Research Journal of Biological Sciences 2 (3): 372-382, 2007

Determination of *In vitro* Antioxidant and Radical Scavenging Activity of *Verbascum oreophilum* C. Koch Var. Joannis (Fam. Scrophulariaceae)

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Abstract: In the present study, Water Extract (WEVO) and Ethanol Extract (EEVO) of *Verbascum oreophilum* C. Koch Var. Joannis (Fam. Scrophulariaceae) was evaluated by employing various in vitro antioxidant assay such as 1, 1-Diphenyl-2-Picryl-Hydrazyl free radical (DPPH) scavenging, total antioxidant activity determination by ferric thiocyanate, total reducing ability determination by Fe^{3+}-Fe^{2+} transformation method, superoxide anion radical scavenging by riboflavin/methionine/illuminate system, hydrogen peroxide scavenging and ferrous ions (Fe^{2+}) chelating activities. WEVO, EEVO inhibited 94.and 95.5% lipid peroxidation of linoleic acid emulsion at 75 μg mL^{-1} concentration, respectively. On the other hand, α-tocopherol indicated inhibition of 70% on peroxidation of linoleic acid emulsion at 75 μg mL^{-1} concentration. In addition, WEVO and EEVO had an effective DPPH scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, Ferric ions (Fe^{3+}) reducing power and Ferrous ions (Fe^{2+}) chelating activities. In addition, those various antioxidant activities were compared to BHA, BHT α-tocopherol and trolox as references antioxidant compounds. Also, total phenolic content in both WEVO and EEVO were determined as gallic acid equivalents. The total phenolics content of WEVO and EEVO were determined by the Folin-Ciocalteu procedure and 48.6 and 97.1 mg gallic acid equivalent of phenols was detected in 1 mg WEBP and EEBP.

Key words: Antioxidant activity, *verbascum oreophilum*, mullein, phenolics content, reducing power, radical scavenging

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. The oxygen consumption inherent in cell growth leads to the generation of a series of Reactive Oxygen Species (ROS) (Barros *et al*., 2006). ROS, which include free radicals such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals (OH•) and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are various forms of activated oxygen. Accordingly, ROS is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into radicals (HOCl, HOBr, O_3, $ONOO^-$, $^1O_2$, H_2O_2). All oxygen radicals are ROS, but not all ROS are oxygen radicals. Peroxynitrite and H_2O_2 are frequently erroneously described in the literature as free radicals, for example. Reactive Nitrogen Species (RNS) is a similar collective term that includes $NO^{\bullet}$ and $NO^{\bullet}_2$ as well as non-radicals such as HNO_2 and N_2O_4. 'Reactive' is not always an appropriate term: H_2O_2, $NO^{\bullet}$ and $O_2^{\bullet-}$ react fast with few molecules, whereas $OH^{\bullet}$ reacts fast with almost everything. Species such as $RO_2^{\bullet}$ $NO_3^{\bullet}$, $RO^{\bullet}$, HOCl, HOBr, $CO_3^{\bullet-}$, $CO_2^{\bullet-}$, $NO_2^{\bullet-}$, $ONOO^-$, NO_2^+ and O_3 have intermediate reactivities (Halliwell, 2006). The interaction of these species with molecules of a lipidic nature produces new radicals: Hydroperoxides and different peroxides (Barros *et al*., 2006).

ROS are continuously produced during normal physiologic events and can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid

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peroxides. ROS is capable of damaging crucial biomolecules such as nucleic acids, lipids, proteins and carbohydrates. Also, ROS and RNS may cause DNA damage that may lead to mutation (Cakir *et al*., 2006). If ROS are not effectively scavenged by cellular constituents, they lead to disease conditions (Halliwell and Gutteridge, 1990; Gulcin *et al*., 2003). ROS may interact with biological systems in a clearly cytotoxic manner. These molecules are exacerbating factors in cellular injury and aging process (Halliwell and Guttridge, 2003; Gulcin *et al*., 2002a, b), prostate and colon cancers, coronary heart disease, atherosclerosis, cancer (Madhavi *et al*., 1996), Alzheimer's disease, diabetes mellitus, hypertension and AIDS (Halliwell and Guttridge, 2003). As a result, ROS and RNS have been implicated in more than 100 diseases, including above mentioned diseases (Gulcin *et al*., 2003; Alho and Leinonen, 1999; Duh *et al*., 1999; Hertog *et al*., 1993; Tanizawa *et al*., 1992).

