Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2016; 8(3); 146-151

ISSN: 0975-5160

Research Article

Evaluation of Antioxidant Activity and Total Phenolic Content of *Eruca sativa* L. Seeds

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Available Online:10th June, 2016

ABSTRACT

Oxidative stress ensues due to the imbalance between the production and elimination of reactive oxygen species eventually leading to cell apoptosis which in turn aggravates chronic disorders like diabetes and its related complications. This study was designed to evaluate the protective effect of *Eruca sativa* L. against oxidative stress. *E. sativa* seeds were procured from the local market and authenticated from NISCAIR, New Delhi. *In vitro* antioxidative potential of *E. sativa* was evaluated by employing various *in vitro* antioxidant methods (total phenol content, total antioxidant capacity, reducing power, DPPH, hydrogen peroxide, nitric oxide and superoxide dismutase scavenging activity. The total phenol content was found to be 216.00 and 229.00 mg/g GAE in EA and EHA and total antioxidant capacity was found to be 111.00 and 230.60 µM/g ascorbic acid equivalents respectively. In the DPPH radical scavenging activity, hydrogen peroxide scavenging activity, nitric oxide scavenging activity and superoxide radical scavenging activity, IC_{50} values of EA and EHA was found to be 3.28 and 3.53 µg/ml, 188.11 and 181.56µg/ml, 73.05 and 64.33 µg/ml and 87.91 and 41.12mg/ml, respectively. The results obtained in this study clearly indicate that *Eruca sativa* L. has a significant potential to use as a natural anti-oxidant agent.

Keywords: *E. sativa;* Antioxidant activity; Total phenol content.

INTRODUCTION

Production of free radicals exceeded (commonly designated as reactive oxygen species, ROS) was ascertained to play multiple important roles in tissue damage and loss of function in a number of tissues and organs[1,2]. Various synthetic and natural antioxidants are being used for attenuating the production of ROS. Synthetic antioxidants like, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have restricted use in foods as they are carcinogenic[3]. Therefore, it is mandatory to evolve natural antioxidants to produce effective and non-toxic amelioration of ROS[4,5].

Eruca sativa L. (Leaves of salad rocket) is a diploid annual herbaceous plant growing up to 80 cm[6,7]. Leaves of salad rocket (*Eruca sativa* L.) are increasingly eaten by humans either alone or as part of mixed salads, and are also used in herbal remedies[8]. There is sporadic information available about phytochemistry and bioactivity of this oily crop[9]. It is known as diuretic, anti-inflammatory, antibacterial[10] and also acts as an anticancer agent[11].

Eruca seeds have high oil contents, protein glucosinolate and Erucic acid contents and commonly used as animal feed in Asia, particularly in India and Pakistan[12]. The major and structurally unique glucosinolate (GLS) in leaves of *Eruca sativa* was identified as 4-mercaptobutyl GLS. 4-methylthiobutyl GLS, 4-methylsulfinylbutyl GLS and 4-(cystein-s-yl) butyl GSL were also present at lower concentrations[13,14]. Three new quercetin glycosides isolated from leaves of *Eruca sativa* (Mill.), namely quercetin 3,3O,4O-tri-O-β-D-glucopyranoside, quercetin 3O-(6-sinapoyl-O-β-D-glucopyranosyl)-3,4O-di-O-β-D-glucopyranoside, and quercetin 3- (2-sinapoyl-O-β-D-glucopyranosyl)-3O-(6-sinapoyl-O-β-D-glucopyranosyl)-4-O-β-D-glucopyranoside[15].

Phytochemical investigations of the aqueous extract of *Eruca sativa* fresh leaves, afforded the presence of nine natural flavonoid compounds which were isolated and identified as kaempferol 3-*O*-(2"-*O*malonyl- β-D-glucopy-ranoside)-4'-*O*-β-D-glucopy-ranoside, kaempferol 3,4'-*O*-diglucopyranoside, rhamnocitrin 3-*O*-(2"-*O*-methylmalonyl-β-D-glucopyranoside)-4'-*O*-β-D-glucopyranoside, 3-*O*glucopyranoside, 4'-*O*-glucopyranoside, rhamnocitrin 3-*O*-glucopyranoside, 4'-*O*glucopyranoside, kaempferol and rhamnocitrin[11].

