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ORIGINAL ARTICLE

Antioxidant activity of clove oil – A powerful antioxidant source

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Abstract The number of methods to measure the antioxidants in botanicals, foods, nutraceuticals and other dietary supplements has increased considerably in the last decade. Clove oil is obtained by distillation of the flowers, stems and leaves of the clove tree. In the present paper, clove oil was evaluated by employing various in vitro antioxidant assay such as α,α-diphenyl-β-picryl-hydrazyl free radical (DPPH·) scavenging, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, 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. Clove oil inhibited 97.3% lipid peroxidation of linoleic acid emulsion at 15 μg/mL concentration. However, under the same conditions, the standard antioxidant compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol and trolox demonstrated inhibition of 95.4, 99.7, 84.6 and 95.6% on peroxidation of linoleic acid emulsion at 45 μg/mL concentration, respectively. In addition, clove oil had an effective DPPH· scavenging, $ABTS^{\cdot+}$ scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, ferric ions (Fe^{3+}) reducing power and ferrous ions (Fe^{2+}) chelating activities. Also, these various antioxidant activities were compared to BHA, BHT, α-tocopherol and trolox as reference antioxidant compounds.

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1. Introduction

Clove oil is obtained by distillation of the flowers, stems and leaves of the clove tree (*Eugenia aromatica* or *Eugenia caryophyllata, Fam. Myrtaceae*) (Anderson et al., 1997; Mylonasa et al., 2005). Clove essential oils were tested for inhibitory activity against important spoilage microorganisms of intermediate moisture foods (Matan et al., 2006). Clove oil has been listed as a "Generally Regarded As Safe" substance by the United States Food and Drug Administration when administered at levels not exceeding 1500 ppm in all food cat-

egories (Jay and Rivers, 1984; Fischer and Dengler, 1990; Anderson et al., 1997; Waterstrat, 1999; Kildeaa et al., 2004). Additionally, the World Health Organisation (WHO) Expert Committee on Food Additives has established the acceptable daily human intake of clove oil at 2.5 mg/kg body weight for humans (Fischer and Dengler, 1990; Nagababu and Lakshmaiah, 1992; Anderson et al., 1997). In the last several years, it has been recognized as an effective anaesthetic for sedating fish for a number of invasive and noninvasive fisheries management and research procedures. It is safe, effective, and relatively inexpensive (Kildeaa et al., 2004). In addition, humans have used clove oil for centuries, as an anaesthetic for toothaches, headaches and joint pain (Shelef, 1983; Soto and Burhanuddin, 1995). Also, it is used throughout the world for applications ranging from food flavoring to local anesthaesia in dentistry profession (Anderson et al., 1997). In addition to its worldwide use as a food flavoring agent, it has also been employed for centuries as a topical analgesic in dentistry (Curtis, 1990; Soto and Burhanuddin, 1995). It was reported that clove oil had antilisteric activity in meat and cheese (Menon and Garg, 2001). Clove oil has been used as an aromatherapy oil, mouth sterilizer or painkiller (Robenorst, 1996).

Clove essential oils have been analysed by GC–MS and 18 components found in essential oils. These components have been tested for antioxidant properties in an egg yolk-based thiobarbituric acid reactive substances (TBARS) assay and also undiluted in a β-carotene agar diffusion assay. The essential oils and the components tested in the TBARS assay have demonstrated some degree of antioxidant activity (Dorman et al., 2000). In addition, the effect of clove oil on the oxidative stability was studied (Nguyen et al., 2000; Gülçin et al., 2004a).

Eugenol (4-allyl-2-methoxyphenol), the active substance, makes up 90–95% of the clove oil (Briozzo et al., 1989), and as a food additive is classified by the FDA to be a substance that is generally regarded as safe (Anderson et al., 1997).

In the past decades, oxidation mechanisms and free radical role in living systems have gained increased attention (Halliwell and Gutteridge, 1989). Oxygen uptake inherent to cell metabolism produces reactive oxygen species (ROS). The reaction of this species with lipid molecules originates peroxyl radicals and their interaction with nucleic acids and proteins conduces to certain alterations and, therefore, functional modifications (Chaillou and Nazareno, 2006). ROS are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. ROS, which include free radicals such as superoxide anion radicals $O_2^{\cdot-}$, hydroxyl radicals ($OH^{\cdot}$) and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are various forms of activated oxygen (Büyükokuroğlu et al., 2001; Gülçin et al., 2003a, 2006a; Gülçin, 2006a).

