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Qualitative Analysis of the *In vitro* Antioxidant Activity of *Amaranthus spinosus*

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Abstract: Oxidative stress caused by reactive oxygen species and free radicals generated in the living cells may cause various chronic, non-communicable diseases. Current research is directed towards finding naturally occurring antioxidants of plant origin. This study was outlined to probe antioxidant potential of ethanolic extract of *Amaranthus spinosus* (ASE) (whole plant) using different antioxidant models. The ASE showed the antioxidant capacity as the inhibition of DPPH radical by 71.21 %. Also the ASE had potent inhibitory effect on scavenging nitric oxide, superoxide (SOD), ABTS radical and hydroxyl radicals by 66.23 %, 61.22 % and 61.68 % respectively. Moreover, the ASE displayed Ferric reducing antioxidant power (FRAP) effect (69.24 %). These findings confirm the biological efficacy of *Amaranthus spinosus* as a potential source of natural antioxidant.

Key words: *Amaranthus spinosus*; FRAP; Reactive oxygen species; Antioxidant activity.

Introduction

The oxidation processes are intrinsic in the management of energy of all living organisms and are therefore, kept under strict control by various cellular mechanisms [14]. However, the production excessive reactive oxygen species (ROS) and the antioxidant protection due to unbalanced mechanisms result in the onset of numerous diseases such as cancer, arteriosclerosis, diabetes and cardiovascular diseases as well as accelerate ageing [1]. The antioxidant molecules are considered as possible protection agents reducing oxidative damage of the human body, when the internal enzymatic mechanisms fail or are inadequately efficient [13]. In the intracellular system of the cell, ROS are produced in cytoplasmic molecules, cytoplasmic proteins, membrane enzymes, peroxisomes, mitochondrial and microsomic electron transport systems [20,21]. These ROS produced by number of exogenous sources are potentially damaging tran-

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sient chemical species [1]. The importance of dietary antioxidant components for chronic disease prevention and health quality improvement has attracted huge research attention since last few decades [3,22].

Amaranthus spinosus Linn. (Family: Amaranthaceae), commonly known as "Kantanotya" in "Bengali", is used as vegetable and cultivated throughout in Bangladesh, India, Sri Lanka and many tropical countries [19]. Plants were freshly collected from Ramna Park Dhaka Bangladesh, identified and authenticated by DR. M.A. Razzaque Shah PhD, Tissue Culture Specialist, BRAC Plant Biotechnology Laboratory, Bangladesh and the voucher specimen no. maintained for the collected samples has been deposited in our laboratory for future references. Moreover, the juice of *Amaranthus spinosus* is used by tribals of Kerala, India to prevent swelling around stomach while the leaves are boiled without salt and consumed for 2-3 days to cure jaundice [16]. The plant possess hepatoprotective, antioxidant activity, aqueous extract has shown significant immunostimulating and stem extract has been credited with antimalarial activity [24]. Hence, the aim of this research is to determine the potential antioxidant efficacy of ethanolic extract of *Amaranthus spinosus* (ASE) by *in vitro* analysis.

Materials and methods

Preparation of the sample

The coarsely powdered *Amaranthus spinosus* plant material (1 kg) was extracted with petroleum ether thrice to remove the fatty material and further marc was extracted thrice with ethanol (50 %, v/v) and concentrated under reduced pressure to yield 6.12 %, w/w (ASE). For *in vitro* studies ASE was dissolved in DMSO. Further dilutions were made to obtain different concentrations and were used for *in vitro* investigations.

Antioxidant Assay

DPPH free radical scavenging assay

The DPPH radical-scavenging capacity of ASE was determined according to the method described by Kao and Chen [23] with some modifications. Briefly, 2 µL of ASE with various concentrations (0.25-2 mg/ml) was added to 198 µL solution of ethanolic DPPH radical solution (0.05 mM). The mixture was mixed vigorously and incubated at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured in triplicate at 517 nm. The scavenging ability was defined as:

Scavenging activity (%) = [1- (Asample - Ablank) /Acontrol]×100

Nitric oxide radical scavenging assay

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Ill osvoy reaction [12]. The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various doses (0-2 mg/ml) of the test solution in a final volume of 1 ml. After incubation for 150 min at 37°C, 500 ml of Griess reagent [sulfanilamide (0.33 % in 20 % glacial acetic acid and napthylethylene diamine dihydrochloride (NED) (0.1 % w/v)) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against ASE. All tests were performed six times. Galic acid was used as a standard.

