EXPERIMENTAL STUDY

In Vitro Evaluation of the Antioxidant Activities of Grape Seed (*Vitis vinifera*) Extract, Blackseed (*Nigella sativa*) Extract and Curcumin

Hesham A. El-Beshbishy* Ph. D, Ahmed M. Mohamadin# Ph. D, Ashraf B. Abdel-Naim@ Ph. D

*Department of Medical Laboratories Technology, College of Applied Medical Sciences**,
Clinical Biochemistry Department, College of Medicine#, Taibah University, Al Madinah Al Munawarah,
Pharmacology and Toxicology Department, College of Pharmacy@
King Abdul Aziz University, Jeddah, Saudi Arabia

Abstract

Objective

Evaluation of *in vitro* antioxidant activities of grape seed (*Vitis vinifera*) extract *GSE*, blackseed (*Nigella sativa*) extract *NSE* and curcumin *CUR*.

Methods

In vitro antioxidative potential of *GSE*, *NSE* and *CUR* was evaluated through determination of total soluble phenolic content and reducing power. Also, the antioxidant activity was measured using linoleic acid peroxidation assay, phosphomolybdenum method, superoxide anion radical scavenging assay and DPPH· free radical-scavenging assay.

Results

GS and *NS* were extracted with 50% ethanol and methanol, respectively. Extraction yield of *GSE* and *NSE* was 127 and 109 mg/g dry weight, respectively. *CUR* elicited greater phenolic contents (115±0.04) than *NSE* (14±0.05) and *GSE* (5.2±0.06) mgGAE/g. Percentage scavenging activity of each sample against DPPH· was 1425%, 600% and 333% for *CUR*, *NSE* and *GSE*, respectively. Percentage superoxide scavenging activity was more pronounced in *CUR* (41%) than *NSE* (32%) and *GSE* (14%). *CUR* exhibited highest percentage antioxidant activity (87.1%) followed by *NSE* (77.6%) and *GSE* (61.4%) using phosphomolybdenum method. In Contrary, increased order of antioxidant activity using FTC-linoleic system was *GSE* > *CUR* > *NSE*. All the mentioned experiments were also conducted using vitamin E as a standard antioxidant. Strong linear regression between the total phenolic contents and above parameters in studied samples were noticed as indicated by high r^2 at $P<0.0001$ that was slightly low in DPPH· scavenging activity than the other analyses.

Conclusions

Our results indicated that, reducing power and antioxidative potentials of *GSE*, *NSE* and *CUR* may be attributed to high total phenolic contents. *GSE*, *NSE* and *CUR* may be valuable as food preservative and nutritional health supplements.

Keywords: *GSE*: Grape seed extract; *NSE*: *Nigella sativa* extract; *CUR*: curcumin, DPPH:diphenylpicrylhydrazyl radical, FTC: ferric thiocyanate, antioxidant, total phenolic content.

Journal of Taibah University Medical Sciences 2009; 4(1): 23-35

Correspondence to:
Dr. Hesham A. El-Beshbishy
Medical Laboratories Technology Department
College of Applied Medical Sciences
Taibah University, ✉ 30001
Al Madinah Al Munawarah, Saudi Arabia
☎ +966 4 6460008
🖷 +966 4 8475790
hesham_elbeshbishy@hotmail.com

Introduction

The role of oxygen radicals has been implicated in several diseases, including cancer, and diabetes mellitus[1]. Antioxidants are vital reducing substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. There is an increasing interest in natural antioxidants that present in medicinal and dietary plants, which might help to prevent oxidative damage[2]. There is a growing interest in natural and safer antioxidants, especially of plant origin[3]. More attention has been paid to the study of the antioxidative and anti-lipid peroxidation activity of natural dietary antioxidant and their protective effects against drug-induced toxicities especially whenever free radical generation is involved[4].

The blackseed (*Nigella sativa*), also known as the black caraway seed, the black cumin and the blessed seed, is an annual herb that grows in countries bordering the Mediterranean sea, Pakistan and India. Although blackseed is not a significant component of the human diet, it is regarded in the Middle East as part of an overall holistic approach to health and is thus incorporated into diets. Nutritional investigations on the blackseed showed that they are a good source of potassium, phosphorous, sodium, iron, zinc, calcium, magnesium, manganese and copper[5]. It has been used traditionally for treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema and as anti-tumor, antihistaminic, antidiabetic and antimicrobial[6]. Functional ingredients of grape seeds (*Vitis vinifera*) include several flavonoids with a phenolic nature such as flavanols (catechin and epicatechin), procyanidins and phenolic acids. Recognition of such health benefits of catechins and procyanidins has led to the use of grape seed extract as a dietary supplement[7]. All these constituents have been reported to exhibit antioxidant activity *in vivo* and *in vitro* as they can scavenge a lot of superoxide radicals and thereby play an important role in the inhibition of carcinogenesis and act as antibacterial agent[8]. Also, these constituents can protect against DNA damage caused by free radicals[7,9]. Phenolics in grapes have been reported to inhibit oxidation of human low density lipoproteins (LDL) *in vitro*[10].

Turmeric, *Curcuma longa* rhizomes, has been widely used for centuries in indigenous medicine for treatment of a variety of inflammatory conditions and other diseases. It has been widely used for the treatment of several diseases from cancer to cystic fibrosis. Epidemiological observations suggested that, turmeric consumption may reduce the risk of some types of cancers and render other protective biological effects in humans[11]. The biological effects of turmeric have been attributed to its active constituent named curcumin CUR (diferuoyl methane) [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6 heptadiene-3,5-dione], that has been widely studied for its anti-inflammatory, anti-angiogenic, antioxidant, wound healing and anticancer effects[12]. CUR exhibits strong antioxidant activity, than vitamins C and E[13].

The aim of this study was to evaluate, investigate and gain insights the *in vitro* antioxidative and free radicals scavenging activity of grape seed extract (*GSE*), black seed (*Nigella sativa*) extract (*NSE*) and curcumin (*CUR*) through using different systems, including total soluble phenolic content, reducing power, antioxidant activity using both linoleic acid peroxidation assay and phosphomolybdenum method, superoxide anion radical scavenging and DPPH free radical-scavenging assay.