ROS are continuously produced during normal physiologic events and can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. ROS is capable of damaging crucial biomolecules such as nucleic acids, lipids, proteins and carbohydrates. Also, ROS and reactive oxygen species (RNS) may cause DNA damage that may lead to mutation (Gülçin et al., 2005, 2006). If ROS are not effectively scavenged by cellular constituents, they lead to disease conditions (Halliwell and Gutteridge, 1990; Gülçin et al., 2003a). ROS may interact with biological systems in a clearly cytotoxic manner. These molecules are exacerbating factors in cellular injury and aging process (Halliwell and Gutteridge, 1989; Gülçin 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 Gutteridge, 1989). As a result, ROS and RNS have been implicated in more than 100 diseases, including the above mentioned diseases (Tanizawa et al., 1992; Gülçin et al., 2003b).

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). Antioxidants are compounds that can delay or inhibit the oxidation of lipid or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu et al., 1998). All aerobic organisms have antioxidant defenses including antioxidant enzymes and foods to remove or repair the damaged molecules (Çakır 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 (Lai et al., 2001; Gülçin, 2007). 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 (Gülçin et al., 2004a; Gülçin, 2005). At present, the most commonly used antioxidants are BHA, BHT, propyl gallate and tert butylhydroquinone. Besides this BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis (Sherwin, 1990). Therefore, there is a growing interest on natural and safer antioxidants (Moure et al., 2001; Gülçin et al., 2006b; Oktay et al., 2003).

Antioxidants act by: removing O_2 or decreasing local O_2 concentrations, removing catalytic metal ions, removing key ROS, e.g. $O_2^{\cdot-}$ and H_2O_2, scavenging initiating radicals, e.g. $OH^{\cdot}$, $RO^{\cdot}$, RO_2^{-}, 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 aim of this study is to investigate the inhibition of lipid peroxidation, ferric ions (Fe^{3+}) reducing antioxidant power assay (FRAP), DPPH$^{\cdot}$ radical scavenging, $ABTS^{\cdot+}$ radical scavenging, superoxide anion radical scavenging in the riboflavin/methionine/illuminate system, hydrogen peroxide scavenging and ferrous ions (Fe^{3+}) chelating activities of clove oil. In addition, the objective of the present study is to also clarify the antioxidant and radical scavenging mechanisms of clove oil. Furthermore, an important goal of this research is to investigate the in vitro antioxidative effects of clove oil as compared with commercial and standard antioxidants such as BHA, BHT, α-tocopherol and trolox commonly used by the food and pharmaceutical industry.

2. Material and methods

2.1. Chemicals

Clove oil is obtained by distillation of the different parts such as flowers, stems and leaves of clove tree. In the present research clove oil was obtained from Magnus Mabec and Reynard USA. Riboflavin, methionine, 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), α,α-diphenyl-β-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. All other chemicals used were in analytical grade and obtained from either Sigma–Aldrich or Merck.

2.2. Radical scavenging activity

Radical scavenging capacity of the tested compounds was determined and compared to that of BHA, BHT, α-tocopherol and trolox by using the DPPH·, $ABTS^{\cdot+}$ and superoxide anion radical scavenging methods.

2.2.1. DPPH free radical scavenging activity

Some pure compounds, with a hydrogen atom or electron donating abilities were measured by the bleaching of a purple coloured methanol solution of DPPH. This spectrophotometric assay uses the stable DPPH·, as a reagent (Burits and Bucar, 2000). The methodology of Blois (1958) previously described by Gülçin (2006a) was used with slight modifications in order to assess the DPPH· free radical scavenging capacity of clove oil, 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 (Ancerewicz et al., 1998). Briefly, 0.1 mM solution of DPPH· was prepared in ethanol and 0.5 ml of this solution was added to 1.5 mL of clove oil solution in ethanol at different concentrations (15–45 μg/mL). These solutions were vortexed thoroughly and incubated in dark. Half an hour later, the absorbance was measured at 517 nm against blank samples. 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):

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

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 clove oil), and A_s is the absorbance in the presence of clove oil (Gülçin, 2007; Elmastaş et al., 2006a). DPPH·, decreases significantly upon exposure to proton radical scavengers (Yamaguchi et al., 1998). IC_{50} which denotes the amount (μg) of clove oil in 1.5 mL solution required to reduce initial concentration of DPPH radicals by 50% was also calculated.