The harmful action of the free radicals can, however, be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism (Kumaran and Karunakaran, 2006). All aerobic organisms have antioxidant defenses including antioxidant enzymes and foods to remove or repair the damaged molecules (Cakir *et al*., 2006). Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food and pharmaceutical products during processing and storage (Halliwell, 1997). Antioxidants can protect the human body from free radicals and ROS effects. They retard the progress of many chronic diseases as well as lipid peroxidation (Pryor, 1991; Kinsella *et al*., 1993; Lai *et al*., 2001). Hence, a need for identifying alternative natural and safe sources of food antioxidants has been created and the search for natural antioxidants, especially of plant origin, has notably increased in recent years (Skerget *et al*., 2005). Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods (Gulcin *et al*., 2004). At the present time, the most commonly used antioxidants are BHA, BHT, propyl gallate and tert-butylhydroquinone. Besides that BHA and BHT have suspected of being responsible for liver damage and carcinogenesis (Wichi, 1988; Sherwin, 1990). Therefore, there is a growing interest on natural and safer antioxidants (Moure *et al*., 2000; Elmastas *et al*., 2005; Oktay *et al*., 2003).

Antioxidants act include: removing O_2 or decreasing local O_2 concentrations, removing catalytic metal ions, removing key ROS, e.g. $O_2^{\bullet-}$ and H_2O_2, scavenging initiating radicals, e.g. OH●, RO●, $RO_2^{\bullet}$, breaking the chain of an initiated sequence, quenching or scavenging singlet oxygen, enhancing endogenous antioxidant defences by up-regulating the expression of the genes encoding the antioxidant enzymes, repairing oxidative damage caused by radicals, increasing elimination of damaged molecules and not repairing excessively damaged molecules in order to minimise introduction of mutations (Gutteridge, 1994; Wood *et al*., 2006).

The genus *Verbascum* is represented in Turkish flora by 232 species, of which 185 are endemic (Davis, 1967, 1975). Antimicrobial (Barbour *et al*., 2004), antiviral (McCutcheon *et al*., 1995) and cytotoxic (Afifi *et al*., 1993) of some members of this genus have been reported. It was reported that *Verbascum sinaiticum* traditionally used in the treatment of various skin disorders and were screened for antimicrobial activity against different strains of bacteria and fungi which are known to cause different types of skin infections (Telci *et al*., 2006). *Verbascum cheiranthifolium* is used for the treatment of several peptic ulcer symptoms such as stomach ache, heartburn (Gürbüz *et al*., 2005). Ucar Turker and Camper reported that *Verbascum thapsus* has been used for the treatment of inflammatory diseases, asthma, spasmodic coughs, diarrhea and other pulmonary problems (Ucar and Camper, 2002). Additionally, the leaves and flowers of *Verbascum thapsus* are reported to have expectorant and demulcent properties which are used to treat respiratory problems such as bronchitis, dry coughs, whooping cough, tuberculosis, asthma and hoarseness (Grieve, 1981; Mabey, 1988).

Some members of this genus are also used for liquor production (Cemek and Kucuk, 2001). In addition, some antioxidant properties such as DPPH free radical scavenging and β-carotene bleaching of *Verbascum wiedemannianum* were reported (Tepe *et al*., 2006). However, we could not find any report in the literature dealing with the antioxidant and radical scavenging properties of *Verbascum oreophilum*.

The aim of this study were to investigate the total antioxidant activity, Ferric ions (Fe^{3+}) Reducing Antioxidant Power assay (FRAP) using the potassium ferricyanide reduction method, DPPH• radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities of WEVO and EEVO. Additionally, an important goal of this research was *in vitro* antioxidative effects of WEVO and EEVO were compared with commercial and standard antioxidants such as BHA, BHT, α-tocopherol and trolox commonly used by the food and pharmaceutical industry.

MATERIALS AND METHODS

Chemicals: Riboflavin, methionine, Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT),

Nitroblue Tetrazolium (NBT), 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), 3-(2-pyridyl)-5, 6-bis (4-phenyl-sulfonic acid)-1, 2, 4-triazine (Ferrozine), linoleic acid, α-tocopherol, polyoxyethylenesorbitan monolaurate (Tween-20) and Trichloroacetic Acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate was purchased from Merck Darmstadt, Germany). All other chemicals used were in analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant material: Air-dried aerial parts of the plant *Verbascum oreophilum* C. Koch Var. Joannis (0.1 kg) were obtained from a local market at Erzurum province, Turkey. The plants were identified by Dr. Ercan Kaya, Atatürk University, Kazım Karabekir Education Faculty, Department of Biology Education.

Plant material and extraction procedures: For preparation of water extraction, 50 g air-dried aerial parts of the plant *Verbascum oreophilum* C. Koch Var. Joannis were grinded Into a fine powder in a mill and was mixed with 1 L boiling water by magnetic stirrer during 15 min. Then the extract was filtered over Whatman No. 1 paper. The filtrates were frozen and lyophilised in lyophilizator at 5 μm Hg pressure at -50°C (Labconco, Freezone 1L).

For ethanol extraction 50 g air-dried aerial parts of the plant *Verbascum oreophilum* C. Koch Var. Joannis were grinded into a fine powder in a mill and was mixed with 500 mL ethanol. The residue was re-extracted under some condition until extraction solvents became colourless. The obtained extracts were filtered over Whatman No. 1 paper and the filtrate was collected, then ethanol was removed by a rotary evaporator (RE 100 Bibby, Stone Staffordshire, England) at 50°C to obtain dry extract. Both extracts were placed in a plastic bottle and then stored at -20°C until used.