Materials and Methods

Chemicals

All the solvents and chemicals used in this study were of highest grade and purchased from Sigma-Aldrich (USA) and Acros (Belgium). 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical, α-tocopherol (vitamin E), ammonium molybdate were purchased from Acros (Belgium). Folin–Ciocalteu reagent (2N), dimethyl sulfoxide (DMSO), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH) and nitroblue tetrazolium (NBT) were

purchased from Sigma-Aldrich (USA). Blackseed and grape seeds were purchased from a local authentic herbal store at Al-Madinah Al-Munawwarah as seeds. *CUR* was purchased as fine white powder from Sigma-Aldrich company (USA).

Samples preparation

Grape seeds (*GS*) were dried in drying oven at 50°C for 3 days. GS ground to fine powder by grinder (Moulinex, model 721, France). About 100g of GS powder was extracted with 500 ml of 50% ethanol and left for one day at room temperature. The extract was then filtered through Büchner funnel followed by removal of ethanol using vacuum rotary evaporator at 40°C. The residue obtained was dissolved in water and kept at 4°C until further use[14]. The selection of 50% ethanol as the organic solvent was based on the fact that ethanol when mixed with water facilitates the solubility of the bioactive compounds compared to pure water[15].

Blackseed (*NS*) were powdered using a grinder (Moulinex, model 721, France). The 100g of fine powdered NS were mixed with 500 ml methanol at room temperature for 30 min. The mixture was left for one day at 4°C and then filtered through Büchner funnel followed by removal of methanol using vacuum rotary evaporator at 40°C to obtain the crude extract that was stored in capped vials in the fridge until further use[16]. Extraction yield for either *GSE* or *NSE* was calculated by subtracting the dried weight of plant material residues after extraction from the weight of the original plant material. The amount of extractable compounds was 127 mg/g dry *GS* powder weight and was 109 mg/g dry black seed powder weight. *CUR* was made as stock solution (10mg of *CUR* dissolved in 0.1 ml DMSO and 10ml ethanol). The working solution was made by diluting the stock solution with phosphate buffer (0.04M, pH 7.0)[17]. Vitamin E, *GSE* and *NSE* were prepared as solution (0.1mg/ml) in 0.1ml DMSO and ethanol.

Total soluble phenolic contents estimation

The total soluble phenolic contents of *GSE*, *NSE* and *CUR* were determined using Folin–Ciocalteu reagent[18]. One ml of each plant extract solution or *CUR* (0.1 mg/ml), was mixed with 7.5 ml Folin–Ciocalteu reagent which was diluted 10× with dist. H_2O. After standing at room temperature for 5 min, 7.5 ml of 60 mg/ml of aqueous Na_2CO_3 solution were added. The mixture was kept at room temperature for 2 hrs and then absorbance was measured at 725nm against blank using Genway spectrophotometer. The results were expressed in gallic acid equivalents (GAE), per g of dry weight from the calibration curve of gallic acid. The experiment was also conducted using vitamin E (0.1mg/ml) as a reference antioxidant. The samples were analyzed in triplicates.

Determination of the reducing power

One ml of each plant extract solution or *CUR* (0.1 mg/ml), was mixed with 2.5ml of 0.2 M sodium phosphate buffer (pH = 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated in water bath at 50°C for 20min. Then, 2.5 ml of 10% trichloroacetic acid were added to the mixture that was centrifuged at 4000 rpm for 10 min. The supernatant (2.5 ml) was then mixed with 2.5 ml dist. H_2O and 0.5 ml of 0.1% $FeCl_3$ solution[19]. The intensity of the blue green color was measured at 700 nm using Genway spectrophotometer. The increase in absorbance of the reaction mixture indicated higher reducing power of the sample. The experiment was also conducted using vitamin E (0.1mg/ml) as a reference antioxidant. The samples were analyzed in triplicates.

DPPH free radical-scavenging assay

A 0.06 mM solution of DPPH· in ethanol was prepared. The initial absorbance of the DPPH· in ethanol was measured at 517 nm and did not change throughout the period of assay. A 0.5 ml solution of the sample at concentration of 0.1 mg/ml was added to 3.5ml of ethanolic DPPH· solution. The change in absorbance at 517 nm was measured at 30 min and free radical

scavenging activity was calculated as inhibition using following equation:-
Percentage DPPH radical scavenging activity =1 - [A_s / A_c] × 100, where A_s: absorbance of the DPPH· solution containing samples. A_c: absorbance of the control solution without sample but with DPPH·[20]. The percentages of DPPH· reduced were plotted against the samples. The experiment was also conducted using vitamin E (0.1mg/ml) as a reference antioxidant. The samples were analyzed in triplicates.

Antioxidant activity using linoleic acid peroxidation (ferric thiocyanate test) assay

The linoleic acid emulsion was prepared by emulsification of 0.28g linoleic acid with 0.28g Tween40 and 50 ml 0.2M phosphate buffer (pH 7.0). Each of the test samples including the *GSE*, *NSE* and *CUR* (0.1 mg/ml), was mixed with 2.5 ml linoleic acid emulsion and equal volume of phosphate buffer. The mixture was incubated at 37°C for 5 days. A negative control was conducted. Aliquots of a volume of 0.1 ml were taken from the incubation mixture every day and mixed with 75% ethanol, 0.1 ml 30% ammonium thiocyanate and 0.1 ml of 20mM $FeCl_2$ in 3.5% HCl and allowed to stand at room temperature for 3 min followed by measuring the intensity of the developed color (resulted from oxidation of Fe^{+2} to Fe^{+3} which form a complex with thiocyanate) at 500 nm using Genway spectrophotometer. Degree of linoleic acid peroxidation was calculated using following equation:-
Percentage Inhibition of lipid peroxidation= 100 - [A_s/ A_c] ×100; where A_s: absorbance of sample; A_c: absorbance of control which contains only linoleic acid emulsion and sodium phosphate buffer[21]. The experiment was also conducted using vitamin E (0.1mg/ml) as a standard antioxidant. The samples were analyzed in triplicates.

Determination of antioxidant activity using phosphomolybdenum assay

An aliquot of 0.2 ml of sample solution (0.1mg/ml) was placed in a 4 ml vial with 2 ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The vials were capped and incubated in water bath at 95°C for 90 min followed by cooling to room temperature and the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity was expressed as mg of α-tocopherol equivalents per g dry weight[22]. The experiment was also conducted using vitamin E (0.1mg/ml) as a reference antioxidant. The samples were analyzed in triplicates.