2.2.2. ABTS radical cation decolourization assay

Radical cation scavenging capacity of clove oil was examined against $ABTS^{\cdot+}$ generated by chemical methods (Re et al., 1999). The spectrophotometric analysis of $ABTS^{\cdot+}$ radical scavenging activity was determined according to method of ABTS also a relatively stable free radical (Shirwaikar et al., 2006). In this method, an antioxidant is added to a pre-formed ABTS radical solution and, after a fixed time period, the remaining $ABTS^{\cdot+}$ is quantified spectrophotometrically (Gülçin et al., 2006a). This method is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of BHA, BHT, α-tocopherol and trolox, a water-soluble α-tocopherol analogue. The $ABTS^{\cdot+}$ was produced by the reaction of 2 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for four hours. Before usage, the $ABTS^{\cdot+}$ solution was diluted to get an absorbance of 0.750 ± 0.025 at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, to 1 mL of $ABTS^{\cdot+}$ solution was added 3 mL of clove oil solution in ethanol at different concentrations (15–45 μg/mL). After thirty minutes, the percentage inhibition of ABTS at 734 nm was calculated for each concentration relative to a blank absorbance. Addition of antioxidant reduced $ABTS^{\cdot+}$ to its colourless form. Solvent blanks were run in each assay. The extent of decolourization is calculated as percentage reduction of absorbance. For preparation of a standard curve, different concentrations of $ABTS^{\cdot+}$ were used. The $ABTS^{\cdot+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (r^2: 0.9841):

$$\text{Absorbance} = 4.6788 \times \left[\text{ABTS}^{\cdot+}\right] + 0.199$$

$ABTS^{\cdot+}$ radical scavenging capability was calculated using the following equation:

$$\text{ABTS}^{\cdot+}\text{scavenging effect}(\%) = \left(1 - \frac{A_s}{A_c}\right) \times 100$$

wherein A_c is the initial concentration of the $ABTS^{\cdot+}$ and A_s is absorbance of the remaining concentration of $ABTS^{\cdot+}$ in the presence of clove oil (Gülçin, 2006b).

2.2.3. Superoxide anion radical scavenging activity

Superoxide radicals were generated by method of Beauchamp and Fridovich (1971) described by Zhishen et al. (1999) with slight modification. Superoxide radicals are generated in riboflavin-methionine-illuminate and assayed by the reduction of NBT to form blue formazan (NBT^{2+}). All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The concentration of clove oil in the reaction mixture was 15 μg/mL. The total volume of the reactant mixture was 3 mL and the concentrations of the riboflavin, methionine and NBT were 1.33×10^{-5}, 4.46×10^{-5} and 8.15×10^{-8} M, respectively. The reactant was illuminated at 25 °C for 40 min. The

photochemically reduced riboflavins generated $O_2^{\cdot-}$ which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank sample. The absorbance was measured at 560 nm. Clove oil was added to the reaction mixture, in which $O_2^{\cdot-}$ was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated by 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 clove oil or standards (Gülçin et al., 2004d, 2006b).

2.3. Total antioxidant activity determination by ferric thiocyanate method in linoleic acid amulsion

The antioxidant activity of clove oil was determined according to the ferric thiocyanate method (FTC) as described by Gülçin (2006a). For stock solutions, 10 mg of clove oil was dissolved in 10 mL ethanol. Then, the solution which contains different concentrations of clove oil (from 15 to 45 μg/mL) 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). For this, 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 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. In the FTC method, during the linoleic acid peroxidation, peroxides are formed and this leads to oxidation of Fe^{+2} to Fe^{+3}. The latter ions form a complex with hiocyanate and this complex has a maximum absorbance at 500 nm. The absorbance of the red colour was measured at 500 nm until it reached a maximum value (Elmastaş et al., 2006b). 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 clove oil were used as blank samples. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

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

where 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 sample clove oil (Gülçin, 2006b; Gülçin et al., 2006a).

2.4. Ferric ions (Fe^{3+}) reducing antioxidant power assay (FRAP)

The ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) method was used to measure the reducing capacity of clove oil. This method was proposed by Oyaizu (1986) with a slight modification (Gülçin, 2006b) which involves the presence of clove oil to reduce the ferricyanide complex to the ferrous form. The FRAP method is based on a redox reaction in which an easily reduced oxidant (Fe^{3+}) is used in stoichiometric excess and antioxidants act as reductants (Benzie and Strain, 1996). Different concentrations of clove oil (15–45 μg/mL) 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. Then, 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.

