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DNA damage protecting activity and *in vitro* antioxidant potential of the methanol extract of Cherry (*Prunus avium* L)

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The characterization of sweet cherry (*Prunus avium* L.) genetic resources in Turkey may help to increase their use in breeding programmers worldwide, as Turkey is the centre of origin of sweet cherry. In the Malatya region, cherry leaves are gathered in April and May, fresh and dried used for stuffing. Components of cherry trees have been used as traditional herbal remedies for various diseases. These components are known to possess antioxidative effects. However, the mechanisms underlying cherry tree component-mediated antioxidative effects remain largely unknown. This study focused on cherry 0-900 Ziraat (Malatya Dalbastı) leaves methanol extract (CLME) and examined antioxidant capacity and DNA damage protecting activity. The antioxidant capacity of these extracts were evaluated using different antioxidant tests, including reducing power, free radical scavenging, hydroxyl radical scavenging, hydrogen peroxide scavenging, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ABTS radical scavenging, deoxyribose assay, β-Carotene bleaching assay and metal chelating activities. The content of total phenolic compounds in CLME was determined using Folin-Ciocalteus reagent and compared with standard antioxidants butylated hydroxytoluene (BHT), Trolox and α-tocopherol. CLME showed a concentration-dependent free radical scavenging capacity and protective DNA strand scission by $OH^{\cdot}$ on pBR322 super coiled plasmid DNA (95 to 97%). Total phenolic effect on DNA cleavage CLME at 400 µg/ml exhibited significant protecting activity against the compounds in the CLME and was determined as gallic acid equivalent 132.17 meq/g dried leaves.

Key words: Cherry 0-900 ziraat (Malatya dalbastı), antioxidant activity, radical scavenging ability, DNA damage, phenolic compounds.

INTRODUCTION

The sweet cherry was originated in the mountains of North Eastern part of Turkey, near the Black Sea region, from where it has spread in Roman times (Zohary and Hopf, 2000). Turkey is one of the leading countries in production and export of sweet cherries (Faostat, 2011). Suitable climatic conditions and excellent export market

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demand have resulted in continuous increase in annual production. *Prunus avium* L is the sweet cherry. Sweet cherry is a vigorous tree with strong apical control with an erect-pyramidal canopy shape; grows to 18 m. In cultivation, sweet cherries are maintained <4 m in height. Leaves are relatively large, elliptic with acute tips, petioles, and strongly veined (Anonymous, 2003). Fruits of these species are not only consumed fresh, but also used to produce jam, jelly, stewed fruit, marmalade, syrup and several types of soft drinks. It is also used for medical purposes due to properties of stalk and fruits. The leaves and seed of these species are used in pharmaceuticals. The tree is also valuable for ornamentation as an evergreen broad leaf plant (Islam, 2002). In addition, stuffed cherry leaves are consumed as food in Malatya.

Free radicals are the molecules with unpaired electrons and commonly called reactive oxygen species (ROS). Free radicals are generated during the process of cellular oxidation, some examples includes superoxide anion, hydrogen peroxide, hydroxyl and nitric oxide radical. These radicals are electrically charged, unstable and highly reactive in nature. It reacts with nucleic acids, mitochondria, proteins and enzymes and resulted in their damage in the cell. However, antioxidant defense system protects the cell from the free radical mediated oxidative stress. When there is over production of free radicals or the failure of an antioxidant defense system, these radicals resulted in tissue injury and cause numerous physiological disorders in the body, cancer, Parkinson's disease, Alzheimer's disease, myocardial infarction and diabetes (Waris and Ahsan, 2006; Zhou et al., 2008; Praticò, 2008; Elahi et al., 2009; Uttara et al., 2009; Wright et al., 2006). It has been proved that a diet rich in antioxidants strengthens the antioxidant defense system and can effectively neutralize the free radicals in body.

Currently, many kinds of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and propyl gallate (PG), which are commonly used in processed foods, are known to have toxic and carcinogenic effects on human health. Natural antioxidants, such as *α*-tocopherol and L-ascorbic acid, are widely used, because they are seen as being safer, but their antioxidant activities are lower than those of synthetic antioxidants (Barlow, 1990). Based on accumulative evidence, in recent decades, tremendous interest has considerably increased in finding natural substances (that is, antioxidants) present in foods or medicinal plants to replace synthetic antioxidants, which are being restricted due to their side effects. On the other hand, polyphenols, used as natural antioxidants, are gaining importance, due to their health benefits for humans, decreasing the risk of cardiovascular and degenerative diseases by reduction of oxidative stress and counteraction of macromolecular oxidation (Bingham et al., 2003; Silva et al., 2004). Thus, much attention has been focused on the investigation of natural antioxidant compounds from plants, which can effectively scavenge ROS.