Determination of anion superoxide-scavenging activity

The superoxide anion was generated in a PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. The reaction mixture, containing 0.2 ml of each sample (0.1mg/ml), 0.2 ml of 60µM PMS, 0.2 ml of 677µM NADH and 0.2 ml of 144µM NBT, was incubated at room temperature for 5 min. Then the absorbance was read at 560 nm. All solutions were prepared in a 0.1M phosphate buffer pH 7.4. The scavenging activity was calculated as follows:-
Percentage inhibition of superoxide ion= [$(A_c - A_s)/A_c$]× 100; where A_c: absorbance of control reaction and A_s: absorbance in presence of sample[23]. The experiment was also conducted using vitamin E (0.1mg/ml) as a reference antioxidant. The samples were analyzed in triplicates.

Data analysis

All data are reported as the mean ± Standard Error (S.E.) of three measurements. The standard error and liner regression coefficient were calculated and the figures were presented using the GraphPad Prism software V. 4.0 (San Diego, USA).

Results

Determination of total soluble phenolic contents and reducing power

The determination of total phenolics based on the absorbance values of *GSE*, *NSE* and *CUR* solution (conc. 0.1mg/ml) that react with Folin-Ciocalteu reagent and followed by comparing with the standard solution of gallic acid equivalents[18]. The standard curve

of gallic acid (**Figure 1A**) was done by using gallic acid concentration ranging from 0.01-0.30mg/ml. The following equation expressed the absorbance of gallic acid standard solution as a function of concentration:- $A = 10.071\ C + 0.314$; whereas *A*: absorbance at 765 nm, and C:gallic acid concentration (mg/ml). *CUR* exhibited the greater phenolic content as mg gallic acid equivalent/g weight (mg GAE/g) for a value of 115±0.04 mg GAE/g (**Figure 1B**). On the other hand, *NSE* and *GSE* produced 14±0.05 mg GAE/g and 5.2±0.06 mg GAE/g, respectively. The reference antioxidant vitamin E used in this experiment elicited a value of total phenolic contents equal 2.0±0.01 mg GAE/g. **Figure 1C** shows the reducing power of *GSE*, *NSE* and *CUR* at concentration of 0.1mg/ml expressed as absorbance at 700 nm, using potassium ferricyanide method. *CUR* exhibited the highest absorbance (0.7±0.04) that was directly proportional to the reducing power followed by *NSE* (0.5±0.08), *GSE* (0.3±0.01) and vitamin E (0.22±0.01).

DPPH free radical-scavenging assay

Free radical scavenging activity of *GSE*, *NSE* and *CUR* (conc. 0.1mg/ml) were tested using the DPPH· Method and the results are shown in **Figure 2**. In this study, the free radical scavenging ability of each sample was evaluated through recording the change of absorbance produced by the reduction of DPPH·. The percentage scavenging activity of each sample against DPPH· were 1425%, 600%, 375% and 333% for *CUR*, *NSE*, vitamin E and *GSE*, respectively.

Antioxidant activity using linoleic acid peroxidation (Ferric thiocyanate test)assay

The absorbencies of the ferric thiocyanate assay system with *GSE*, *NSE* and *CUR* as well as blank reaction and vitamin E at 500nm were plotted against time intervals (**Figure 3**). It was noticed that the absorbance directly proportional with the time, the highest increase was pronounced after 120 hrs for each system. The absorbance at 500nm was 1.1, 0.86, 0.65, 0.36, 0.29 for blank, *GSE*, *NSE*, vitamin E, *CUR*, respectively. As expected, the highest absorbance was noticed for the blank reaction at all time intervals. The ratios of absorbance of *GSE*, *NSE*, *CUR* and vitamin E at 24, 48, 72, 96 and 120 hrs were presented in **Table 1**.

Determination of antioxidant activity using phosphomolybdenum assay

The antioxidant activity of *GSE*, *NSE* and *CUR* at concentration of 0.1mg/ml was measured using the phosphomolybdenum method. It seemed that *CUR* exhibited the highest antioxidant activity (87.1%) followed by *NSE* (77.6%), *GSE* (61.4%) and Vitamin E (78%) as shown in **Figure 4A**.

Determination of anion superoxide-scavenging activity

The percentage superoxide scavenging activities of the *GSE*, *NSE* and *CUR* at concentration of 0.1mg/ml on superoxide radicals are shown in **Figure 4B.** The increasing order of the percentage superoxide scavenging activities was *CUR* (41%) > *NSE* (32) > *GSE* (14%). Vitamin E produced a percentage = 19%.

Linear regression analysis

Linear regression analysis of total soluble phenolic content (mgGAE/g) and: percentage DPPH· scavenging activity of *GSE* and *NSE* (**Figure 5A**), percentage DPPH· scavenging activity of *CUR* (Fig. 5B), absorbance at 500nm using the FTC-linoleic system for detection of antioxidant activity of *GSE*, *NSE* and *CUR* (**Figure 5C**), absorbance at 500nm using the PMS-NADH system for detection of superoxide scavenging activity of *GSE*, *NSE* and *CUR* (**Figure 5D**), and percentage of antioxidant activity using the phosphomolybedenum method (**Figure 5E**). All data showed strong linear regression of total phenolic content and the above parameters in studied samples as indicated by high r^2 at $P<0.0001$ ranging from +0.7535 to 0.9999. In contrary, the degree of linear regression was slightly low in case of percentage DPPH· scavenging activity than the other analyses i.e. r^2 at $P<0.0001$=+0.3136 for *CUR*, +0.4394 for *GSE*. However, the r^2 at $P<0.0001$ in case of *NSE* = + 0.8522.