2.5. Ferrous ions (Fe^{2+}) chelating activity

The chelating of ferrous ions by clove oil was estimated by the method of Dinis et al. (1994) described previously Gülçin et al. (2003a), wherein the Fe^{2+}-chelating ability of clove oil was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm. Clove oil (15 μg/mL) 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 ten minutes. 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 clove oil or standards. The control contains only $FeCl_2$ and ferrozine, complex formation molecules (Gülçin et al., 2004b; 2006b).

2.6. 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). Clove oil at 15 μg/mL 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 contained 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: 0.9895):

$$\text{Absorbance} = 0.038 \times [H_2O_2] + 0.4397$$

The percentage of H_2O_2 scavenging of clove oil 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 clove oil or standards (Elmastaş et al., 2005; Gülçin et al., 2006a).

2.7. 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.

3. Results and discussion

A large number of methods have been developed to evaluate total antioxidant capacity of food and dietary supplements, herbal extracts or pure compounds. Nevertheless, few of them have been used widely due to the difficulty of measuring total antioxidant capacity owing to limitations associated with methodological issues and free radical sources (Prior et al., 2005; Schauss et al., 2006). In this study, the antioxidant activity of the clove oil was compared to BHA, BHT, α-tocopherol and its water-soluble analogue trolox. The antioxidant activity of the clove oil, BHA, BHT, α-tocopherol and trolox has been evaluated in a series of in vitro tests: DPPH· free radical scavenging, $ABTS^{\cdot+}$ 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 were done.

3.1. Total antioxidant activity determination in linoleic acid emulsion was by ferric thiocyanate method

Lipid peroxidation process 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 had oxidized by air during the experimental period, was indirectly measured. Ferrous chloride and thiocyanate react with each other to produce ferrous thiocyanate by means of hydroperoxide (Inatani et al., 1983).

Total antioxidant activity of clove oil, BHA, BHT, α-tocopherol and trolox was determined by the ferric thiocyanate method in the linoleic acid system. Clove oil exhibited effective antioxidant activity in this system. The effect of different concentrations (15–45 μg/mL) of clove oil on lipid peroxidation of linoleic acid emulsion are shown in Fig. 1 and was found to be 97.9, 99.4 and 99.7% and their activities are greater than that found in 45 μg/mL concentration of BHA (95.5%), α-tocopherol (84.6%) and trolox (95.6%), but the same with BHT (99.7%). The autoxidation of linoleic acid emulsion without clove oil or standard compounds was accompanied by a rapid increase of peroxide value. Consequently, these results clearly indicated that clove oil had effective and powerful antioxidant activity by ferric thiocyanate. At the same concentration (45 μg/mL), inhibition effect of clove oil and standard compounds on linoleic acid emulsion exhibited the following order: clove oil = BHT > BHA > trolox > α-tocopherol.

3.2. 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; Arabshahi-Delouee and Urooj, 2007). 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's total capacity to withstand free radical stress (Wood et al., 2006). FRAP assay takes advantage of an electron transfer reaction in which a ferric salt is used as an oxidant (Benzie

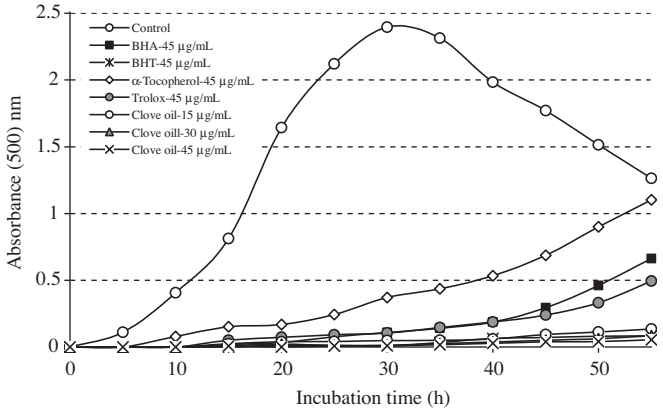

Figure 1 Total antioxidant activities of different concentrations (15–45 μg/mL) of clove oil and standard antioxidant compounds such as BHA, BHT, α-tocopherol and trolox at the concentration of 45 μg/mL (BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene).