Antioxidant evaluation

Determination of total phenolic compounds by folin-ciocalteu reagent: The amount of total phenolic contents in the WEVO and EEVO was determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977). Folin-Ciocalteu is a method used for the determination of total phenolic compounds. Gallic acid was used as a standard phenolic compound. Briefly, 1 mL of extract solution contains 1 mg extracts, in a volumetric flask diluted with distilled water (46 mL). One millilitre of Folin-Ciocalteu reagent was added and the content of the flask mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2%) was added and then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. The amount of total phenolic compounds in WEVO and EEVO determined as microgram of Gallic Acid Equivalent (GAE) using an equation that was obtained from standard gallic acid graph (r^2:0.9217):

$$\text{Absorbance}_{(760\ nm)} = 0.0014 \times \text{Total Phenols [GAE (μg)]}$$

Total antioxidant activity determination by ferric thiocyanate method: The antioxidant activity of WEVO, EEVO and standards was determined according to the ferric thiocyanate method as described by Gülçin (2006). For stock solutions, 10 mg of WEVO and EEVO was dissolved in 10 mL ethanol. Then, the solution which contains different concentration of WEVO or EEVO (from 25 to 75 μg mL^{-1}) solution in 2.5 mL of sodium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion in sodium phosphate buffer (0.04 M, pH 7.0). Therefore, 5 mL of the linoleic acid emulsion was prepared by mixing and homogenising 15.5 μL of linoleic acid, 17.5 mg of Tween-20 as emulsifier and 5 mL phosphate buffer (pH 7.0). On the other hand, 5 mL of control was composed of 2.5 mL of linoleic acid emulsion and 2.5 mL, 0.04 M sodium phosphate buffer (pH 7.0). The mixed solution (5 mL) was incubated at 37°C in polyethylene flask. The peroxide levels were determined by reading the absorbance at 500 nm in a spectrophotometer (Shimadzu, UV-1208 UV-VIS Spectrophotometer, Japan) after reaction with $FeCl_2$ and thiocyanate at intervals during incubation. During the linoleic acid peroxidation, peroxides are formed and that leads to oxidation of Fe^{2+}-Fe^{3+}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. The absorbance of the red colour was measured at 500 nm until reaching a maximum value. This step was repeated every 5 h. The percentage inhibition values were calculated at this point (30 h). High absorbance indicates high linoleic acid emulsion peroxidation. The solutions without WEVO or EEVO were used as blank samples. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was calculated by following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = 100 - \left(\frac{A_S}{A_C} \times 100\right)$$

in here A_C is the absorbance of control reaction which contains only linoleic acid emulsion and sodium phosphate buffer and A_S is the absorbance in the presence of WEVO and EEVO sample or standard compounds.

Ferric ions (Fe^{3+}) Reducing Antioxidant Power assay (FRAP): The reducing power of WEVO and EEVO was determined by the method of Oyaizu (1986) with slight modification (41). Different concentrations of WEVO and

EEVO (25-75 μg mL^{-1}) in 1 mL of distilled water were mixed with sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10%) were added to the mixture. The 2.5 mL of this solution was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates an increase of reduction capability.

Ferrous ions (Fe^{2+}) chelating activity: The chelating of ferrous ions by WEVO and EEVO was estimated by the method of Dinis *et al.* (1994) wherein the Fe^{2+}-chelating ability of WEVO and EEVO was monitored by the absorbance of the ferrous iron-ferrozine complex at 562 nm. Briefly, WEVO or EEVO (from 25-75 μg mL^{-1}) in 0.4 mL was added to a solution of 2 mM $FeCl_2$ (0.2 mL). The reaction was initiated by the addition of 5 mm ferrozine (0.4 mL) and total volume was adjusted to 4 mL of ethanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by using the formula given below:

$$\text{Ferrous ions chelating effect (\%)} = \left(1 - \frac{A_S}{A_C}\right) \times 100$$

where A_C is the absorbance of control and A_S is the absorbance in the presence of WEVO or EEVO or standards. The control contains $FeCl_2$ and ferrozine, complex formation molecules.

Hydrogen peroxide scavenging activity: The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch *et al.* (1989). For this aim, a solution of H_2O_2 (43 mm) was prepared in phosphate buffer (0.1 M, pH 7.4). WEVO and EEVO at the 25 μg mL^{-1} concentration in 3.4 mL phosphate buffer was added to 0.6 mL of H_2O_2 solution (0.6 mL, 43 mm). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing the sodium phosphate buffer without H_2O_2. The concentration of hydrogen peroxide (mm) in the assay medium was determined using a standard curve (r^2:0.9217):

$$\text{Absorbance} = 0.006 \times [H_2O_2] + 0.578$$

The percentage of H_2O_2 scavenging of WEVO, EEVO and standard compounds was calculated using the following equation:

$$H_2O_2 \text{ scavenging effect (\%)} = \left(1 - \frac{A_S}{A_C}\right) \times 100$$

Where A_C is the absorbance of the control and A_S is the absorbance in the presence of the sample WEVO, EEVO or standards (Elmastas *et al.*, 2006).