Compositional characteristics and antioxidant components, such as oxygen radical absorbance capacity (ORAC) using fluorescein (FL), phenolic acids, and total contents of phenolics, anthocyanins, and carotenoids, of cherry laurel varieties and pekmez were investigated (Alasalvar et al., 2005). However, relatively little or no information is available on free-radical scavenging activities, reducing power, and inhibition of oxidation of human low-density lipoprotein (LDL) cholesterol of cherry laurel fruit and its pekmez (Kolayli et al., 2003).

Cherry laurel fruit and its concentrated juice (pekmez) were examined for their antioxidant activities using different free-radical scavenging activity tests [hydrogen peroxide, superoxide radical, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical], together with reducing power and inhibition of oxidation of human low-density lipoprotein cholesterol. On a fresh weight basis, pekmez exhibited a significantly ($P < 0.01$) higher antioxidant activity than that of cherry laurel fruit in most cases. However, on a dry weight basis hydrogen peroxide and DPPH radical scavenging activities, and reducing power were significantly higher ($P < 0.01$) in cherry laurel fruit than in its pekmez, with some exceptions, thus indicating possible destruction of antioxidative compounds during pekmez production (liyana-Pathirana et al., 2006).

There have been only a few studies on the pharmacological effects of cherry leaves. Cherry leaves extract (CLE) was shown to have antioxidant activity through reduction of ROS production that showed a phenolic glucoside isolated from CLE exhibited peroxynitrite scavenging (Jung et al., 2002; Jung et al., 2005). Thus, previous studies *in vitro* showed that CLE has antioxidative effects. However, the mechanisms underlying CLE mediated antioxidative effects remain largely unknown. CLE showed that it prevented t-BOOH-induced reduction of thioredoxin-2 (Trx2), but not thioredoxin-1 (Trx1) and Trx reductases (TrxR1 and TrxR2) protein expression. CLE prevents tert-butyl hydroperoxide (t-BOOH)-induced reduction in (Trx2) expression, promotion of ROS production, activation of p38 kinase, and increase in DNA damage and protects against cell death (Taguchi et al., 2011).

The aim of this study was to investigate the antioxidant and DNA damage protecting activity of a methanol extract of cherry leaves.

MATERIALS AND METHODS

Chemicals

pBR322 super coiled plasmid DNA, agarose, ethidium bromide, Tris-Borat-EDTA gel buffer, bromophenol blue, and EDTA were purchased from Vivantis; ammonium molybdate tetrahydrate, aluminum chloride hexahydrate, sodium nitrite, sodium carbonate

decahydrate, trolox, α-tocopherol, ethanol, methanol, ethyl acetate, DPPH, Tween 20, hydrogen peroxide, linoleic acid, and trans-beta-carotene were purchased from Sigma-Aldrich; chloroform, potassium ferricyanide, sodium thiosulfate, iron chloride tetrahydrate, $FeSO_4$, sulphuric acid, β-mercapto ethanol, $MnCl_2$, Iron (III) chloride, and sodium salicylate were purchased from Merck; trichloroacetic acid (Alfa Aesar) and BHT were purchased from Safc; methanol, $K_3Fe(CN)_6$, ferrozine, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) were purchased from (Fluka), 2-Deoxy-D-Ribose were purchased from Alfa Aesar; potassium dihydrogen phosphate was purchased from Riedel.

Plant and extraction

Sweet cherry cultivars 0-900 Ziraat (Dalbastı) grown in Malatya region of Turkey were used in this study. Cherry leaves were collected from the area of Malatya Fruit Research Institute, Malatya, Turkey in the first week of June, 2009. Leaves were dried for 10 days at room temperature. Cherry leaves (20 g) were ground in an electric blender and then incubated into a glass flask with 300 ml of methanol in the dark for 3 days on a magnetic stirrer, at 25°C, filtered, and concentrated by using a rotary evaporator (İka RV 05 Basic 1B). The crude methanol extract of cherry leaves (1,23 g), as a green color, was obtained in dark glass bottles at 4°C until use.

Determination of total phenolic compounds

The content of total phenolic compounds in the methanol extract of cherry leaves was determined using Folin-Ciocalteus reagent according to the method of Singleton et al. (1999). Crude methanol extract (40 µl) of cherry leaves (1 mg/ml) was mixed with 200 µl Folin-Ciocalteus reagent and 1160 µl of distilled water, followed by 600 µl 20% sodium carbonate (Na_2CO_3) at 3 min later. The mixture was shaken for 2 h at room temperature and absorbance was measured at 765 nm. All tests were performed in triplicate. Gallic acid was used as a standard. The concentration of total phenolic compounds in cherry leaves extracts was determined as a µg of gallic acid equivalents per 1 mg of extract using the following equation obtained from a standard gallic acid graph (R^2 = 0.9878):

Absorbance = 0.0012 × gallic acid (µg).