Superoxide radical scavenging assay

This activity was measured by the reduction of NBT according to a previously reported method [9]. The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 200 µL reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 ìM), NBT (50 µM), PMS (15 µM) and various concentrations (0.25-2 mg/ml) of sample solution. After incubation for 15 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. All tests were performed six times. Galic acid was used as positive control.

Hydroxyl radical scavenging

This was assayed as described by Elizabeth and Rao [8] with a slight modification. The assay is based

on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe3+-ascorbate-EDTA-H2O2 system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH2PO4-KOH buffer (20 mM, pH 7.4); FeCl3 (100 µM); EDTA (100 µM); H2O2 (1.0 mM); ascorbic acid (100 ìM) and various concentrations (3-100 µg/ml) of the ASE. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8 % TCA, then 1 ml 1 % aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Qercetin, a classical OH^- Scavenger, was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

ABTS radical scavenging assay

The antioxidant capacity of ASE was estimated in terms of the ABTS radical scavenging activity following the procedure described by Delgado-Andrade *et al*. [7]. Briefly, ABTS was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12-16 h before use. The ABTS+ solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance at 730 nm of 0.70±0.02. After the addition of 2 µL of ASE to 198 µL of diluted ABTS+ solution, incubated in dark for 30 min. All samples were analyzed in triplicate. The ABTS radical-scavenging activity of the samples was expressed as

$$S\ \% = (Acontrol - Asample)/Acontrol) \times 100$$

Where, Acontrol is the absorbance of the blank control (ABTS solution without test sample) and Asample is the absorbance of the test sample (ASE).

FRAP (Ferric reducing antioxidant power) assay

The FRAP method used is from Benzie and Strain with little modifications [11]. The FRAP solution was prepared in a proportion of 10:1:1 of acetate buffer (300 mM) (pH 3.6), 2,4,6-tripyridyl-s-triazine (TPTZ) (10 mM) in HCl (40 mM) and FeCl3 (20 mM), respectively. FRAP solution was incubated at 37°C for 30 minutes before mixing with the appropriate dilution of ASE. The absorbance was measured at 593nm using microplate reader. Results were expressed as mg of ascorbic acid equivalents (µM).

Statistical analysis

All data were expressed as the mean ± SD. Analysis of variance using one-way ANOVA was performed to test the significance of differences between means at the 5 % level of significance using the statistical analysis software, SAS (SAS 9.1 Version, NC, USA).

Result and discussion

DPPH scavenging activity

The results of this assay showed significant decrease in the concentration of DPPH radicals due to the scavenging ability of ASE and standard compound, ascorbic acid (Figure 1). The scavenging effect of ASE and standard compound at the concentration of 100 µg/ml, on the DPPH radical was found to be 67.61 % and 91.97 % respectively. The results were concentration-dependent and statistically significant ($p < 0.05$). Similarly findings on DPPH radical scavenging activities of various plant extracts have also been observed previously [2,10]. Recently Kaur *et al*.[18] also reported DPPH radical scavenging activity of ethanolic extracts of some selected medicinal plants with a maximum inhibitory effect on inhibiting DPPH radicals by 81.80 %.

Nitric oxide scavenging activity

Nitric oxide as a cell signaling molecule has been associated with a variety of physiological processes in the human body. It transmits signals from vascular endothelial cells to vascular smooth muscle cells and causes vasodilatation 4. It plays a vital role in physiological functions in respiratory, immune, neuromuscular and other systems. Nitric oxide also regulates the release of neurotransmitter, neuronal excitability, learning and memory processes as well as inflammatory bowel syndrome, sepsis, septic shock, cephalalgia, dementia, multiple sclerosis and stroke [4].