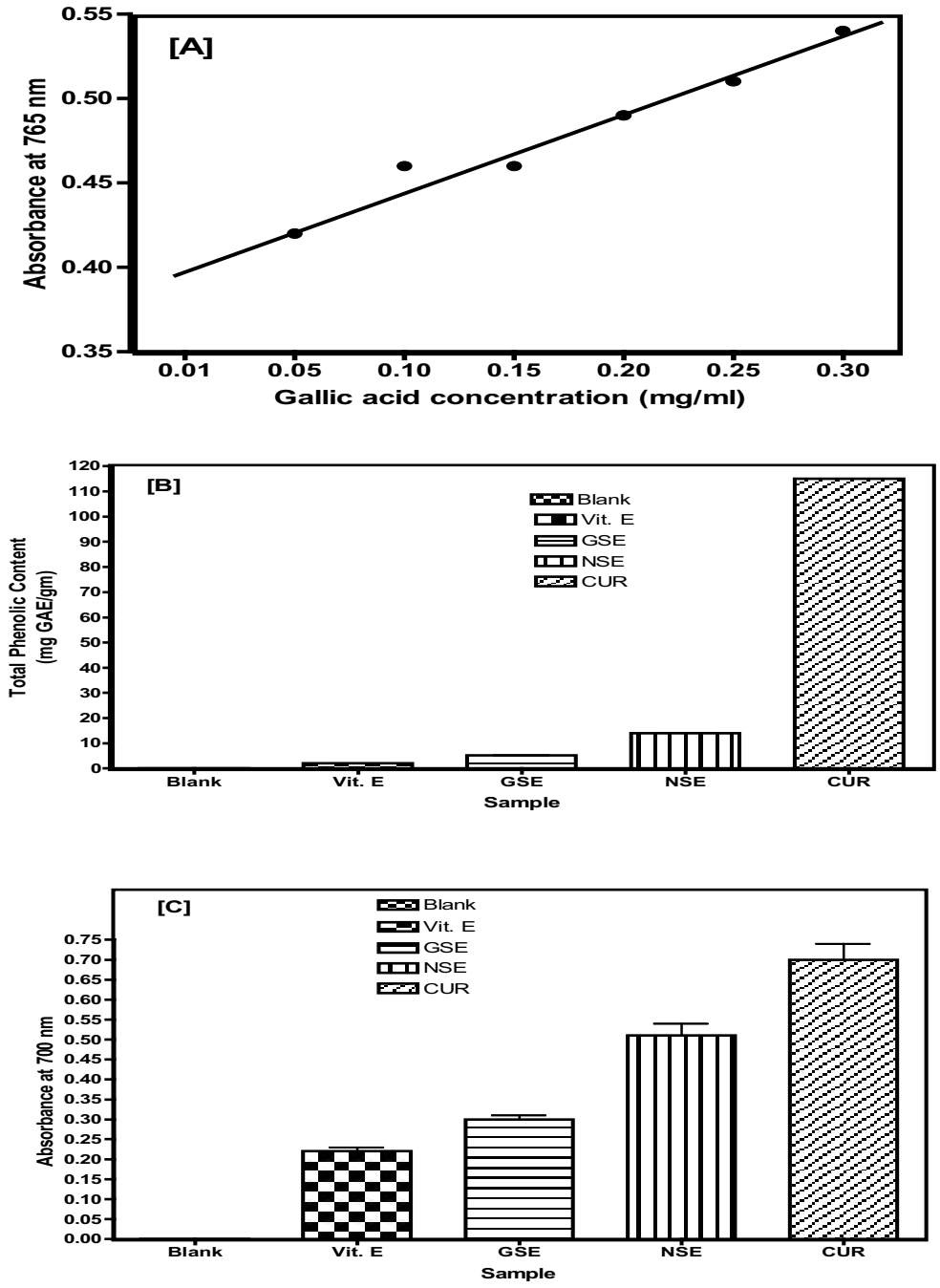

Figure 1: [A] *Gallic acid standard curve using gallic acid concentrations ranging from 0.01-0.30mg/ml.* **[B]** *Total soluble phenolic contents of GSE, NSE and CUR (0.1mg/ml each) determined by Folin-Ciocalteu reagent expressed as gallic acid equivalents (GAE) utilizing absorbance versus concentration curve for gallic acid.* **[C]***Reducing power of GSE, NSE and CUR (0.1mg/ml each) determined using potassium ferricyanide method and expressed as absorbance at 700 nm. Vitamin E (0.1mg/ml) was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as mean ± S.E.*

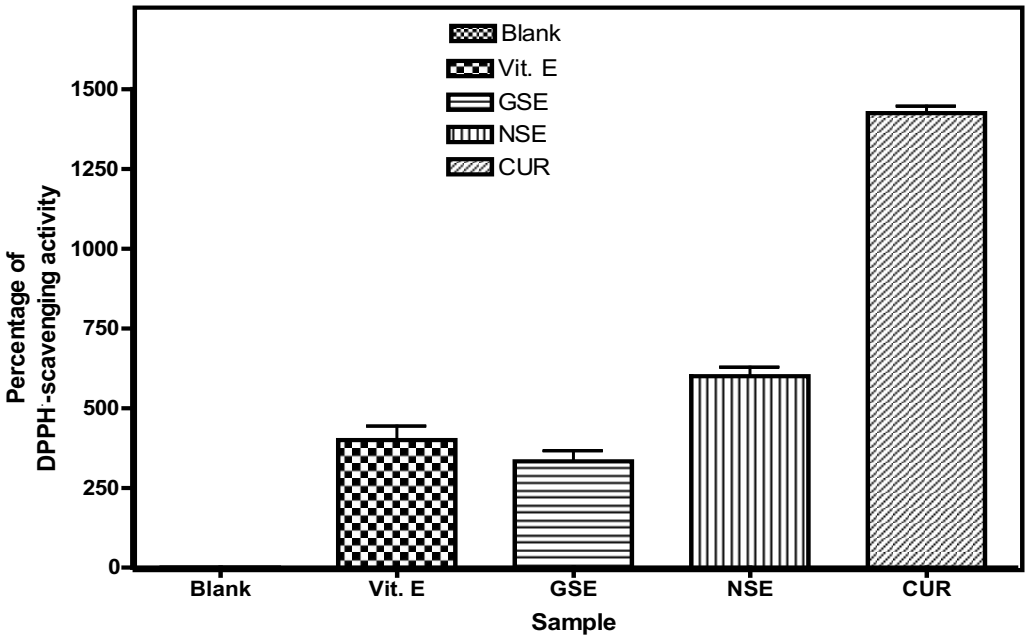

Figure 2: *DPPH radical scavenging activity of GSE, NSE and CUR (0.1mg/ml each) determined using diphenyl picrylhydrazyl radical and expressed as % inhibition of DPPH radical. Vitamin E (0.1mg/ml) was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as mean ±S.E.*

