 160 µg/ml was found to be 52.18% whereas for the chloroform extract was found to be 53.24% while ascorbic acid shows 64.09% inhibition. IC_{50} value of hydroalcoholic extract of *Allium cepa* was found to be 120µg/ml whereas for the chloroform extract IC_{50} value was found to be 90µg/ml. The IC_{50} of ascorbic acid was found to be 42µg/ml. (Figure 7)

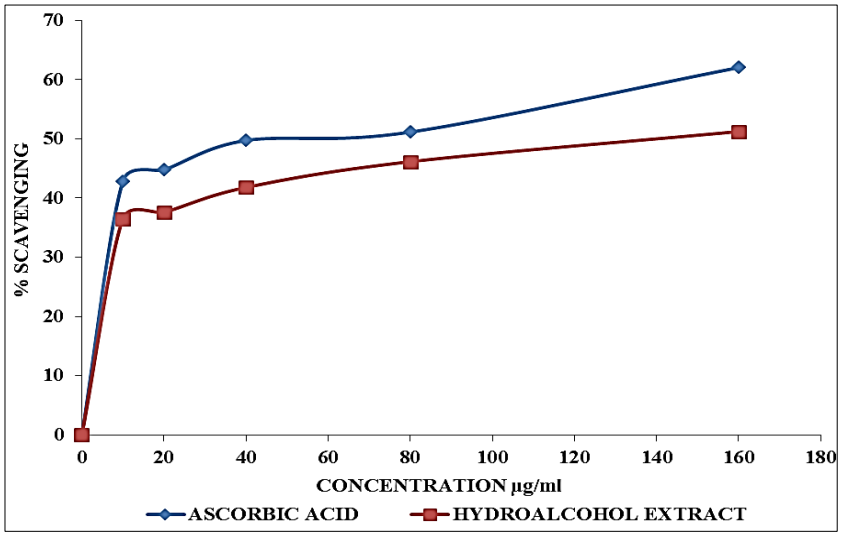

Fig 3: Effect of ascorbic acid and hydroalcoholic extract of *Allium cepa* on hydrogen peroxide scavenging assay

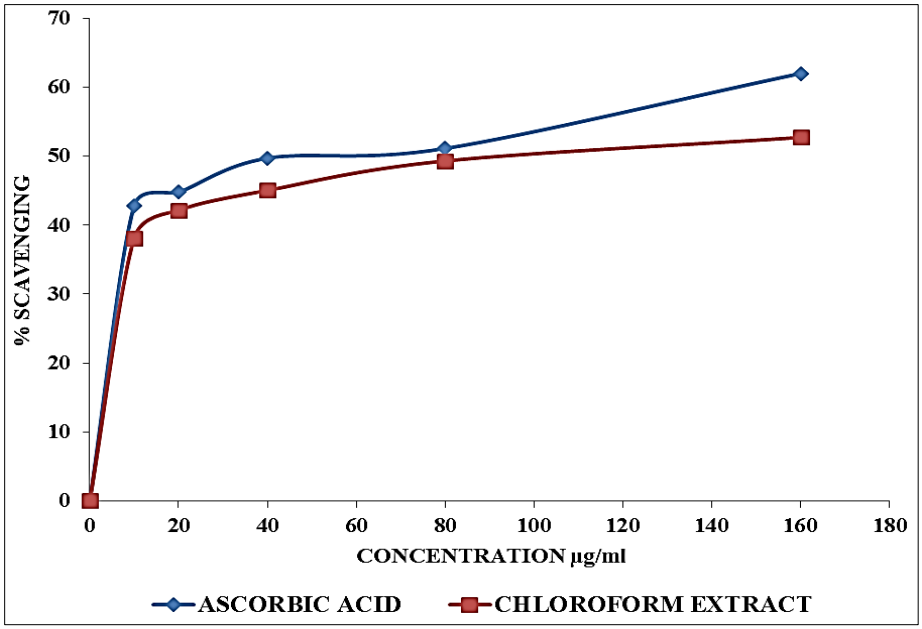

Fig 4: Effect of ascorbic acid and chloroform extract of *Allium cepa* on hydrogen peroxide scavenging assay

Effect of hydroalcoholic and chloroform extract of *Allium cepa* on DPPH free radical scavenging activity

The free radical scavenging activity of the hydroalcoholic and chloroform extract of *Allium cepa* was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (2,2-diphenyl-2-picryl hydrazyl). The method is based on the reduction of 5, 5 dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione (GSH) to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm by using a commercial kit. Degree of inhibition of DPPH by monitoring the decrease in absorbance measured at 517 nm. Ascorbic acid was used as the reference compound.