E. sativa is known to ameliorate hyperglycemia and also considering the potential flavonoidal moieties present in the plant gives the idea that it can potentially treat diabetes as well as the complications associated with diabetes[16]. In the present study *in vitro* antioxidative potential of alcoholic (EAE) and hydro-alcohol (40%) (EHE) extracts of *E. sativa were* evaluated by using DPPH free radical-scavenging assay, nitric oxide scavenging assay, hydrogen peroxide scavenging assay, reducing power, superoxide radical scavenging activity, total phenolic content by Folin-Ciocalteau method and total antioxidant activity by phosphomolybdenum method.

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Table 1: Preliminary phytochemical screening of alcohol and hydroalcohol extract of *E. sativa*

S. No.	Chemical constituent	EA	EHA
1.	Alkaloids	-	-
2.	Carbohydrates	+	+
3.	Glycosides	-	-
4.	Saponins	-	+
5.	Fixed oils and fats	-	-
6.	Terpenoids	+	-
7.	Phenolic compounds	-	+
8.	Tannins	-	-
9.	Flavonoids	-	+
10.	Proteins	-	-
11.	Amino acids	-	-

MATERIAL AND METHODS

Materials and reagents

Seeds of *Eruca sativa* L. were procured from local market. Taxonomic identification was done by Dr. Sunita Garg, NISCAIR, New Delhi, India and the plant specimen (NISCAIR/RHMD/Consult/2013/2296/76) has been submitted in Herbarium for future records. 2,2-diphenyl-1-picrylhydrazyl (DPPH), NADPH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BSA, Nitroblue tetrazolium (NBT), Folin-Ciocalteau reagent, *N*-(1-Naphthyl) ethylenediamide dihydrochloride were purchased from Molychem Pvt. Ltd., India. All other chemicals and reagents used were of analytical grade.

Preparation of extracts

Powdered seeds (500 g) were extracted sequentially with petroleum ether, chloroform, alcohol and hydro-alcohol (40%) using soxhlet apparatus. The extracts were concentrated under reduced pressure at 40˚C. The dried extract thus obtained was used directly or re-dissolved in ethanol or water for the determination of antioxidant activity.

Preliminary phytochemical screening

The alcohol and hydro-alcohol extract *L. usitatissimum* were used to test the chemical compounds, including alkaloids, carbohydrates, fixed oils and fats, terpenoids, phenols, tannins, glycosides, saponins, proteins, amino-acids and flavonoids in accordance with the methods of Trease and Evans, Harborne with a slight modification[17,18].

Determination of total phenol content

Total phenolic content in the EA and EHA were determined with Folin-Ciocalteau reagent using gallic acid as a standard phenolic compound. Sample was diluted appropriately to obtain absorbance in the range of calibration curve. An aliquot of 1 ml of sample solution was mixed with 1 ml of Folin–Ciocalteau reagent. 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 measured at 760 nm. The concentration of total phenolic compounds in the extract was obtained as mg of gallic acid equivalent (GAE) per gram dry weight[19].

Total antioxidant capacity (TAOC)

An aliquot of 0.3 ml of EA, EHA and propyl gallate (25, 50, 100, 200 and 400 µg/ml) were mixed with 3 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank 0.3 ml of methanol was used instead of sample. The tubes were capped with aluminium foil and incubated in boiling water bath at 95˚C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm against a blank. Ascorbic acid was used as a standard. Total antioxidant capacity was expressed as equivalents of ascorbic acid (µmol/g) [20].

DPPH scavenging activity

The DPPH radical scavenging ability of EA and EHA was evaluated according to the method of Shimada et al., 1992[21]. The prepared extracts were re-dissolved in alcohol and different concentration (3.0 ml; 1-5 µg/ml) in each reaction set, were mixed with 1.0 ml of 0.1 mM of DPPH in ethanol. The mixture was incubated in the dark for 30 min at room temperature. 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:

$$\% \text{ Inhibition} = \frac{A_0 - A_t}{A_0} \times 100$$

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.

Hydrogen peroxide scavenging activity

The activity was determined according to the method of Ruch et al., 1989[22]. An aliquot of 40 mM H_2O_2 solution (0.6 ml) was mixed with various concentrations of the extracts (EA and EHA; 10-320 µg/ml). To the mixture 2.4 ml of phosphate buffer (0.1 M, pH 7.4) was added and the mixture was shaken vigorously and incubated at room temperature for 10 min. Then, the absorbance of the reaction mixture was determined at 230 nm. V_C was used as the positive control. The H_2O_2 scavenging activity was calculated as follows:

$$\% \text{ Inhibition} = 1 - \left(\frac{A_1 - A_2}{A_0}\right) \times 100$$

Where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample and A_2 is the absorbance of the sample only (phosphate buffer instead of H_2O_2 solution). The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of H_2O_2.