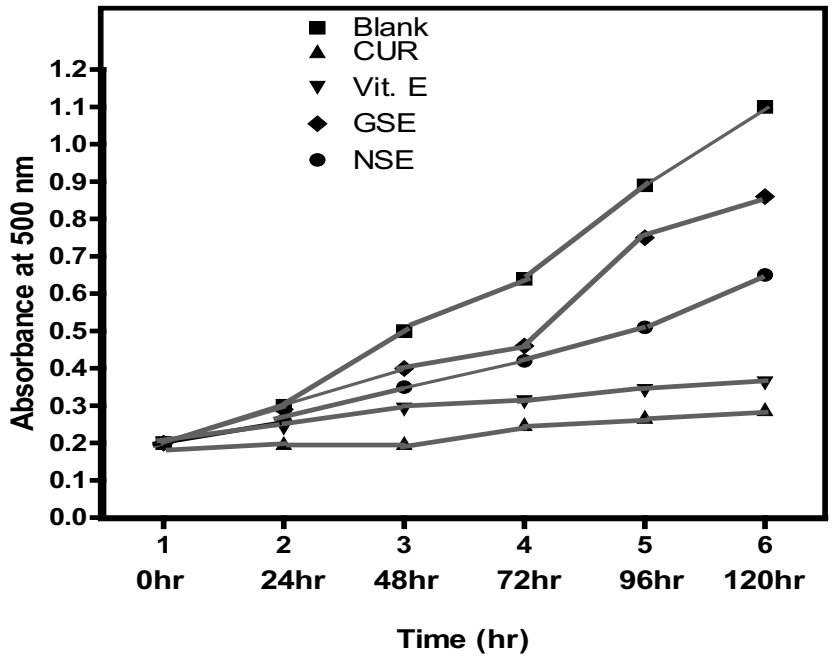

Figure 3: *Absorbance versus time plots for the antioxidant activities of GSE, NSE and CUR (0.1mg/ml each) at 500 nm using the ferric thiocyanate/ linoleic acid peroxidation in vitro assay. Vitamin E (0.1mg/ml) was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as mean ± S.E.*

Table 1: Ratios of absorbance of *GSE*, *NSE* and *CUR* (0.1mg/ml for each) to the absorbance of control system at 500 nm determined by linoleic acid / Ferric thiocyanate peroxidation *in vitro* assay.

Sample	$A_{sample} / A_{control}$				
	24hr	48hr	72hr	96hr	120hr
GSE	0.66 ± 0.12	0.40 ± 0.10	0.39 ± 0.12	0.30 ± 0.10	0.26 ± 0.04
NSE	0.96±0.18	0.80 ± 0.13	0.72 ± 0.16	0.84 ± 0.16	0.78 ± 0.13
CUR	0.86 ± 0.2	0.70 ± 0.14	0.65 ± 0.11	0.57 ± 0.09	0.60 ± 0.90
Vit. E	0.80 ± 0.16	0.58 ± 0.07	0.48 ± 0.09	0.38 ± 0.86	0.33 ± 0.06

Vitamin E (0.1mg/ml) was used as a reference antioxidant as a positive control.
The samples were analyzed in triplicates. Values were expressed as mean ± S.E.

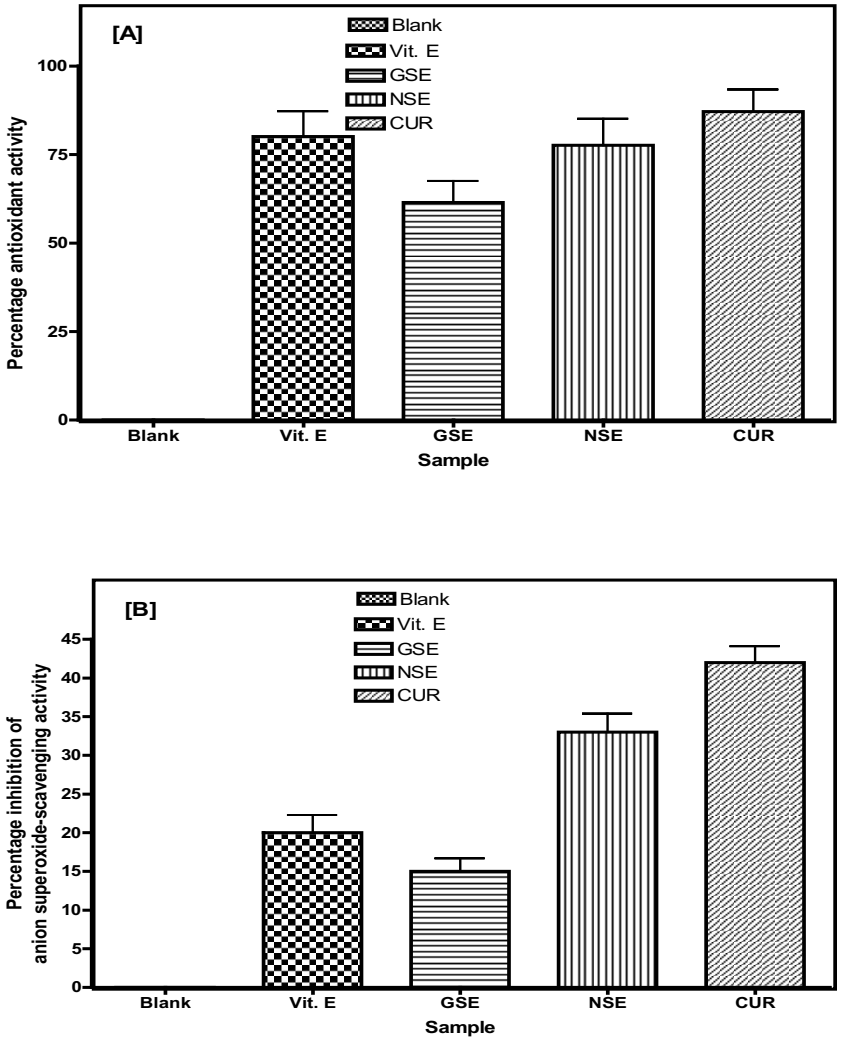

Figure 4: [A]*Percentage of antioxidant activity of CUR, GSE and NSE (0.1mg/ml each) using the phosphomolybedenum method.* **[B]** *Percentage inhibition of superoxide scavenging activity GSE, NSE and CUR (0.1mg/ml each) determined using PMS-NADH system. Expressed as % inhibition of superoxide radical. Vitamin E (0.1mg/ml) was used as reference antioxidant. The samples were analyzed in triplicate. All the measured values were expressed as mean ± S.E.*