Radical scavenging activity was expressed as inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ inhibition} = (A_0 - A_t)/A_0 \times 100$$

Where A_0 was the absorbance of control (blank without extract) and A_t was the absorbance in presence of extract. All the tests were performed in triplicate and graph was plotted with mean values.

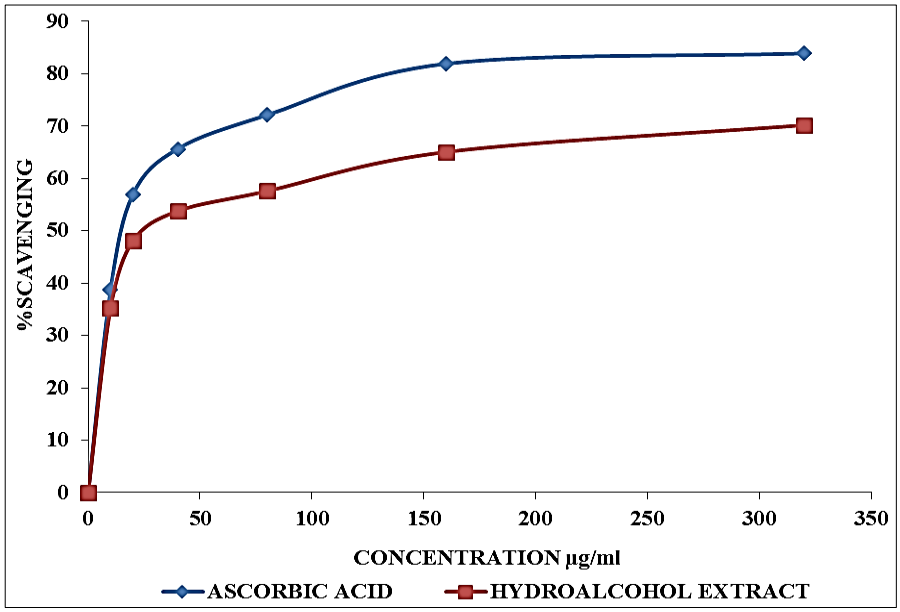

Fig 5: Effect of ascorbic acid and hydroalcoholicic extract of *Allium cepa* on % scavenging of DPPH

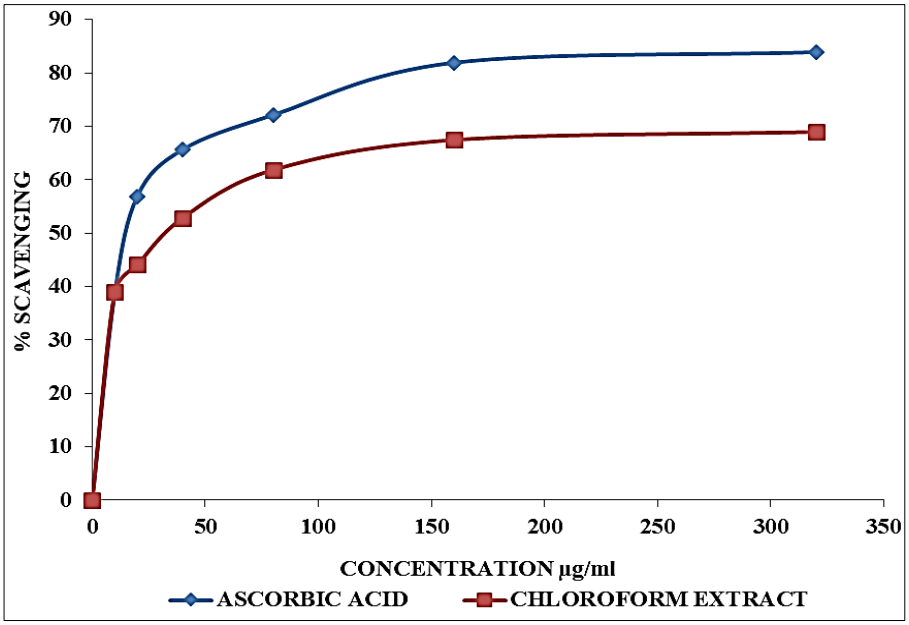

Fig 6: Effect of ascorbic acid and chloroform extract of *Allium cepa* on hydrogen peroxide scavenging assay % scavenging of DPPH