Reducing power assay

The Fe^{3+}-reducing power of EA and EHA was determined according to the method of Oyaizu (1986)[23]. Different concentrations (10-320 µg/ml) of extracts (2.5 ml) were mixed with 2.5 ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50˚C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid (*w/v*) was added and the mixture centrifuged at 1000 rpm for 8 min. The supernatant (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1% of

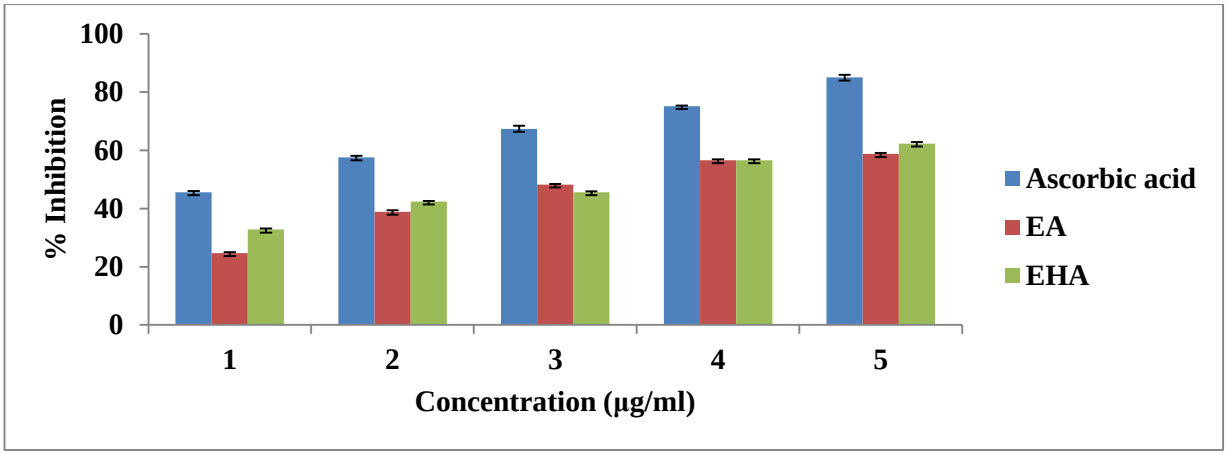

Figure 1: DPPH radical scavenging activity of alcohol and hydroalcohol extract of *Eruca sativa*. EA: *E. sativa* alcohol extract; EHA: *E. sativa* hydroalcohol extract. Values are mean ±SD for n=3

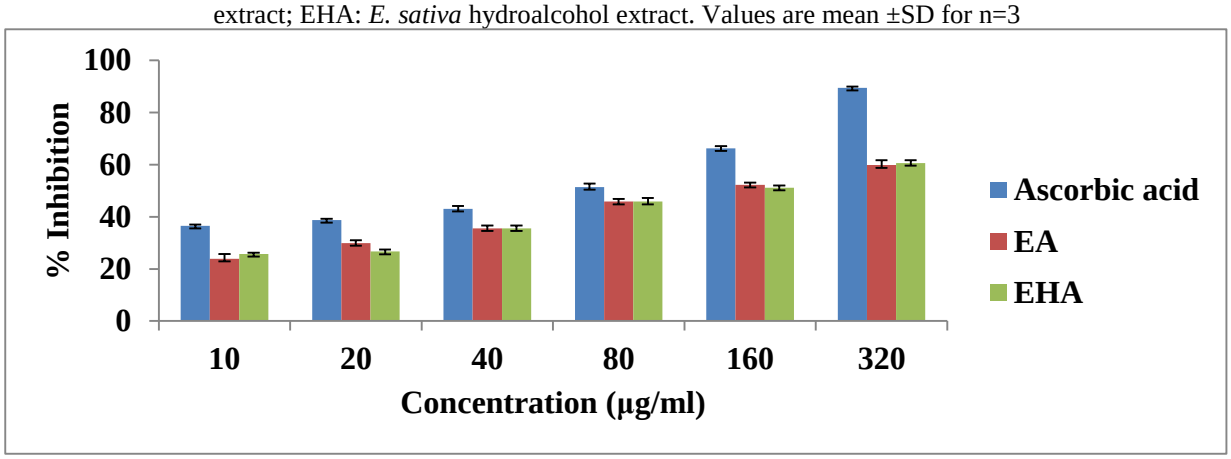

Figure 2: Hydrogen peroxide scavenging activity of the alcohol and hydroalcohol extract of *Eruca sativa*. EA: *E. sativa* alcohol extract; EHA: *E. sativa* hydroalcohol extract. Values are mean ±SD for n=3