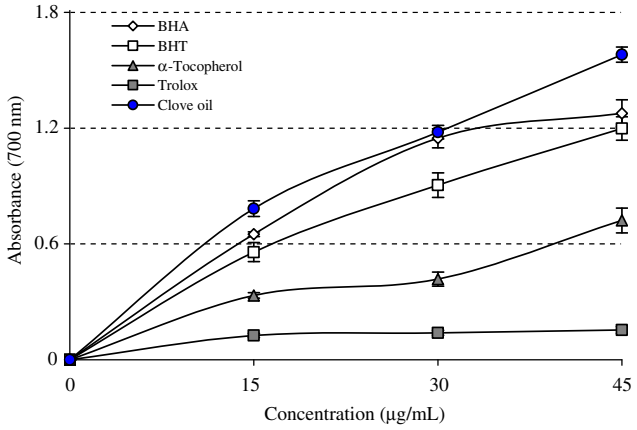

Figure 2 Total reductive potential of different concentrations (15–45 μg/mL) of clove oil (r^2: 0.9677) and reference antioxidants; BHA, BHT, α-tocopherol and trolox determined by FRAP method (BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene).

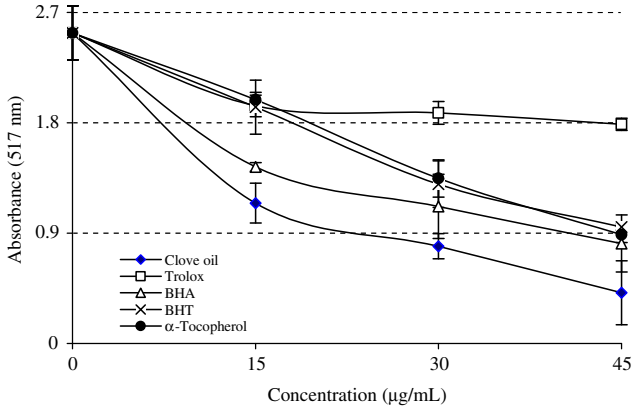

Figure 3 DPPH free radical scavenging activity of different concentrations (15–45 μg/mL) of clove oil (r^2: 0.8786) and reference antioxidants; BHA, BHT, α-tocopherol and trolox (BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene; DPPH·: α,α-diphenyl-β-picryl-hydrazyl free radical).

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.

Fig. 2 show that clove oil had effective reducing power using the potassium ferricyanide reduction method when compared to the standards (BHA, BHT, α-tocopherol and trolox). For the measurements of the reductive ability of clove oil, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of clove oil using the method of Oyaizu (1986). At different concentrations (15-45 μg/mL), clove oil demonstrated powerful reducing ability (r^2: 0.9677) and these differences were statistically very significant ($p < 0.01$). The reducing power of clove oil, BHA, BHT, α-tocopherol and trolox increased steadily with increasing concentration of samples. Reducing power of clove oil and standard compounds exhibited the following order: Clove oil > BHA ≈ BHT > α-tocopherol > trolox. The results on reducing power demonstrate the electron donor properties of clove oil 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.

3.3. Ferrous ions (Fe^{2+}) chelating capacity

Elemental species such as ferrous iron (Fe^{2+}) can facilitate the production of ROS within animal and human systems; hence, the ability of substances to chelate iron can be a valuable antioxidant capability. 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 clove oil, BHA, BHT, α-tocopherol and trolox are shown in Table 1. The chelating effect of ferrous ions (Fe^{2+}) by the clove oil and standards was determined according to the method of Dinis et al. (1994). Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. 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.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

Ferric ions (Fe^{3+}) also produce radicals from peroxides although the rate is tenfold lesser than that of ferrous (Fe^{2+}) ions (Miller, 1996). Ferrous ions (Fe^{2+}) are the most powerful pro-oxidants among the various species of metal ions (Halliwell and Gutteridge, 1984). Minimising 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

Table 1 Hydrogen peroxide (H_2O_2) scavenging activity, metal chelating activity on ferrous ion (Fe^{2+}) and superoxide anion radical ($O_2^{\cdot-}$) scavenging activity by riboflavin/methionine/illuminate system of clove oil and standard antioxidant compounds such as BHA, BHT, α-tocopherol and trolox at the concentration of 15 μg/mL (BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene).