The percentage of scavenging effect on the DPPH radical increases with increase in the concentrations of extract from 10 – 320 µg/ml. HA and CH extract decolorized DPPH due to its hydrogen donating ability. The percentage of inhibition in hydroalcoholic extract varies from 38.42 % (at 10µg/ml) to 67.21% (at 320 µg/ml) whereas chloroform extract varies from 35.45% (at 10µg/ml) to 64.3% (at 320µg/ml). Whereas in case of ascorbic acid percentage inhibition was found to be 37.35% – 86.47% inhibition.

In DPPH free radical scavenging assay, IC_{50} value of hydroalcoholic extract of NA was found 28µg/ml and chloroform extract of NA was 34µg/ml found while that of ascorbic acid was found to be 16.1µg/ml.

Discussion

Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. The phenolic compounds mainly produce their antioxidant action by adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994) [17]. The most widespread and diverse phenolics are the flavonoids and possess significant antioxidant activity towards a number of easily oxidizable compounds (Robards *et al.*, 1999) [18]. In the present study, *in-vitro* anti-oxidant activity of *Allium cepa* was carried out by using reducing power assay, hydrogen peroxide scavenging activity, total phenol content method and DPPH free radical scavenging assay. In case of total phenolic content in hydroethanol extract and chloroform extract was found to be 19.42 mg/g and 28.60 mg/g as Gallic acid equivalent (GAE) which was also reported in previous study (Srinivasan, 2011) showed 1.37mg/100mg sample in hydroalcoholic extract. In reducing power assay, EC_{50} value for hydroethanol and chloroform extract was found to be 30µg/ml and 43µg/ml respectively while that of ascorbic acid as found to be 18µg/ml due to presence of the reducers (antioxidants) causes the conversion of ferric ions/ ferricyanide complex used in the study to ferrous form and may be attributed to hydrogen donation from phenolic compounds (Shimada *et al.*, 1992) [19].

In hydrogen peroxide scavenging assay, the percentage inhibition of hydroalcoholic extract on H_2O_2 at 160 µg/ml was found to be 52.18% whereas for the chloroform extract was found to be 53.24% while s ascorbic acid shows 64.09% inhibition. IC_{50} value of hydroalcohol extract of *Allium cepa* was found to be 120µg/ml whereas for the chloroform extract IC_{50} value was found to be 90µg/ml. The IC_{50} of ascorbic acid was found to be 42µg/ml. DPPH is a stable free radical and antioxidant on interaction with DPPH neutralize its free radical character and discoloration indicates the scavenging activity of the extract (Sochor *et al.*, 2010) [20]. The free radical scavenging activity of the hydroalcohol and chloroform extract of *Allium cepa* was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (2,2-diphenyl-2-picryl hydrazyl).

The method is based on the reduction of 5, 5 dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione (GSH) to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm by using a commercial kit. The percentage of scavenging effect on the DPPH radical increases with increase in the concentrations of extract from 10 – 320 µg/ml. HA and CH extract decolorized DPPH due to its hydrogen donating ability. The percentage of inhibition in hydroalcohol extract varies from 38.42 % (at 10µg/ml) to 67.21% (at 320 µg/ml) whereas chloroform extract varies from 35.45% (at 10µg/ml) to 64.3% (at 320µg/ml). Whereas in case of ascorbic acid percentage inhibition was found to be 37.35% – 86.47% inhibition.

In DPPH free radical scavenging assay, IC_{50}value of hydroalcohol extract of NA was found 28µg/ml and chloroform extract of NA was 34µg/ml found while that of ascorbic acid was found to be 16.1µg/ml. From all above studies, antioxidant or free radical scavenging activity of *Allium cepa* is due to the presence of flavonoids & terpenoids.

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