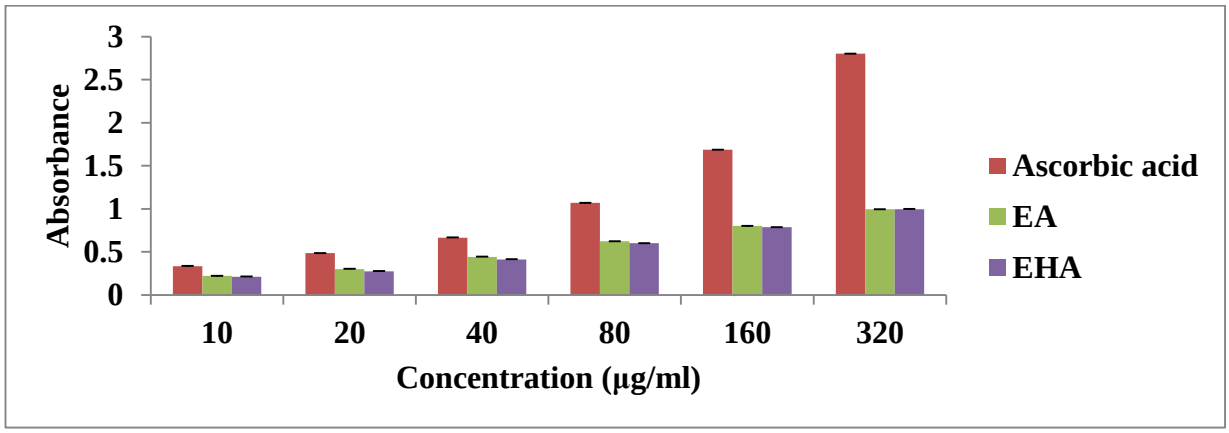

Figure 3: Reducing power assay of the alcohol and hydroalcohol extract of *Eruca sativa*. EA: *E. sativa* alcohol extract; EHA: *E. sativa* hydroalcohol extract. Values are mean ±SD for n=3

ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. The assay was carried out in triplicate and the results expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph plotted between absorbance at 700 nm against extract concentration. Ascorbic acid was used as standard.

Nitric oxide scavenging activity

At physiological pH, aqueous solution of sodium nitroprusside spontaneously generates nitric oxide[24] which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. The reaction mixture of 5mM Sodium nitroprusside in phosphate buffer saline (PBS) and 3.0 ml of different concentrations (10-320 µg/ml) of the EA and EHA was incubated at 25˚C for 150 min. After incubation,

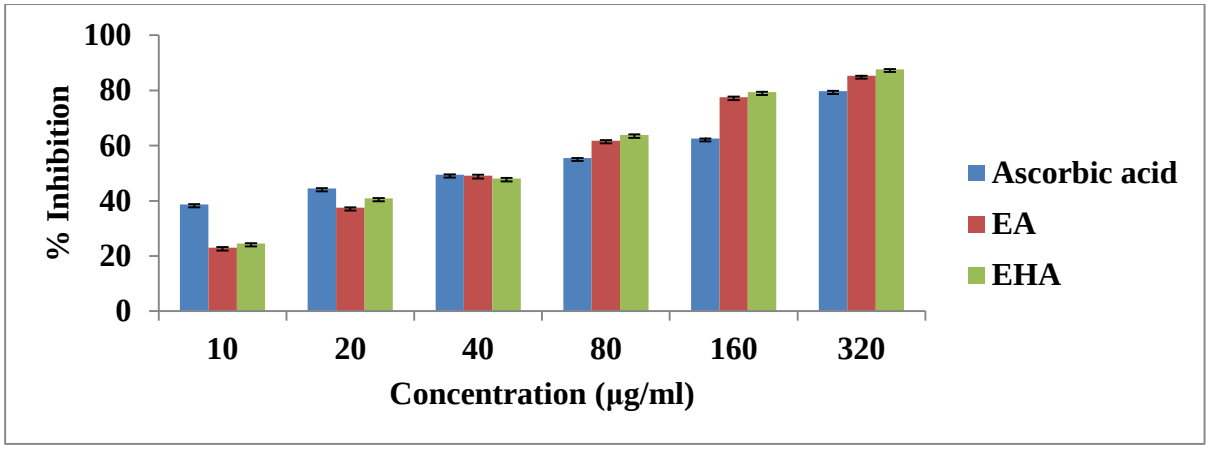

Figure 4: Nitric oxide scavenging activity of the alcohol and hydroalcohol extract of *Eruca sativa*. EA: *E. sativa* alcohol extract; EHA: *E. sativa* hydroalcohol extract. Values are mean ±SD for n=3