	H_2O_2 scavenging activity (%)	Metal chelating activity (%)	Superoxide scavenging activity (%)
BHA	13.6 ± 3.5	69.9 ± 7.5	75.3 ± 6.5
BHT	16.7 ± 4.1	60.0 ± 9.3	70.2 ± 7.1
Trolox	25.6 ± 3.3	45.2 ± 6.2	16.0 ± 1.9
α-Tocopherol	13.6 ± 2.9	31.3 ± 5.5	22.2 ± 3.3
Clove oil	22.9 ± 2.3	58.2 ± 1.7	57.0 ± 0.4

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 colour 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 clove oil was assessed for its ability to compete with ferozzine for ferrous ions (Fe^{2+}) in the solution. In this assay, clove oil interfered with the formation of ferrous and ferrozine complex suggesting that they have chelating activity and are capable of capturing ferrous ion before ferrozine.

As can be seen Table 1, the difference between 15 μg/mL concentration and the control values was statistically significant ($p < 0.01$). In additon, clove oil exhibited 58.2 ± 1.7% ferrous ions chelating activity at 15 μg/mL concentration. On the other hand, the percentages of ferrous ions (Fe^{2+}) chelating capacity of the same concentration of BHA, BHT, α-tocopherol and trolox were found as 69.9 ± 7.5, 60.0 ± 9.3, 31.3 ± 5.5 and 45.2 ± 6.2%, respectively. These results show that the ferrous ion chelating effect of clove oil was lower than that of BHA ($p > 0.05$) and similar to BHT ($p > 0.05$) but higher than α-tocopherol ($p < 0.05$) and trolox ($p < 0.05$), statistically.

Metal chelating capacity was significant since it reduced the concentration of the catalyzing 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 Table 1 reveal that clove oil demonstrates a marked capacity for iron binding, suggesting that its main action as peroxidation protector may be related to its iron binding capacity.

3.4. Hydrogen peroxide scavenging activity

Biological systems can produce hydrogen peroxide (MacDonald-Wicks et al., 2006). It is also produced from polyphenol-rich beverages under quasi-physiological conditions and increases in amount with the incubation time (Chai et al., 2003). Furthermore, hydrogen peroxide 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 phagocytes (MacDonald-Wicks et al., 2006). It is known that H_2O_2 is toxic and induces cell death in vitro (Aoshima et al., 2004). Hydrogen peroxide can attack many cellular energy-producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Hyslop et al., 1988). The ability of clove oil to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (1989) as shown in Table 1 and compared with that of BHA, BHT α-tocopherol and trolox as standards. Hydrogen peroxide scavenging activity of clove oil at the used concentration (15 μg/mL) was found to be 22.9 ± 2.3%. On the other hand, BHA, BHT, α-tocopherol and trolox exhibited 13.6 ± 3.5, 16.7 ± 4.1, 13.6 ± 2.9 and 25.6 ± 3.3% hydrogen peroxide scavenging activity respectively at the same concentration. These results showed that clove oil had an effective hydrogen peroxide scavenging activity. At the above concentration, the hydrogen peroxide scavenging effect of clove oil and four standard compounds decreased in the order of trolox ≈ clove oil > BHT > BHA ≈ α-tocopherol. Hydrogen peroxide itself is not very reactive; however it can sometimes be toxic to cells 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 the protection of pharmaceutical and food systems (Gülçin, 2006b).

3.5. 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). Assays based on the use of $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals are among the most popular spectrophotometric methods for determination of the antioxidant capacity of food, beverages and vegetable extracts (Bendini et al., 2006). Both the chromogen radical compounds can directly react with antioxidants. Additionally, $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ scavenging methods have been used to evaluate the antioxidant activity of compounds due to their simple, rapid, sensitive, and reproducible procedure (Özcelik et al., 2003). However, Awika et al. (2003) have recently reported superiority of $ABTS^{\cdot+}$ assay over $DPPH^{\cdot}$, as $ABTS^{\cdot+}$ assay is operable over a wide range of pH, is inexpensive, and more rapid than that of $DPPH^{\cdot}$ assay.