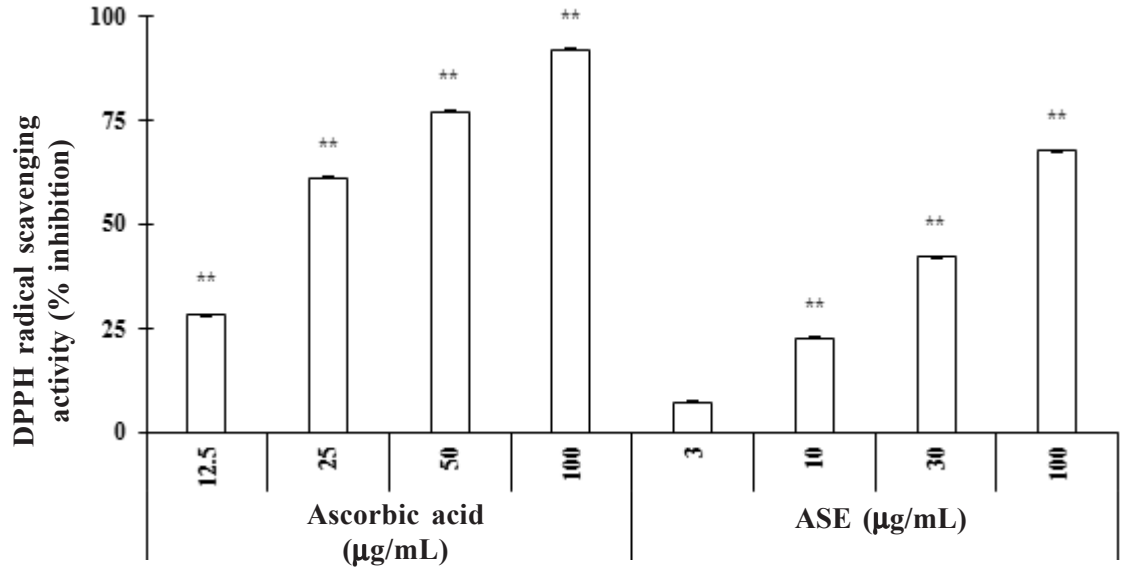

Fig. 1. DPPH radical scavenging activity of ethanolic extract of *Amaranthus spinosus* (ASE) and standard antioxidant compound, ascorbic acid

Although the mechanisms by which nitric oxide may inhibit lipid peroxidation are not clearly understood, one possible mechanism relates to the ability of nitric oxide to terminate propagation of lipid peroxidation reactions. Figure 2 shows scavenging effect of ASE and standard compound galic acid on the nitric oxide radicals at the concentration of 100 µg/ml which was found to be 72.10 % and 87.80 % respectively. The results were statistically significant ($p < 0.05$) and concentration dependent.

Superoxide scavenging activity

The body generate highly toxic species superoxide radical (O^{2-}) by various biological and metabolic reactions. Although relatively weak superoxide oxidants display only limited chemical reactivity, they are potential precursors of a highly reactive species including hydrogen peroxide, hydroxyl radical and singlet oxygen, causing lipid peroxidation [17]. Therefore, superoxide radical scavenging capacity is the first line of defense mechanism in the human being against oxidative

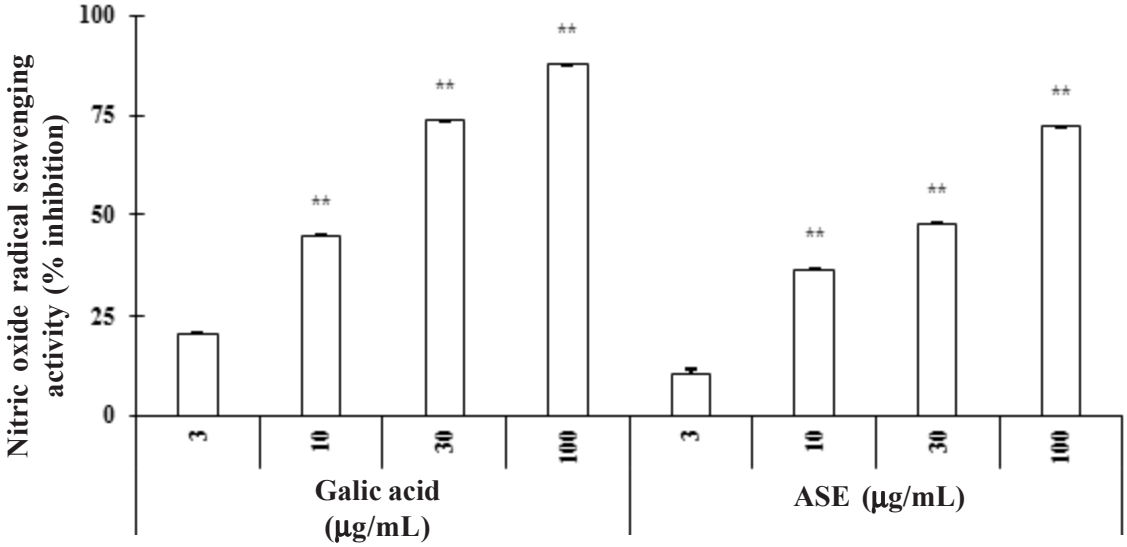

Fig. 2. Nitric oxide radical scavenging activity of ethanolic extract of *Amaranthus spinosus* (ASE) and standard antioxidant compound, gallic acid