Radical scavenging activity: The total radical scavenging capacity of the tested compounds was determined and compared to that of BHA, BHT, α-tocopherol and trolox by using the DPPH● and superoxide anion radical scavenging methods.

DPPH free radical scavenging activity: The hydrogen atom or electron donation abilities of some pure compounds were measured by the bleaching of a purple coloured methanol solution of DPPH. This spectrophotometric assay uses the stable radical, 1,1-Diphenyl-2-Picrylhydrazyl (DPPH●), the methodology of Blois (1958) previously described by Gülçin (2006) was used with slight modifications in order to assess the DPPH• free radical scavenging capacity of WEVO and EEVO. Wherein the bleaching rate of a stable free radical, DPPH• is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH• absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. When a hydrogen atom or electron was transferred to the odd electron in DPPH•, the absorbance at 517 nm decreased proportionally to the increases of non-radical forms of DPPH. Briefly, 0.1 mm solution of DPPH• was prepared in ethanol and 0.5 mL of this solution was added 1.5 mL of WEVO and EEVO solution in ethanol at different concentrations (25-75 μg mL^{-1}). These solutions were vortexed thoroughly and incubated in dark. A half hour later, the absorbance was measured at 517 nm against blank samles. Lower absorbance of the reaction mixture indicates higher DPPH• free radical scavenging activity. A standard curve was prepared using different concentrations of DPPH•. The DPPH• concentration scavenging capacity was expressed as mm in the reaction medium and calculated from the calibration curve determined by linear regression (r^2:0.9845):

$$\text{Absorbance} = 9.692 \times [\text{DPPH•}] + 0.215$$

The capability to scavenge the DPPH• radical was calculated using the following equation:

$$\text{DPPH}\cdot \text{ scavenging effect (\%)} = \left(1 - \frac{A_S}{A_C}\right) \times 100$$

Where A_C is the absorbance of the control which contains 0.5 mL control reaction (containing DPPH• solution except the WEVO or EEVO) and A_S is the absorbance in the presence of WEVO and EEVO (Gulcin *et al*., 2004). DPPH•, decreases significantly upon exposure to proton radical scavengers (Yamaguchi *et al*., 1998).

Superoxide anion radical scavenging activity: Superoxide anion scavenging activity of WEVO and EEVO was based on the method described by Liu *et al.* (1997) with a slight modification (Oktay *et al*., 2003). The superoxide radicals were generated in 3 mL of Tris-HCl buffer (16 mm, pH 8.0) containing 1 mL of Nitroblue Tetrazolium (NBT) (50 mm) solution, 1 mL NADH (78 mm) solution and sample solution of black pepper extracts (75 mg mL^{-1}) in water were mixed. The reaction was started by adding 1 mL of Phenazine Methosulphate (PMS) solution (10 mm) to the solution mixture. Then, reaction mixture was incubated at room temperature for 5 min. The absorbance was measured at 560 nm in a spectrophotometer against blank samples. L-Ascorbic acid was used as a control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$O_2^{\cdot -} \text{ scavenging effect (\%)} = \left(1 - \frac{A_S}{A_C}\right) \times 100$$

Where A_C is the absorbance of the control and A_S is the absorbance of WEVO, EEVO or standards.

Statistical analysis: The experimental results were performed in triplicate. The data were recorded as mean±standard deviation and analysed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's Multiple Range tests. $p<0.05$ was regarded as significant and $p<0.01$ was very significant.

RESULTS AND DISCUSSION

Natural antioxidants were closely related to their biofunctionalities, such as the reduction of chronic diseases like DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacteria growth which is often associated with the termination of free radical propagation in biological systems (Covacci *et al*., 2001; Zhu *et al*., 2002). Antioxidant capacity is widely used as a parameter for medicinal bioactive components. In this study, the antioxidant activity of the WEVO and EEVO was compared to BHA, BHT and α-tocopherol. The antioxidant activity of the WEVO and EEVO and standards has been evaluated in a series of *in vitro* tests: DPPH• free radical scavenging, total antioxidant activity by ferric thiocyanate method, reducing power, scavenging of superoxide anion radical-generated non-enzymatic system, hydrogen peroxide scavenging and metal chelating activities.

Table 1 shows the yields and total phenolic contents of WEVO and EEVO. The antioxidant effect of plant phenolics has been studied in relation to the prevention of coronary diseases and cancer, as well as age-related degenerative brain disorders (Parry and Bolwell, 2000). In addition, it was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen *et al*., 1993; Gulcin, 2005). As it can be seen in Table 1, 48.6 and 97.1 μg GAE of phenols was detected in 1 mg of WEBP and EEBP. According to the recent reports, a highly positive relationship between total phenols and antioxidant activity was found in many plant species (Yamaguchi *et al*., 1998).