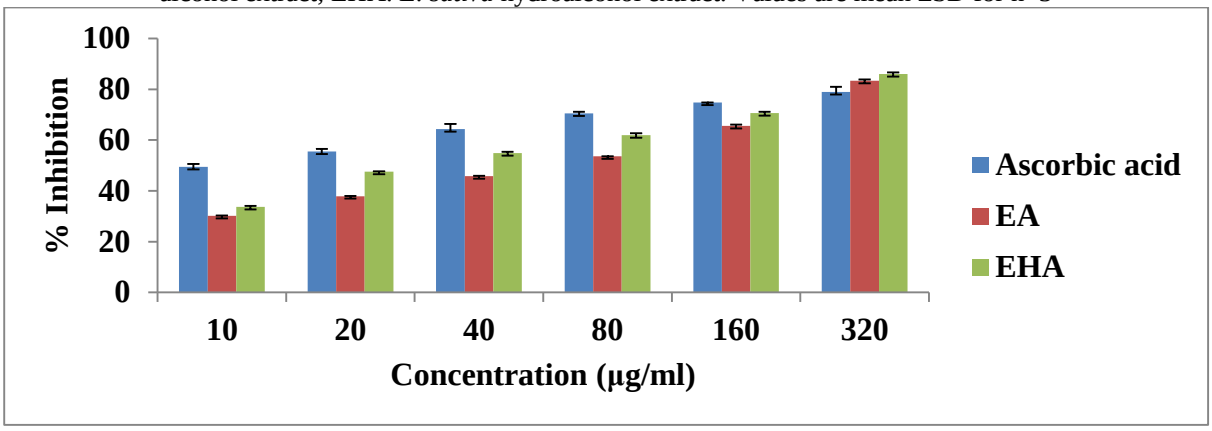

Figure 5: Superoxide radical scavenging activity of the alcohol and hydroalcohol extract of *Eruca sativa*. EA: *E. sativa* alcohol extract; EHA: *E. sativa* hydroalcohol extract. Values are mean ±SD for n=3

the samples were added to Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The pink chromophore generated during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was measured at 546 nm.

Ascorbic acid was used as positive control. The percentage of inhibition was measured by the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_t}{A_0} \times 100$$

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.

Superoxide radical scavenging activity

The activity was measured by the reduction of NBT (nitroblue tetrazolium reagent) method as described by Shukla et al., 2009[25]. The method is based on generation of superoxide radical (O^{2-}) by auto-oxidation of hydroxylamine hydrochloride in presence of NBT, which gets reduced to nitrite. Nitrite in presence of EDTA gives a color that was measured at 560 nm. Different concentrations of extract (10-320 µg/ml) were taken in a test tube. To this, reaction mixture consisting of 1 ml of (50 mM) sodium carbonate, 0.4 ml of (24 mM) NBT and 0.2 ml of 0.1 mM EDTA solutions were added to the test tube and immediate reading was taken at 560 nm. After incubating the reaction mixture at 25˚C for 15 min, about 0.4 ml of (1 mM) of hydroxylamine hydrochloride was added to initiate the reaction and reduction of NBT was measured at 560 nm. Ascorbic acid was used as the positive control. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage of inhibition was calculated according to the following equation:

$$\% \text{ Inhibition} = \frac{A_0 - A_t}{A_0} \times 100$$

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

RESULTS

Phytochemical screening: Preliminary phytochemical screening of EA and EHA showed the presence of carbohydrates, phenolic compounds, terpenoids and flavonoids (see Table 1).

Antioxidant activity of EA and EHA

Results obtained in the present study revealed a considerable level of phenols in the seed extracts of *E. sativa*. Total phenol content present in EA and EHA was

found to be 216 and 229 mg/g Gallic Acid Equivalent (GAE) respectively. Total antioxidant capacity reflects the total antioxidant defence system. The phosphomolybdenum method used in the present study involves the reduction of molybdenum VI (Mo^{6+}) to a green Mo^{5+} complex in acidic medium. Total antioxidant capacity of EA and EHA was found to be 111 and 230.6 µM/g ascorbic acid equivalent respectively.