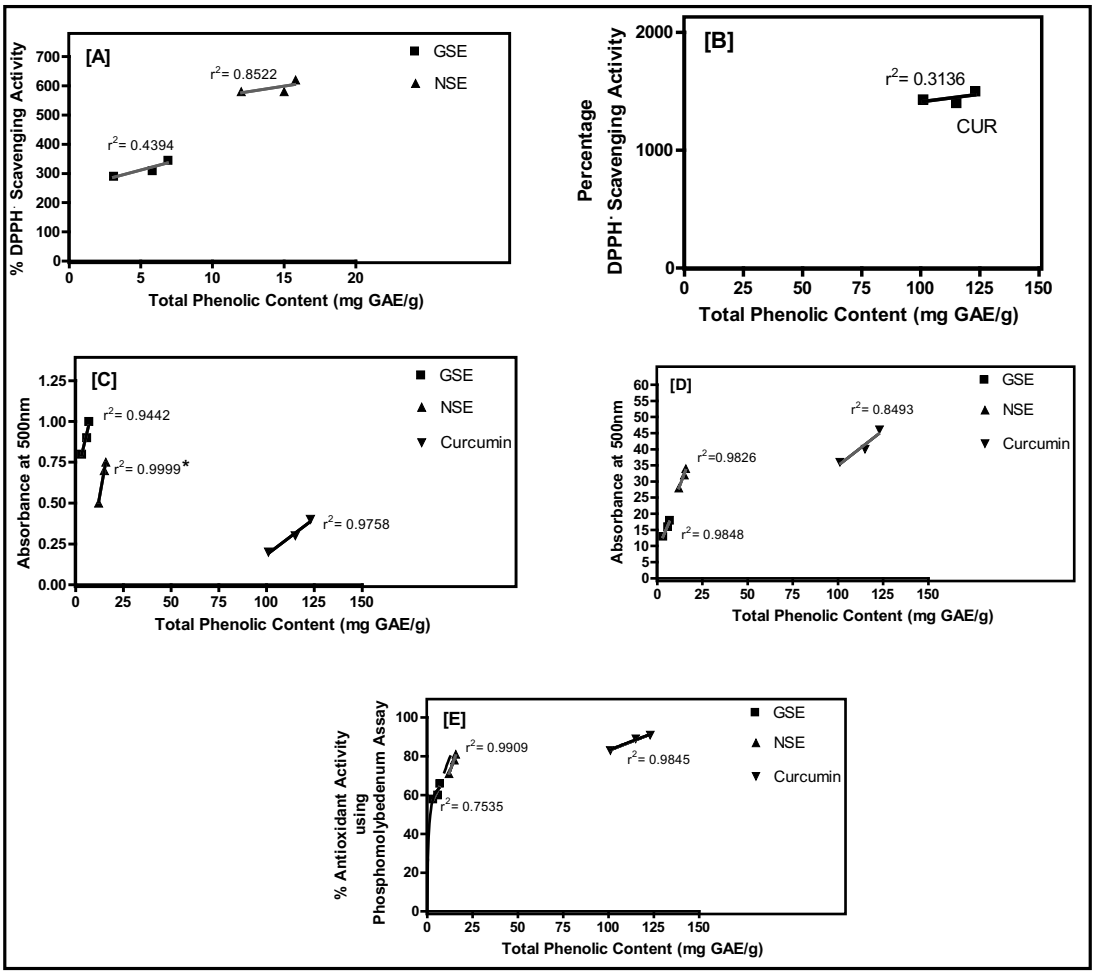

Figure 5: *Linear regression analysis of total soluble phenolic contents (mg GAE/g) and:* ***[A]*** *DPPH· scavenging activity of GSE andNSE (r^2= 0.4394, 0.8522, respectively; P<0.0001).* ***[B]*** *DPPH· scavenging activity of curcumin (r^2= 0.3136; P<0.0001).* ***[C]*** *absorbance at 500nm using the FTC-linoleic system for detection of antioxidant activity of GSE, NSE and curcumin (r^2= 0.9442, 0.9999,0.9758; respectively; P<0.0001).* ***[D]*** *absorbance at 500nm using the PMS-NADH system for detection of superoxide scavenging activity of GSE, NSE and curcumin (r^2= 0.9848, 0.9826, 0.8493; respectively; P<0.0001).* ***[E]*** *antioxidant activity GSE, NSE and curcumin (r^2= 0.7535, 0.9909, 0.9845; respectively; P<0.0001) using the phosphomolybedenum method. All data showed strong linear regression between total phenolic content and the above parameters in all studied samples. The samples were analyzed in triplicates. Values were expressed raw data. DPPH·: diphenyl picrylhydrazyl radical; PMS-NADH: phenazine methosulfate-nicotinamide adenine dinucleotide, FTC: ferric thiocyanate.*

Discussion

Phenolic compounds are hydroxylated derivatives of benzoic and cinnamic acids that are responsible for the antioxidant activity[24]. In general, the antiradical and antioxidant activities of plant extract are associated to the phenolic content[25]. The phosphomolybdate complex can be reduced to blue products by the phenolic compounds[26]. The reason that, *CUR* elicited the higher total phenolic content may be due to the fact that it contains two phenolic groups. It is clear that, the number of phenolic groups present in the structure of an antioxidant molecule is not always the only factor to determine its antioxidant activity. Positions of the phenolic groups,

presence of other functional groups in the molecules such as double bonds and conjugation to phenolic and ketone groups, also play important roles in antioxidant activities and have been reported by another study that studied the activity relationship of antioxidant activity of flavonoids and phenolic acid[27]. In general, the mentioned factors can be presented as the tension at the phenolic groups. Unlike, *GSE* and *NSE*, the two phenolic groups of *CUR* are attached to different unadjacent benzene rings that give the two OHs the mobility to work freely without hindrance. Additionally, polarity and hydrophobicity of antioxidants plays an important factor in the antioxidant activity especially in the biomembrane systems[28].

The antioxidant activity has been reported to be associated with the reducing power[29]. *CUR* exhibited the highest absorbance that was directly proportional to the reducing power followed by *NSE*, *GSE* and vit. E. The antioxidant action of reductones (a class of compounds that has been associated with the reducing property) is based on the breaking of the free radical chain by donating a hydrogen atom. Reductones also react with certain precursors of peroxides, thus preventing peroxide formation[30]. The data presented in this study indicated that, the marked antioxidant activity of *CUR*, *NSE* and to some extent *GSE* seemed to be as a result of their reducing power. The grape seed flavanol and procyanidin compounds may act in a similar fashion as reductones by donating electrons and reacting with free radicals to convert them to more stable products and terminating the free radical chain[8]. This postulate can be applicable in case of *NSE* and *CUR*.