Total antioxidant activity determination in linoleic acid emulsion by ferric thiocyanate method: Lipid peroxidation contains a series of free radical-mediated chain reaction processes and is also associated with several types of biological damage (Perry *et al*., 2000). The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation which is the primary product of lipid oxidation. In this assay, hydroperoxide produced by linoleic acid added to the reaction mixture, which has oxidized by air during the experimental period, was indirectly measured. Ferrous chloride and thiocyanate react with each other to produce ferrous thiocyanate (red colour) by means of hydroperoxide (Siddhuraju *et al*., 2002).

Total antioxidant activity of WEVO, EEVO, BHA, BHT and α-tocopherol was determined by the ferric thiocyanate method in the linoleic acid system. WEVO and EEVO exhibited effective antioxidant activity in this system. The effect of different oncentrations

Table 1: Yield and total phenolic contents in percent of WEVO and EEVO (WEVO: Water Extract of *Verbascum oreophilum* C. Koch Var. Joannis and EEVO: Ethanol Extract of *Verbascum oreophilum* C. Koch Var. Joannis)

	Yield (%)	Total phenolic compounds (μg)
WEVO	17.6	48.9
EEVO	3.4	97.1

(25-75 μg mL^{-1}) of WEVO and EEVO on lipid peroxidation of linoleic acid emulsion are shown in Fig. 1 and 2. The effect of 75 μg mL^{-1} concentration of WEVO and EEVO was found to be 94.0 and 95.5% and their activities are greater than same concentration of α-tocopherol (70.4). The autoxidation of linoleic acid emulsion without WEVO and EEVO or α-tocopherol was accompanied by a rapid increase of peroxide value. Consequently, these results clearly indicate that WEVO and EEVO had effective and powerfull antioxidant activity by ferric thiocyanate.

Ferric ions (Fe^{3+}) Reducing Antioxidant Power assay (FRAP): Different studies have indicated that the electron donation capacity, reflecting the reducing power, of bioactive compounds is associated with antioxidant activity (Siddhuraju *et al*., 2002). Antioxidants can be explained as reductants and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al*., 2002). There are a number of assays designed to measure overall antioxidant activity or reducing potential, as an indication of host total capacity to withstand freeradical stress (Zhu *et al*., 2002) (Wood *et al*., 2006). The Ferric Ion Reducing Antioxidant Power (FRAP) assay takes advantage of an electron transfer reaction in which a ferric salt is used as an oxidant (Benzie and Strain, 1996). In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

As can be seen from Fig. 3, WEVO and EEVO had effective reducing power using the potassium ferricyanide reduction method when compared to the standard (α-tocopherol). For the measurements of the reductive ability of WEVO and EEVO, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of WEVO and EEVO using the method of Oyaizu (1986). At different concentrations (25-75 μg mL^{-1}), WEVO (R^2:0.9859) and EEVO (R^2:0.9908) demonstrated powerfull reducing ability and these differences were statistically very significant ($p<0.01$). The reducing power of WEVO, EEVO and

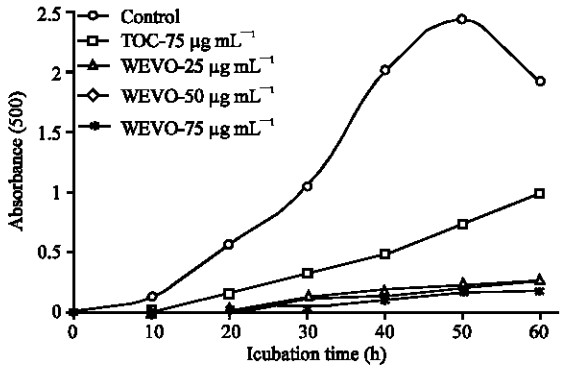

Fig 1: Total antioxidant activities of different concentrations (25-75 μg mL^{-1}) of WEVO and 75 μg mL^{-1} concentration of α-tocopherol (WEVO: Water Extract of *Verbascum oreophilum* C. Koch Var. Joannis)

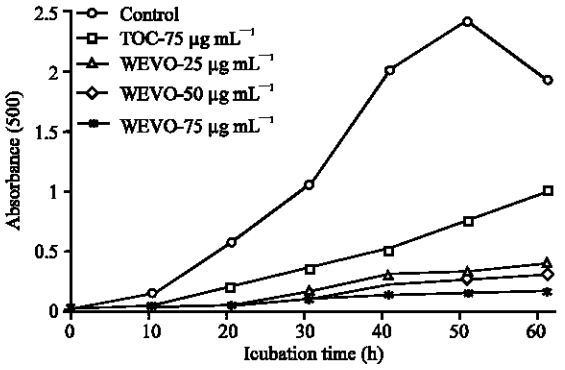

Fig. 2: Total antioxidant activities of different concentrations (25-75 μg mL^{-1}) of EEVO and 75 μg mL^{-1} concentration of α-tocopherol (EEVO: Ethanol Extract of *Verbascum oreophilum* C. Koch Var. Joannis)