stress. Superoxide anion is an oxygen-centered radical with a selective reactivity [17]. It has also been reported that antioxidant properties of some plant products are effective mainly via scavenging of superoxide anion radical [6]. In this assay, superoxide anions derived from dissolved oxygen by the PMS/NADH system reduce NBT.

Figure 3 shows significant ($p < 0.05$) and concentration-dependent inhibition of superoxide radical generation by ethanolic extract of *Amaranthus spinosus* (ASE) of all concentrations of 100 µg/ml and the standard compound tested. At the concentration of 100 µg/ml, the superoxide radical inhibitory effect of 100 µg/ml, and Galic acid was found to be 57.17 %, and 81.77 %, respectively.

Hydroxyl radical scavenging

This assay shows the abilities of the extract and standard quercetin to inhibit hydroxyl radical-mediated deoxyribose degradation in Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results are shown in figure 4. At 100 µg/ml, the percentage inhibition values were 78.03 % for ethanolic extract of *Amaranthus spinosus*

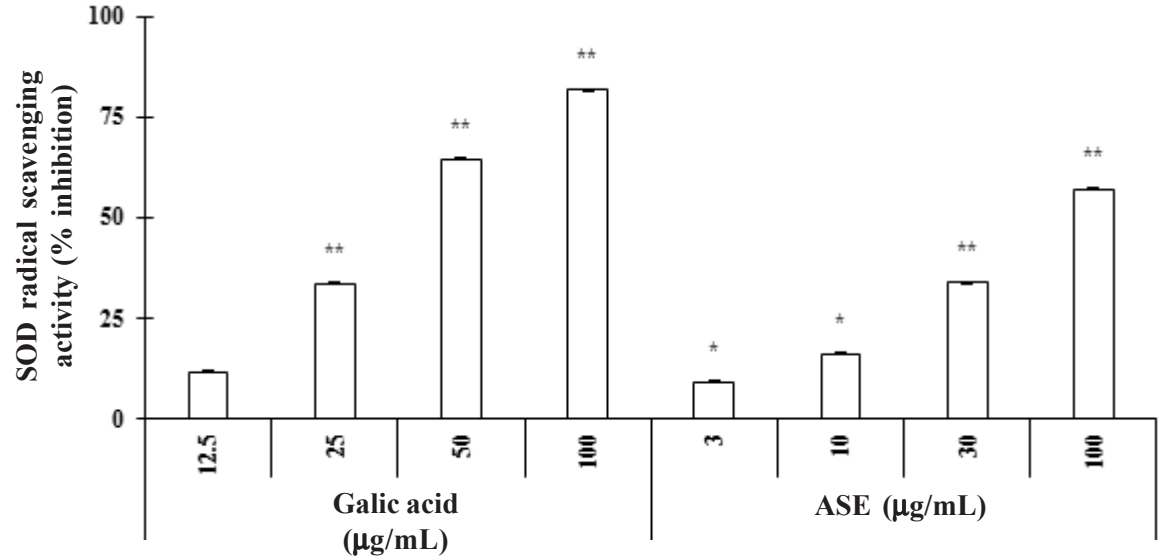

Fig. 3. Superoxide radical scavenging activity of ethanolic extract of *Amaranthus spinosus* (ASE) and standard antioxidant compound, gallic acid