Scavenging activity of DPPH radical

The free radical scavenging activity of CLME was measured by DPPH using the previously reported procedure (Yen and Chien, 2000). Briefly, 0.1 mM solution of DPPH in methanol was prepared. Then, 1 ml of this solution was added to 3 ml of CLME solution at different concentrations (0.050 to 0.50 mg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance was measured at 517 nm in a spectrophotometer (Shimadzu, UV/Visible Recording, Kyoto, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The radical scavenging activity was calculated as follows:

Scavenging effect (%) = [(A_{517} of control-A_{517} of sample/A_{517} of control)] × 100.

where BHT, α-tocopherol and Trolox were used as positive controls.

ABTS radical cation decolorization assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging activity was determined according to the method of Re et al. (1999). 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 BHT, a-tocopherol and trolox, a water-soluble a-tocopherol analogue. The $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 12 h. Before usage, the $ABTS^{\bullet+}$ solution was diluted to get an absorbance of 0.750 ± 0.025 at 734 nm with ethanol. Then, 2.3 ml of $ABTS^{\bullet+}$ solution was added 100 µl of CLME solution at different concentrations (0.10 to 0.30 mg/ml). After 30 min, the percentage inhibition of 734 nm was calculated for each concentration relative to a blank absorbance. Solvent blanks were run in each assay. The extent of decolorization is calculated as percentage reduction of absorbance. The scavenging capability of $ABTS^{\bullet+}$ radical was calculated using the following equation:

$ABTS^{\bullet+}$ scavenging effect (%) = $(1- A_S/A_C) \times 100$

where A_C is the initial concentration of the $ABTS^{\bullet+}$ and A_S is absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of CLME (Gülçin et al., 2010).

Determination of reducing power

The reducing power of CLME solution was determined according to the method of Oyaizu (1986). Different concentrations of CLME solution (0.050, 0.10 and 0.25 mg) in 1 ml of distilled water were mixed with 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. A portion (2.5 ml) of TCA (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) 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 (Shimadzu, UV/Visible Recording). Higher absorbance of the reaction mixture indicated greater reducing power. Butylated hydroxytoluene and α-tocopherol were used as standards.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenger ability was measured according to a literature procedure with a few modifications (Smirnoff and Cumbes, 1989). Hydroxyl radical was generated from Fenton reaction between 1.5 mM $FeSO_4$ and 6 mM H_2O_2 (1.4:1, v/v) at 37°C for 30 min before the assay, and was detected by their ability to hydroxylate salicylate. The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and varied concentrations of the extracts (0.10, 0.20, and 0.30 mg/ml). After incubation for 1 h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The scavenging activity of hydroxyl radical effect was calculated as follows: $[1-(A_1-A_2)/A_0] \times 100$, where A_0 is absorbance of the control (without extract) and A_1 is the absorbance in the presence of the extract, A_2 is the absorbance without sodium salicylate.

Hydrogen peroxide (H_2O_2) scavenging activity assay

Hydrogen peroxide scavenging activity of CLME and standards was assayed by the method of Zhao et al. (2006). H_2O_2 (1.0 ml, 0.1 mM) and 1.0 ml of various concentrations (0.15, 0.30, and 0.45 mg/ml) of the extract were mixed, followed by 100 µl 3% ammonium molybdate, 10 ml H_2SO_4 (2 M) and 7.0 ml KI (1.8 M). The mixed solution was titrated with $Na_2S_2O_3$ (5 mM) until the yellow color

disappeared. The percentage scavenging effect was calculated as:

Hydrogen peroxide scavenging rate (%) = $[V_C-V_S/V_C) \times 100$,

where V_C was the volume of $Na_2S_2O_3$ solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), V_S was the volume of $Na_2S_2O_3$ solution used in the presence of CMLE. Ascorbic acid was used as standard.

Deoxyribose assay

The reaction mixture, containing methanol extract of CLME (0,02 to 0.10 mg/ml) was incubated with deoxyribose (10 mM), H_2O_2 (50 mM), $FeCl_3$ (10 µM), EDTA (1 mM) and ascorbic acid (10 mM) in potassium phosphate buffer (50 mM, pH 7.4) for 60 min at 37°C (Halliwell et al., 1987). Then, reaction was terminated by adding 1 ml of 10% TBA (1% w/v) and 1 ml of TCA (2% w/v) and then heating the tubes in a boiling water-bath for 15 min. The contents were cooled and the absorbance of the mixture was measured at 532 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose:

Inhibition (%) = [(Ac-As)/Ac] × 100,

where Ac is the absorbance of the control and As is the absorbance in the presence of samples of extracts.