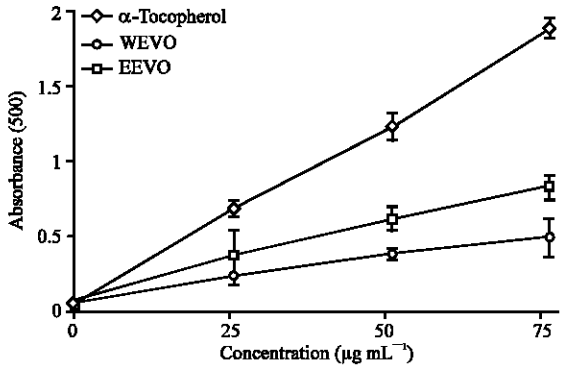

Fig. 3: Total reductive potential of different concentrations (25-75 μg mL^{-1}) of WEVO and EEVO and reference antioxidant; α-tocopherol (WEVO: Water Extract of *Verbascum oreophilum* C. Koch Var. Joannis and EEVO: Ethanol Extract of *Verbascum oreophilum* C. Koch Var. Joannis)

α-tocopherol increased steadily with increasing concentration of samples. Reducing power of WEVO, EEVO and standard compounds exhibited the following order: α-tocopherol>EEVO>WEVO. The results on reducing power demonstrate the electron donor properties of WEVO and EEVO thereby neutralizing free radicals by forming stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging.

Ferrous ions (Fe^{2+}) chelating capacity: Because elemental species such as Ferrous iron (Fe^{2+}) can facilitate the production of ROS within animal and human systems, the ability of substances to chelate iron can be a valuable antioxidant capability (45). Iron, in nature, can be found as either Ferrous (Fe^{2+}) or Ferric ion (Fe^{3+}), with the latter form of ferric ion predominating in foods. Ferrous ions (Fe^{2+}) chelation may render important antioxidative effects by retarding metal-catalyzed oxidation.

Ferrous ions (Fe^{2+}) chelating activities of WEVO and EEVO, BHA, BHT, α-tocopherol and trolox are shown in Table 1. The chelating effect of Ferrous ions (Fe^{2+}) by the WEVO and EEVO and standards was determined according to the method of Dinis (1994). Iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity among transition metals. The effective Ferrous ions (Fe^{2+}) chelators may also afford protection against oxidative damage by removing iron (Fe^{+2}) that may otherwise participate in HO• generating Fenton type reactions.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\bullet \text{ (Fenton reaction)}$$

Ferric (Fe^{3+}) ions also produce radicals from peroxides although the rate is tenfold less than that of ferrous (Fe^{2+}) ions. Ferrous ions (Fe^{2+}) are the most powerful pro-oxidant among the various species of metal ions (Halliwell and Gutteridge, 1984). Minimizing ferrous (Fe^{2+}) ions may afford protection against oxidative damage by inhibiting production of ROS and lipid peroxidation. Ferrozine can quantitatively form complexes with Fe^{2+} in this method, In the presence of chelating agents the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of color reduction therefore allows estimating the metal chelating activity of the coexisting chelator. Lower absorbance indicates higher metal chelating activity. Metal chelation is an important antioxidant property (Kehrer, 2000) and hence WEVO and EEVO were assessed for its ability to compete with ferozzine for Ferrous ions (Fe^{2+}) in the solution. In this assay, WEVO and EEVO are interfered with the formation of Ferrous ions (Fe^{2+}) and ferrozine complex. It was suggesting that they have chelating activity and are able to capture ferrous ion before ferrozine.

The difference between 25 μg mL^{-1} concentration and the control values was statistically significant ($p<0.01$). In additon, as can be seen in Fig. 4, WEVO and EEVO exhibited marked chelation of ferrous ion at all used concentrations ($p<0.01$). On the other hand, the percentages of ferrous ions (Fe^{2+}) chelating capacity of same concentration (75 μg mL^{-1}) of WEVO, EEVO and α-tocopherol were found as 51, 71 and 43%, respectively. These results show that the Ferrous ion (Fe^{2+}) chelating effect of WEVO and EEVO was higher than α-tocopherol ($p<0.05$).

Metal chelating capacity was significant since it reduced the concentration of the catalysing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. The data obtained from Fig. 4 reveal that WEVO and EEVO demonstrates a marked capacity for iron binding, suggesting that their main action as peroxidation protector may be related to its iron binding capacity.