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 their ability to scavenge synthetic free radicals, using a variety of radical-generating systems and methods for detection of the oxidation end-point. $ABTS^{\cdot+}$ or $DPPH^{\cdot}$ radical-scavenging methods are common spectrophotometric procedures for determining the antioxidant capacities of components. When an antioxidant is added to the radicals there is a degree of decolourization owing to the presence of the antioxidants which reverses the formation of the $DPPH^{\cdot}$ radical and $ABTS^{\cdot+}$ cation:

$$DPPH^{\cdot} + AH \rightarrow DPPH_2 + A^{\cdot}$$

$$ABTS^{\cdot+} + AH \rightarrow ABTS^{+} + A^{\cdot}$$

These chromogens (the violet DPPH radical and the blue green ABTS radical cation) are easy to use, have a high sensitivity, and allow for rapid analysis of the antioxidant activity of a large number of samples. These assays have been applied to determine the antioxidant activity of pure components (Awika et al., 2003; van den Berg et al., 2000; Yu et al., 2002). In this study, three radical scavenging methods were used to assess the determination of potential radical scavenging activities of clove oil, namely $ABTS^{\cdot+}$ radical scavenging, DPPH radical scavenging and· superoxide anion radical scavenging activity.

DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances (Özcelik et al., 2003). In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow col-

oured 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 the decrease in the absorbance of DPPH$^{\cdot}$ at 517 nm. resulting in a colour change from purple to yellow, the absorbance decreased when the DPPH$^{\cdot}$ was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH$^{\cdot}$ 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 (Matthäus, 2002). Fig. 4 illustrates a significant decrease ($p < 0.01$) in the concentration of DPPH radical due to the scavenging ability of clove oil and standards. BHA, BHT, α-tocopherol and trolox were used as references for radical scavengers. The scavenging effect of clove oil and standards on the DPPH radical decreased in the order of clove oil > BHT > α-tocopherol > BHA > trolox, which were 83.6, 67.8, 64.9, 62.5 and 29.4%, at the concentration of 45 μg/mL, respectively. DPPH free radical scavenging activity of clove oil also increased with an increasing concentration (r^2: 0.8786). EC_{50} value for clove oil was found as 21.50 μg/mL. Lower EC_{50} indicates a higher DPPH free radical scavenging activity. 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). Proposed reaction based on the analysis of the DPPH and clove oil molecule is summarized in Fig. 3.

Bleaching of a preformed solution of the blue-green radical cation ABTS$^{\cdot+}$ has been extensively used to evaluate the antioxidant capacity of complex mixtures and individual compounds. The reaction of the preformed radical with free-radical scavengers can be easily monitored by following the decay of the sample absorbance at 734 nm. The ABTS radical cation can be prepared employing different oxidants. Results obtained using $K_2S_2O_8$ as oxidant show that the presence of peroxodisulfate increases the rate of ABTS$^{\cdot+}$ autobleaching in a concentration-dependent manner. ABTS$^{\cdot+}$ radicals were generated in the ABTS/$K_2S_2O_8$ system:

$$S_2O_8^{2-} + \text{ABTS} \rightarrow SO_4^{2-} + SO_4^{-\cdot} + \text{ABTS}^{\cdot+}$$

where the scission of the peroxodisulfate could take place after the electron transfer. In the presence of excess ABTS, the sulfate radical will react according to

$$SO_4^{-\cdot} + 2\text{ABTS} \rightarrow SO_4^{2-} + 2\text{ABTS}^{\cdot+}$$

leading to a whole reaction represented by

$$S_2O_8^{2-} + 2\text{ABTS} \rightarrow 2SO_4^{2-} + 2\text{ABTS}^{\cdot+}$$

ABTS$^{\cdot+}$ radicals are more reactive than DPPH radicals and unlike the reactions with DPPH radical which involve H atom transfer, the reactions with ABTS$^{\cdot+}$ radicals involve electron transfer process (Kaviarasan et al., 2007). Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of pure substances, solutions, aqueous mixtures and beverages (Miller, 1996). A more appropriate format for the assay is a decolourization technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants. The improved technique for the generation of ABTS$^{\cdot+}$ described here involves the direct production of the blue/green ABTS$^{\cdot+}$ chromophore through the reaction between ABTS and potassium persulfate. ABTS$^{\cdot+}$, the oxidant, was generated by potassium persulfate oxidation of ABTS2− and the radical cation is measured spectrophotometrically. This is a direct generation of a stable form of radical prior to the reaction with antioxidants, to create a blue–green ABTS$^{\cdot+}$ chromophore (MacDonald-Wicks et al., 2006).