Plants play a significant role in health promotion through free radical scavenging activity[31]. DPPH· is a stable radical showing a maximum absorbance at 515 nm. In DPPH· assay, the antioxidant were able to reduce the stable radical DPPH to the yellow-colored diphenyl picrylhydrazone[32]. The method based on the reduction of DPPH· in alcoholic solution in the presence of a hydrogen-donating antioxidant due to formation of the non-radical form DPPH-H in the reaction. DPPH· is usually used as a reagent to evaluate free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[19]. The disappearance of the DPPH radical absorption at 515 nm by the action of antioxidants is taken as a measure of antioxidant activity. Radical scavenging activity of *GSE*, *NSE* and *CUR* (conc. 0.1mg/ml) were tested using the DPPH· method. In this study, the free radical scavenging ability of each sample was evaluated through recording the change of absorbance produced by the reduction of DPPH·. The results obtained indicated that, higher percentage of DPPH· scavenging activity in case of *CUR* may be attributed to the high reducing power and higher total phenolic contents present in *CUR*.

The ferric thiocyanate test determines the antioxidant activity through measurement of the amount of peroxides formed in linoleic acid emulsion of antioxidant during incubation[33]. The antioxidant activity pattern was different to those obtained by the other experiments conducted in this study. The increasing order of antioxidant was *GSE* > *CUR* > *NSE*. The explanation of these findings can be clarified on the basis of the fact cleared, that the mentioned these variations were attributed to the structural factors of the antioxidants. Hydrophobicity of antioxidants plays an important role in their solubility in the oil. The hydrophobic antioxidant mixed well with the oil better than the hydrophilic antioxidant[32]. When the structure of thymoquinone (*NSE* main constituent), procyanidin (*GSE* main component) and *CUR* were compared, it was noticed that procyanidin tends to form dimmers[7], so that, the hydrophilic-OH group was not free. The structure of *CUR* has two free hydrophilic-OH groups[34], so that the hydrophilicity of *CUR* molecule seemed to be more pronounced than procyanidin. The thymoquinone was shown the more hydrophilic and low phenolic content than *CUR*, so that *NSE* exhibited the highest absorbance ratio. This was confirmed by low antioxidant activity of *NSE* compared to *CUR*.

The antioxidant activity of *GSE*, *NSE* and *CUR* at concentration of 0.1mg/ml was

measured using the phosphomolybdenum method. It seemed that, *CUR* exhibited highest antioxidant activity followed by vitamin E, *NSE* and *GSE*. The phosphomolybdenum method based on the reduction of Mo^{+4} to Mo^{+5} by the antioxidant compounds and the formation of green Mo^{+5} complex that can be absorbed at 695 nm[35]. The antioxidant breaks the free radical chain by donating a hydrogen atom[30]. This may depend on the presence of polyphenols that was freely present in case of *CUR* than thymoquinone or procyanidin.

Superoxide has been observed to directly initiate lipid peroxidation. It has also been reported that antioxidant properties of some flavonoids are effective mainly through scavenging of superoxide anion radical[36]. Superoxide anions derived from dissolved oxygen by the PMS-NADH system will 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. Antioxidants are able to inhibit the blue NBT formation[35]. The decrease of absorbance in presence of antioxidants indicates consumption of superoxide anion in the reaction mixture. The increasing order of the percentage superoxide scavenging activities was *CUR* > *NSE* > vitamin E >*GSE*. These effects may be attributed to the antioxidant activity of the *CUR*, *NSE* and *GSE*.

Linear regression analysis of total soluble phenolic content (mgGAE/g) and: percentage DPPH· scavenging activity of *GSE* and *NSE*, percentage DPPH· scavenging activity of *CUR*, absorbance at 500nm using the FTC-linoleic system for detection of antioxidant activity of *GSE*, *NSE* and *CUR*, absorbance at 500nm using the PMS-NADH system for detection of superoxide scavenging activity of *GSE*, *NSE* & *CUR* and percentage of antioxidant activity using the phosphomolybedenum method. All data showed strong linear regression of total phenolic content and the above parameters in studied samples as indicated by high r^2 at $P<0.0001$. The degree of linear regression was slightly low in case of percentage DPPH scavenging activity than the other analyses.

Conclusion

CUR was found to be an effective antioxidant more than *NSE* and *GSE*, by the use of different *in vitro* assays such as determination of total soluble phenolic content, reducing power, DPPH· scavenging activity, superoxide scavenging activity and the antioxidant activity assays using the phosphomolybdenum method. However, determination of the antioxidant activity using ferric thiocyanate/linoleic system revealed higher antioxidant power in case of *GSE* due to hydrophobicity of *GSE* that allow proper dissolution in the linoleic acid. Based on results achieved, we can concluded that *GSE*, *NSE* and *CUR* have the potency to act as a powerful antioxidant and can be used for decreasing the lipid peroxidation thereby may be valuable as food preservative and natural nutritional health supplements.

Acknowledgement

This research was financially supported by a research grant no. 109/428, funded by the deanship of scientific research, Taibah University, Al Madinah Al Munawwara, Saudi Arabia.

References

1. Halliwell B, Gutteridge J. Oxygen toxicity, oxygen radicals, transition metals and diseases. **Biochem J 1984;** 219: 1- 4
2. Silva B, Ferreres F, Malva J, Dias A. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. **Food Chem 2005;** 90: 157-167
3. Gülçin I, Mshvildadze V, Gepdiremen A. Antioxidant activity of a triterpenoid glycoside isolated from the berries of *Hedera colchica*: 3-O-(d-glucopyranosyl)-hederagenin. **Phytotherapy Res 2006;** 20: 130–134