Hydrogen peroxide scavenging activity: Biological systems can produce hydrogen peroxide. Hydrogen peroxide can attack many cellular energy-producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Hyslop *et al*., 1988). It can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. It is used in the respiratory burst of activated

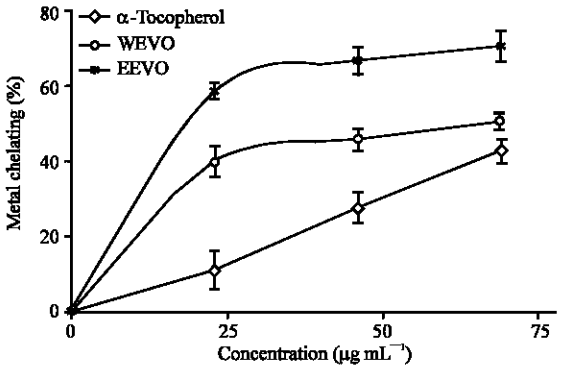

Fig. 4: Metal chelating effect of different concentrations of WEVO, EEVO and α-tocopherol on ferrous ions (Fe^{2+}) (WEVO: Water Extract of *Verbascum oreophilum* C. Koch Var. Joannis and EEVO: Ethanol Extract of *Verbascum oreophilum* C. Koch Var. Joannis)

phagocytes (McDonald *et al*., 2006). The ability of WEVO and EEVO to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.* (1989) as shown in Fig. 5 and compared with that of BHA, BHT and α-tocopherol as standards. Hydrogen peroxide scavenging activity of WEVO and EEVO at the used concentration (25 μg mL^{-1}) was found to be 56±6.3 and 48±5.1%. On the other hand, BHA, BHT, α-tocopherol scavenged 88±4.2, 97±2.2 and 93±3.1% hydrogen peroxide at the same concentration, respectively. At the above concentration, the hydrogen peroxide scavenging effect of WEVO and EEVO and three standard compounds decreased in the order of BHT > α-tocopherol>BHA>WEVO>EEVO. Hydrogen peroxide itself is not very reactive; however it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. Addition of hydrogen peroxide to cells in culture can lead to transition metal ion-dependent OH radicals mediated oxidative DNA damage. Levels of hydrogen peroxide at or below about 20-50 mg seem to have limited cytotoxicity to many cell types. Thus, removing hydrogen peroxide as well as superoxide anion is very important for protection of farmaceutical and food systems (Gulcin *et al*., 2004).

Radical scavenging activity: The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products (Soares *et al*., 1997). Assay

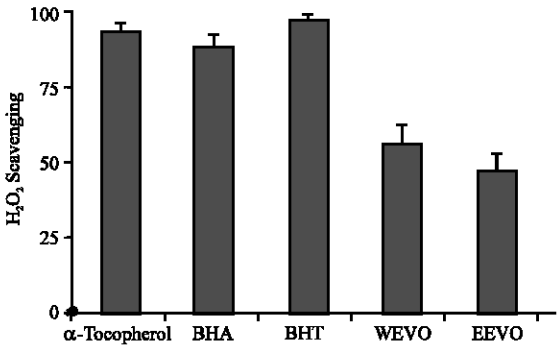

Fig. 5: Hydrogen peroxide scavenging effect of same concentrations of WEVO, EEVO BHA, BHT and α-tocopherol (WEVO: Water extract of *Verbascum oreophilum* C. Koch Var. Joannis and EEVO: Ethanol extract of *Verbascum oreophilum* C. Koch Var. Joannis, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene)

based upon the use of DPPH• is the most popular spectrophotometric methods for determination of the antioxidant capacity of food, beverages and vegetable extracts (Bendini *et al*., 2006). This chromogen radical compound can directly react with antioxidants. Additionally, DPPH• scavenging method have been used to evaluate the antioxidant activity of compounds due to the simple, rapid, sensitive and reproducible procedure (Ozcelik *et al*., 2003).

Radical scavenging activity is very important due to the deleterious role of free radicals in foods and in biological systems. Diverse methods are currently used to assess the antioxidant activity of plant phenolic compounds. Chemical assays are based on the ability to scavenge synthetic free radicals, using a variety of radical-generating systems and methods for detection of the oxidation end-point. DPPH• radical scavenging method is one of the most common spectrophotometric procedure for determining the antioxidant capacities of components. This chromogen (the violet DPPH radical) is easy to use, has a high sensitivity and allow for rapid analysis of the antioxidant activity of a large number of samples. This assay has been applied to determine the antioxidant activity of pure components (Awika *et al*., 2003; Van *et al*., 2000; Yu *et al*., 2002).

In this study, two radical scavenging methods were used to assess the detrermination of potential radical scavenging activities of WEVO and EEVO, namely DPPH radical scavenging and superoxide anion radical scavenging activity. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants (Oyaizu, 1986). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al*., 1997).