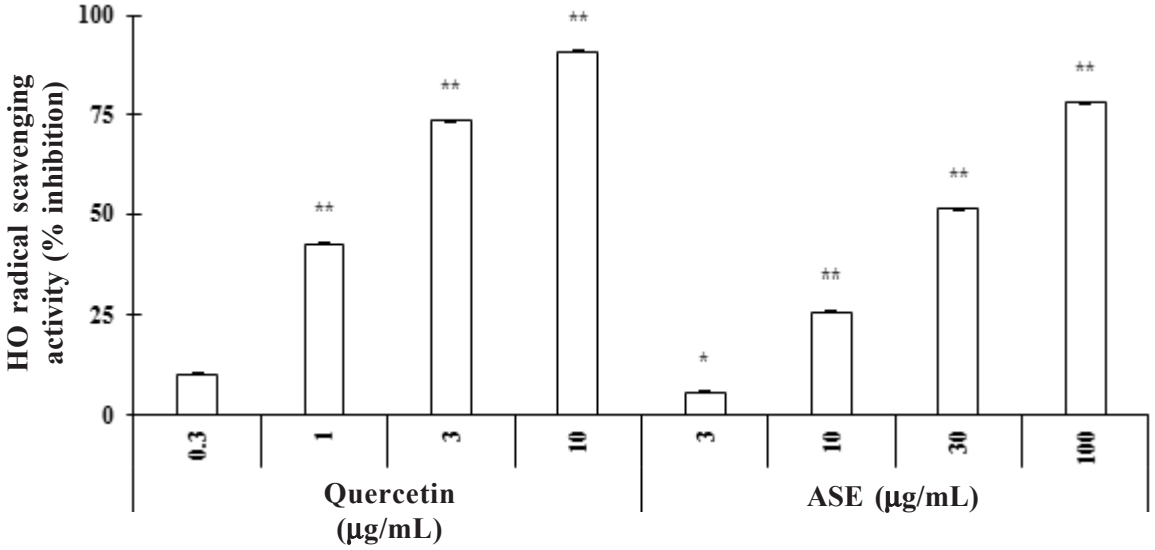

Fig. 4. Hydroxyl radical scavenging activity of ethanolic extract of *Amaranthus spinosus* (ASE) and standard antioxidant compound, quercetin

(ASE). Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage [5]. They were produced in this study by incubating ferric-EDTA with ascorbic acid and H_2O_2 at pH 7.4, and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA)-like product. This compound forms a pink chromogen upon heating with TBA at low pH [15]. When ethanolic extract of *Amaranthus spinosus* (ASE) was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction.

ABTS radical scavenging assay

The results of this assay showed significant decrease in the concentration of ABTS radicals due to the scavenging ability of ASE and standard compound, ascorbic acid **(**Figure 5). The scavenging effect of ASE and standard compound at the concentration of 100 µg/ml, on the ABTS radical was found to be 65.10 % and 92.18 % respectively. The results were concentration-dependent and statistically significant ($p < 0.05$).

FRAP (Ferric reducing antioxidant power) assay

FRAP method was used to present rather quick and simple method measuring antioxidant presents in the ethanolic extract of *Amaranthus spinosus* (ASE). The FRAP assay is based on the ability of phenolics to reduce yellow ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (FE(II)-TPTZ) by the action of electron donating antioxidants [11]. The result of blue color measured spectrophotometrically at 593 nm is taken as linearly related to the total reducing capacity of electron-donating antioxidants. As shown in Figure. 6, ethanol extract showed significant different with all solvent used in this study ($p<0.05$). Absorbance values measured in the extracts varied from 3 to 100 µg/ml. ASE showed the 688 % antioxidant activity in FRAP assay which proved that ASE contained substantial ferric reducing activities compared to the standard ascorbic acid.

Conclusion

On the basis of the results obtained in this study, it is concluded that ethanolic extract of *Amaranthus spinosus* (ASE), containing alkaloids, glycosides, terpenoids, saponin, phenols and steroids as essential phytochemicals, exhibited significant antioxidant and free radical scavenging activities. Moreover, ASE exerted potent antioxidant activity as reducing power ability. Antioxidant activity of herbal extracts is of considerable interest to food industry which is looking for such compounds with significant biological potential to be used as alternatives to synthetic and conventional food preservation system. Hence, these findings indi-