The reduction capacity of DPPH was determined by the decrease in absorbance at 517 nm (Figure 1). On the DPPH radical, EA and EHA had significant effect with increasing concentration from 1-5 µg/ml. Scavenging effect of ascorbic acid was higher than that of extracts. The IC_{50} value of EA and EHA were 3.28 and 3.53 µg/ml respectively while that of ascorbic was found to be 1.43 µg/ml. A higher DPPH radical scavenging activity is associated with lower IC_{50} value. The loss of H_2O_2 can be measured at 230 nm when it is incubated with a scavenger. Figure 2 shows the scavenging ability of EA and EHA in comparison with ascorbic acid on H_2O_2 at different concentrations. The IC_{50} value of EA and EHA was found to be 188.11 and 181.56 µg/ml respectively while that of ascorbic acid was found to be 80 µg/ml.

Reducing power may serve as a significant indicator of potential antioxidant activity. In this method reducing power was determined based on the ability of the extract to reduce ferric ions/ferricyanide complex to ferrous ion complex. The formation of ferrous ion complex was observed by the formation of Perl's Prussian blue color at 700 nm (Figure 3). EC_{50} (effective concentration at which the absorbance is 0.5) was calculated from the calibration curve and was found to be 92.5 and 104.5 µg/ml for EA and EHA respectively and 21.42 µg/ml for ascorbic acid.

Various concentrations of EA and EHA (10-320 µg/ml) showed significant inhibition against NO radical in a dose dependant manner. The concentration of EA and EHA required for 50 % inhibition (IC_{50}) was found to be 73.05 and 64.33 µg/ml respectively whereas that of ascorbic acid was found to be 63.55 µg/ml (Figure 4). Moreover, EA and EHA had significant activity against superoxide radicals in a dose dependant manner (Figure 5). IC_{50} of ascorbic acid was found to be 27.96 µg/ml and that of EAE and EHE was 87.91 and 41.12 µg/ml respectively.

DISCUSSION

In the present study, we have investigated the different fractions of *E. sativa* seeds for total phenol content and *in vitro* antioxidant properties. Natural antioxidants may be useful in preventing deleterious effects of oxidative stress. Redox capacity of polyphenolic compounds plays a major role in quenching free radicals. Phenolic compounds from plants are known to be good natural anti-oxidants. However, the activity of synthetic anti-oxidants was often observed to be higher than that of natural anti-oxidants[26]. Phenolic compounds, at certain concentrations, markedly slowed down the rate of conjugated diene formation. The interests of phenolics are increasing in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food[27].

DPPH free radical scavenging method has been widely adopted as a tool for estimating the free radical scavenging activities of lipophilic radicals. A chain of lipophilic radicals is initiated by the lipid auto-oxidation[28]. Hydrogen peroxide (H_2O_2) is generated *in vivo* by several oxidase enzymes. H_2O_2 *via* $OH\cdot$ acts as a messenger molecule in the synthesis and activation of inflammatory mediators[29]. EA and EHA were found to possess potent DPPH and hydrogen peroxide scavenging properties. Nitric oxide (NO) is an essential regulating molecule required for several physiological processes like vasodilation, nerve transmission and control of blood pressure[30]. Excess generation of NO leads to a chain of reactions *via* formation of peroxynitrite ions that are extremely toxic and can be detrimental to human health[31]. The plant products have the property to counteract the effect of excessive NO generation. Superoxide radical is a harmful free radical acting as a precursor of more reactive oxidative species like oxygen and hydroxyl radicals that contributes to tissue damage and various other pathological conditions[30]. Results showed that O^{2-} scavenging activity of EHA was higher than EA. Recent studies have shown that phenolic compounds, particularly flavonoids and catechins, are important antioxidants and superoxide scavengers[32].

CONCLUSION

The present study suggests that *E. sativa* L. has higher amount of phenolic compounds which scavenge free radicals. *E. sativa* L. exhibited strong *in vitro* antioxidant activity *via* scavenging DPPH, H_2O_2, NO and superoxide dismutase radicals. This profound protective effect of *E. sativa* may suggest its extensive use as antioxidant and future studies are directed for their *in vivo* evaluation in diseases resulting from oxidative stress *viz.*, diabetes and its complications.

Conflict of Interest

We declare that we have no conflict of interest.

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