With this method it was possible to determine the antiradical power of an antioxidant by measuring of a decrease in the absorbance of DPPH• at 517 nm. Resulting a color change from purple to yellow, the absorbance decreased when the DPPH• was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH• molecule. In the radical form, this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule (Matthaus, 2002). Figure 6 illustrates a significant decrease ($p<0.01$) in the concentration of DPPH radical

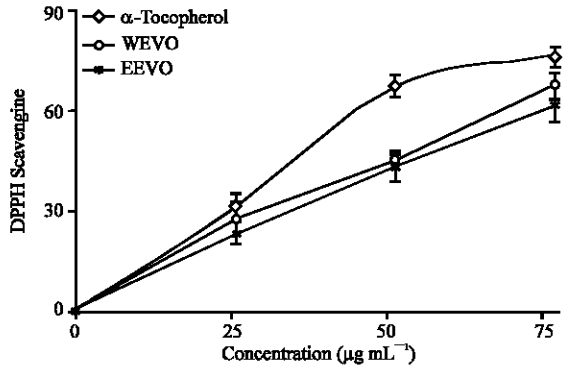

Fig. 6: DPPH free radical scavenging activity of different concentrations (15-45 μg mL^{-1}) of WEVO and EEVO and reference antioxidants; BHA, BHT, α-tocopherol and trolox [WEVO: Water Extract of *Verbascum oreophilum* C. Koch Var. Joannis and EEVO: Ethanol extract of *Verbascum oreophilum* C. KOCH var. joannis, BHA: Butylated Hydroxyanisole, BHT: Butylated Hydroxytoluene; DPPH•: 1, 1- Diphenyl- 2- Picryl- Hydrazyl free radical)

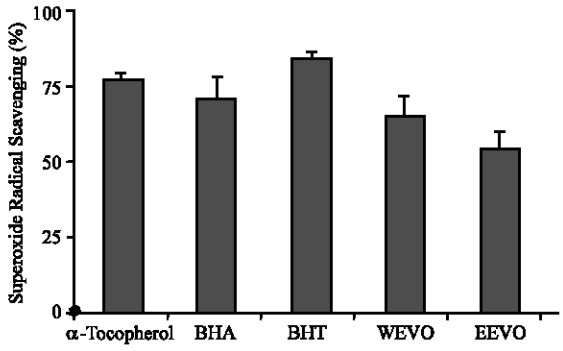

Fig. 7: Superoxide anion radical scavenging activity of 25 μg mL^{-1} concentration of WEVO and EEVO and reference antioxidants; BHA, BHT and α-tocopherol [WEVO: Water Extract of *Verbascum oreophilum* C. Koch Var. Joannis and EEVO: Ethanol Extract of *Verbascum oreophilum* C. Koch Var. Joannis BHA: Butylated hydroxyanisole, BHT: Butylated Hydroxytoluene;)

due to the scavenging ability of WEVO, EEVO and α-tocopherol (r^2:0.9516). The scavenging effect of WEVO (r^2:0.9931), EEVO (r^2:0.9969) and α-tocopherol on the DPPH radical decreased in the order of α-tocopherol >WEVO EEVO, which were 78, 69 and 63%, at the concentration of 75 μg mL^{-1}, respectively. DPPH free radical scavenging activity of WEVO and EEVO also increased with an increasing concentration. Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening the radical scavenging activity of specific compounds (Amarowicz *et al*., 2004).

Superoxides are oxygen-centred radicals with selective reactivity. Although relatively weak oxidants, superoxides exhibit limited chemical reactivity, but can generate more dangerous species, including singlet oxygen and hydroxyl radicals, which cause the peroxidation of lipids (Halliwell and Chirico, 1993). These species are produced by a number of enzyme systems in autooxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complex such as cytochrome c. Superoxide anions are a precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage (Halliwell and Guttridge, 1984). Also, it is easily formed by radiolysis of water in the presence of oxygen and formate, which allows accurate reaction rate constants to be measured (McDonalds *et al*., 2006). It has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Also, superoxide has been observed to directly initiate lipid peroxidation (Wickens, 2001). It has also been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical (Yen and Dhu, 1994). Superoxide anion plays an important role in the formation of other ROS such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA (Pietta, 2000). Superoxide radicals are normally formed first and their effects can be magnified because it produces other kinds of free radicals and oxidizing agents (Liy *et al*., 1997). Superoxide anions derived from dissolved oxygen by riboflavin/methionine/illuminate system and reduce NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the blue NBT formation (Cos *et al*., 1998). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Figure 7 shows the inhibition percentage of superoxide radical generation by 25 μg mL^{-1} concentration of WEVO, EEVO and standards. As seen in Fig. 7, the percentage inhibition of superoxide anion radical generation by 25 μg mL^{-1} concentration of WEVO and EEVO was found as 63.2±6.8 and 52.6±7.3%. On the other hand, at the same concentration, BHA, BHT and α-tocopherol and trolox exhibited 69.6±6.3, 82.2±2.1 and 75.4±2.2% superoxide anion radical scavenging activity, respectively.

CONCLUSION

According to data obtained from the present study, WEVO and EEVO was found to be an effective antioxidant and radical scavenging activity in different *in vitro* assay such as inhibition of linoleic acid peroxidation, Ferric ions (Fe^{3+}) Reducing Antioxidant Power assay (FRAP), DPPH• radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and ferrous ions (Fe^{2+}) chelating activities when it is compared to standard antioxidant compounds such as BHA, BHT and α-tocopherol. Based on the discussion above, it can be used for minimizing or preventing lipid oxidation in pharmaceutical products, retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the shelf life of pharmaceuticals.

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