4. El-Beshbishy H. Hepatoprotective effect of green tea (*Camellia sinensis*) extract against tamoxifen-induced liver injury in rats. **J Biochem and Mol Biol 2005;** 38(5): 363-370
5. Al-Jassir M. Chemical composition and microflora of black cumin (*Nigella sativa* L.) seeds growing in Saudi Arabia. **Food Chem 1992;** 45: 239-242
6. Burits M, Bucar F. Antioxidant activity of *Nigella sativa* eseential oil. **Phytotherapy Res 2000;** 14: 323-328
7. Yilmaz Y, Toledo R. Health aspects of functional grape seed constituents. **Trendds in Food Sci 2004;** 15: 422-433
8. Jayaprakasha G, Selvi T, Sakariah K. Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. **Food Res Int 2003;** 36: 117-122
9. El-Alfy A, Ahmed A, Fatani A. Protective effect of red grape seeds proanthocyanidins against induction of diabetes by alloxan in rats. **Pharmacol Res 2005;** 52: 264–270
10. Teissedre P, Frankel E, Waterhouse A, Peleg H, German J. Inhibition of *in vitro* human LDL oxidation by phenolic antioxidants from grapes and wines. **J Sci of Food and Agri 1996;** 70: 55-61
11. Cousins M, Adelberg J, Chenb F. Antioxidant capacity of fresh and dried rhizomes from four clones of turmeric (*Curcuma longa* L.) grown *in vitro*. **Indian Crop Prod 2007;** 25: 129–135
12. Maheshwari R, Singh A, Gaddipati J, Srimal, R. Multiple biological activities of curcumin: A short review. **Life Sci. 2006;** 78(18): 2081-2087
13. Ghoneim I, Abdel-Naim A, Khalifa A, El-Denshary, E. Protective effects of curcumin against ischemia/reperfusion insult in rat forebrain. **Pharmacol Res 2002;** 46(3): 273-279
14. Ahn H, Jeona T, Lee J, Hwang S, Lim Y, Park D. Antioxidative activity of persimmon and grape seed extract: *in vitro* and *in vivo*. **Nutrition Res 2002;** 22: 1265–1273
15. Nawaz H, Shi J, Mittal G, Kakuda Y. Extraction of polyphenols from grape seeds and concentration by ultrafilterarion. **Sep and Puri Technol 2006;** 48: 176-181
16. Bourgou S, Ksouri R, Bellila A, Skandrani I, Falleh H, Marzouk B. Phenolic composition and biological activities of Tunisian *Nigella sativa* L. shoots and roots. **Comptes Rendus Biologies 2008;** 331: 48–55
17. Gülçin I. Antioxidant and antiradical activities of L-carnitine. **Life Sci 2006;** 78: 803–811
18. Erkan N, Ayranci G, Ayranci E. Antioxidant activities of rosemary (*Rosmarinus officinalis* L.) extract, blackseed (*Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. **Food Chem 2008;** 110: 76-82
19. Oyaizu M. Studies on products of browning reaction: Antioxidative activity of products of browning reaction. **Jap J Nutr 1986;** 44: 307–315
20. Leong P, Shui G. An investigation of antioxidant capacity of fruits in Singapore markets. **Food Chem 2002;** 76: 69–75
21. Yen G, Hsieh C. Antioxidative activity of extracts from Du-zhong (*Eucommia ulmoides*) toward various peroxidation models in vitro. **J Agri Food Chem 1998;** 46: 3952-3957
22. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitative of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. **Analytical Biochem 1999;** 269: 337–341
23. Ozsoy N, Can A, Yanardag R, Akev N. Antioxidant activity of Smilax excels L. leaf extracts. **Food chem. 2008;** 110: 571-583
24. Khattak K, Simposon T, Ihasnullah. A. Effect of gamma irradiation on the extraction yield, total phenolic content and free radical-scavenging activity of *Nigella sativa* seed. **Food Chem 2008;** 110: 967-972
25. Göktürk B, Sagdic O, Ozkan, G, Cetin, S. Determination of antibacterial effects and total phenolic contents of grape (*Vitis vinifera* L.) seed extracts. **Int J Food Sci Technol 2006;** 41: 799-804

26. Vasco C, Ruales J, Kamal-Eldin A. Total phenolic compounds and antioxidant capacities of major fruits from Ecuador. **Food Chem 2008;** 111(4): 816-823
27. Rice-Evans A, Miller J, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. **Free Rad Biol and Med 1996;** 20(7): 933–956
28. Wu C, Huang M, Lin Y, Ju H, Ching H. Antioxidant properties of *Cortex fraxini* and its simple coumarins. **Food Chem 2007;** 104: 1464–1471
29. Tanaka M, Kuie C, Nagashima Y, Taguchi T. Application of antioxidative maillard reaction products from histidine and glucose to sardine products. **Nippon Suisan Gakkaishi 1988;** 54: 1409-1414
30. Gordon M. The mechanism of antioxidant action in vitro. In: B. J. F. Hudson (Ed.). **Food antioxidants, 1990;** 1-18 London: Elsevier Applies Science.
31. Sánchez-Moreno C. Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. **Foods Sci Technol Int 2002;** 8: 121-137
32. Frankel E, Meyer A. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. **J Sci Food and Agri 2000;** 80: 1925–1941
33. Singh G, Maurya S, de Lampasona M, Catalan C. A comparison of chemical, antioxidant and antimicrobial studies of cinnamon leaf and bark volatile oils, oleoresins and their constituents. **Food Chem Toxicol 2007;** 45: 1650–1661
34. Tuba K, Gülçin I. Antioxidant and radical scavenging properties of curcumin. **Chemico-Biological Interactions 2008;** 174: 27–37
35. Parejo I, Viladomat F, Bastida, J. Comparison between the radical scavenging activity and antioxidant activity of six distilled and non-distilled Mediterranean herbs and aromatic plants. **J Agri Food Chem 2002;** 50: 6882–6890
36. Yen G, Duh P. Scavenging effect of methanolic extract of peanut hulls on free radical and active oxygen species. **J Agri Food Chem 1994;** 42: 629–632
37. Frankel E, Meyer A. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. **J Sci Food and Agri 2000;** 80: 1925–1941
38. Singh G, Maurya S, de Lampasona M, Catalan C. A comparison of chemical, antioxidant and antimicrobial studies of cinnamon leaf and bark volatile oils, oleoresins and their constituents. **Food Chem Toxicol 2007;** 45: 1650–1661
39. Tuba K, Gülçin I. Antioxidant and radical scavenging properties of curcumin. **Chemico-Biological Interactions 2008;** 174: 27–37
40. Parejo I, Viladomat F, Bastida, J. Comparison between the radical scavenging activity and antioxidant activity of six distilled and non-distilled Mediterranean herbs and aromatic plants. **J Agri Food Chem 2002;** 50: 6882–6890
41. Yen G, Duh P. Scavenging effect of methanolic extract of peanut hulls on free radical and active oxygen species. **J Agri Food Chem 1994;** 42: 629–632