All the tested compounds exhibited affectual radical cation scavenging activity. As seen in Fig. 4, clove oil had an effective ABTS$^{\cdot+}$ radical scavenging activity in a concentration-dependent manner (15–45 μg/mL, (r^2: 0.8011). EC_{50} value for clove

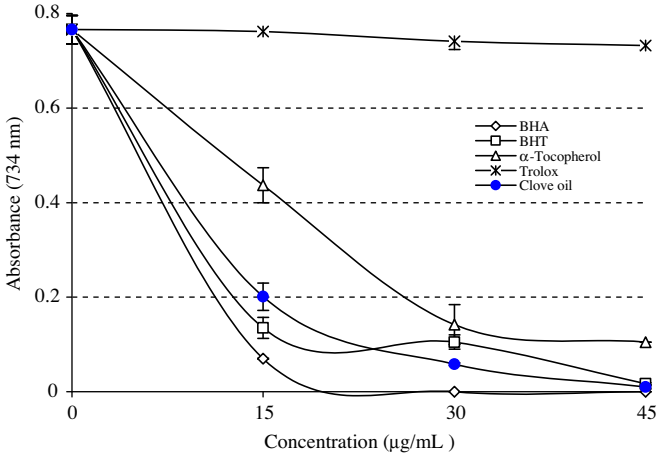

Figure 4 ABTS radical scavenging activity of different concentrations (15–45 μg/mL) of clove oil (r^2: 0.8011) and reference antioxidants; BHA, BHT, α-tocopherol and trolox (BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene; ABTS$^{\cdot+}$: 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

oil was found as 14.83 μg/mL. There is a significant decrease ($p < 0.01$) in all concentrations of $ABTS^{\cdot+}$ due to the scavenging capacity of all clove oil concentrations. Also, the scavenging effect of clove oil and standards on the $ABTS^{\cdot+}$ decreased in the order: BHA = clove oil ≈ BHT > α-tocopherol > trolox, which were 100, 98.7, 97.8, 86.3 and 4.4%, at the concentration of 45 μg/mL, respectively. Also, no significant differences in $ABTS^{\cdot+}$ scavenging potential could be determined among clove oil, BHA and BHT ($p > 0.05$).

Numerous biological reactions generate superoxide radical which is a highly toxic species. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potential precursors of a highly reactive species, such as hydroxyl radical, and thus the study of the scavenging of this radical is important (Kanatt et al., 2007). Therefore, superoxide anion scavenging capacity in the living organisms is the first line of defense against oxidative stress (Schauss et al., 2006). Superoxide anion is an oxygen-centred radical with a selective reactivity. Although relatively weak oxidants, superoxides exhibit only 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 the potential of reacting with biological macromolecules and thereby inducing tissue damage (Halliwell and Gutteridge, 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 (MacDonald-Wicks 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 Duh, 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 theyproduce other kinds of free radicals and oxidizing agents (Liu et al., 1997). Superoxide anions are derived from dissolved oxygen in 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; Parejo et al., 2002). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Table 1 shows the inhibition percentage of superoxide radical generation by 15 μg/mL concentration of clove oil and standards. The inhibition of superoxide radical generation of clove oil is higher than α-tocopherol and trolox but lower than BHA and BHT. As can be seen in Table 1, the percentage inhibition of superoxide anion radical generation by 15 μg/mL concentration of clove oil was found as 57.0 ± 0.4%. On the other hand, at the same concentration, BHA, BHT and α-tocopherol and trolox exhibited 75.3 ± 6.5, 70.2 ± 7.1, 22.2 ± 3.3 and 16.0 ± 1.9% superoxide anion radical scavenging activity, respectively. According to these results, clove oil had a higher superoxide anion radical scavenging activity than α-tocopherol ($p < 0.01$) and trolox ($p < 0.01$) but lower than that of BHA and BHT.

4. Conclusion

Clove oil has been recognised as a "Generally Regarded As Safe" substance by the United States Food and Drug Administration when administered at levels not exceeding 1500 ppm in all food categories. Additionally, the World Health Organisation (WHO) Expert Committee on Food Additives has established the acceptable daily human intake of clove oil at 2.5 mg/kg body weight for humans (Kildeaa et al., 2004). This consumption level of clove oil can reduce many health risks. According to data obtained from the present study, clove oil was found to be an effective antioxidant in different in vitro assays including reducing power, DPPH radical ABTS radical and superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities when it is compared to standard antioxidant compounds such as α-BHA, BHT, tocopherol, a natural antioxidant, and trolox which is a water-soluble analogue of tocopherol. Based on the discussion above, it can be used for minimising or preventing lipid oxidation in food and pharmaceutical products, retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the shelf life of food and pharmaceuticals.

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