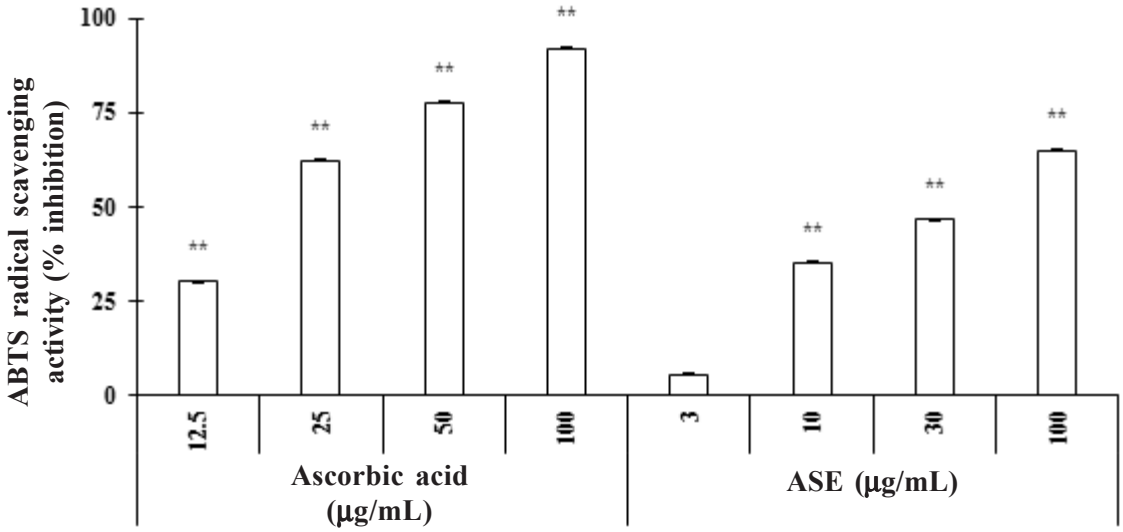

Fig. 5. ABTS radical scavenging activity of ethanolic extract of *Amaranthus spinosus* (ASE) and standard antioxidant compound, ascorbic acid

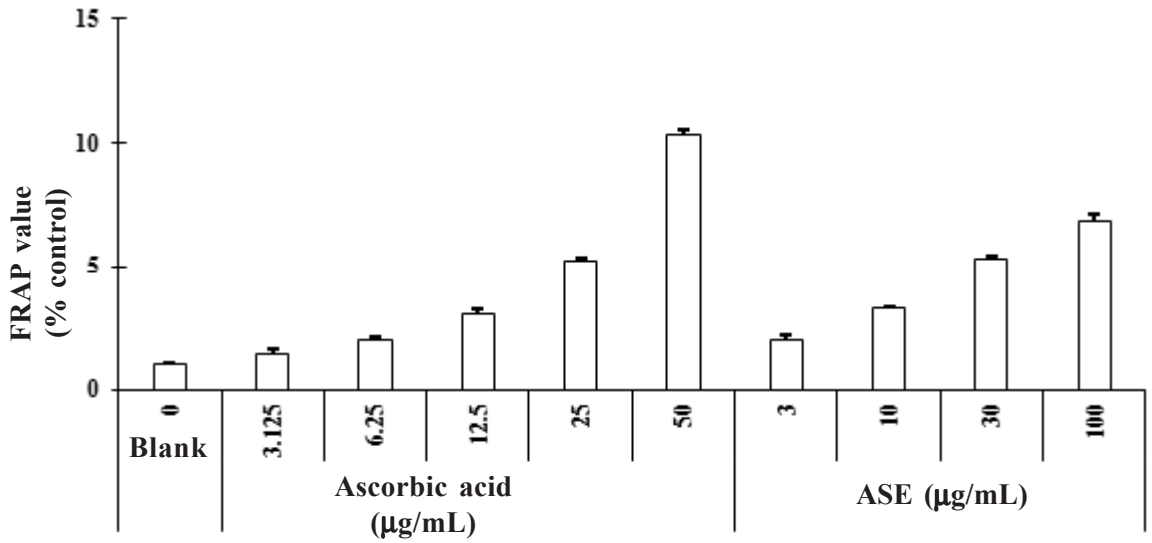

Fig. 6. Ferric reducing antioxidant power of ethanolic extract of *Amaranthus spinosus* (ASE) and standard antioxidant compound, gallic acid

cate that ASE can be a source of natural antioxidant for using in food industry against oxidative deterioration as well as a useful therapeutic agent in the prevention of oxidative stress-related degenerative diseases. Further, studies are planned to isolated individual biologically active constituents from ASE in order to establish their precise *in vivo* antioxidant mode of action.

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