β-Carotene bleaching test

The test was carried out following the spectrophotometric method of Miller (1971), based on the ability of the different extracts to decrease the oxidative bleaching on β-carotene in a β-carotene/linoleic acid emulsion, was used. 2.0 mg sample of crystalline β-carotene was dissolved in 10 ml of chloroform and 1 ml of this solution was added to 20 µl of linoleic acid and 200 µl of Tween- 20 in a round-bottom flask. After removing the chloroform in a rotary evaporator under vacuum at 40°C for 5 min, 50 ml distilled water was added to the residue, with vigorous stirring in order to form an emulsion. Five milliliters of this emulsion was added to each tube containing extracts (0.05 mg/ml). A zero reading was taken at 470 nm on the reaction mixture in each tube immediately after addition of the emulsion to the antioxidant solution. A control sample with distilled water instead of extract was also analyzed for antioxidant activity. The tubes were then stoppered and placed in a water bath at 50°C. Subsequent readings were taken at regular intervals until the carotene had been decolorized (about 90 min). All determinations were performed in duplicate. As standard Trolox, α-tocopherol, BHT and ascorbic acid were used. The percentage inhibition was calculated from the data with the slightly modified formula (Kulisic at al., 2004).

Inhibition (%) = $[(A_{S(90)} - A_{C(90)})/(A_{C(0)}-A_{C(90)})] \times 100$

where $A_{S(90)}$ is the absorbance of the antioxidant at t=90 min, $A_{C(90)}$ is the absorbance of the control at t=90 min, and $A_{C(0)}$ is the absorbance of the control at t=0 min.

Effect of cherry leaves methanol extract (CLME) on pBR322 super coiled plasmid DNA scission induced by hydroxyl radical

DNA damage protective activities of CLME were checked on pBR322 super coiled plasmid DNA (Vivantis). Plasmid DNA was oxidized with H_2O_2 + UV treatment in the presence of CLME and checked on 1% agarose after modification (Attaguile et al., 2000).

In brief, the experiments were performed in a volume of 10 µl in a microcentrifuge tube containing 200 ng of plasmid DNA in phosphate buffer (7.14 mmol phosphate and 14.29 mmol NaCl), pH 7.4, H_2O_2 was added at a final concentration of 2.5 mmol/L with and without 1 µl of 0.20 and 0.40 mg/ml methanol extract. The reactions were initiated by UV irradiation and continued for 5 min on the surface of a UV transilluminator (8000 µW cm^{-1}) at 300 nm at room temperature. After irradiation, the reaction mixture (10 µl) with gel loading dye was placed on 1% agarose gel for electrophoresis. Electrophoresis was performed at 40 V for 3 h in the presence of ethidium bromide (10 mg/ml). Untreated pBR322 super coiled plasmid DNA was used as a control in each run of gel electrophoresis along with partial treatment, that is, only ultraviolet (UV) treatment and only H_2O_2. Percentage inhibition of the DNA strand scission was calculated as follows:

Inhibition (%) = I−[(Sm + a − Sc)/(Sm − Sc)]

where Sm+a is the percentage remaining super coiled after treatment with mix plus agent, Sc is the percentage remaining super coiled in control untreated plasmid, and Sm is the percentage remaining super coiled mix without agent (Fukuhara and Miyata, 1998).

Densitometric analysis of treated and control pBR322 super coiled plasmid DNA

Gel was scanned on the gel documentation system (Gel-Doc-XR, BioRad, Hercules, CA). Bands on the gels were quantified discovery series Quantity One program (version 4.5.2, BioRad).

Statistical analysis

The antioxidant data in the present study were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between the means was determined by the Duncan's multiple range tests at 95% least significant difference ($P < 0.05$).

RESULTS AND DISCUSSION

Polyphenolic contents of the extracts

Phenolic compounds are known as powerful chain breaking antioxidants (Shahidi and Wanasundara, 1992). Phenols are very important plant constituents, because of their scavenging ability due to their hydroxyl groups (Hatano et al., 1989). The phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1 g daily from a diet rich in fruits and vegetables (Tanaka et al., 1998). The total amount of phenolic content in 1 g methanol extract of cherry leaves 116.4 ± 35.0 mg gallic acid equivalent of phenols was detected.

Antioxidant activity

DPPH radical scavenging activity

The free-radical scavenging activity of methanol extract of CLME was tested by their ability to bleach the stable

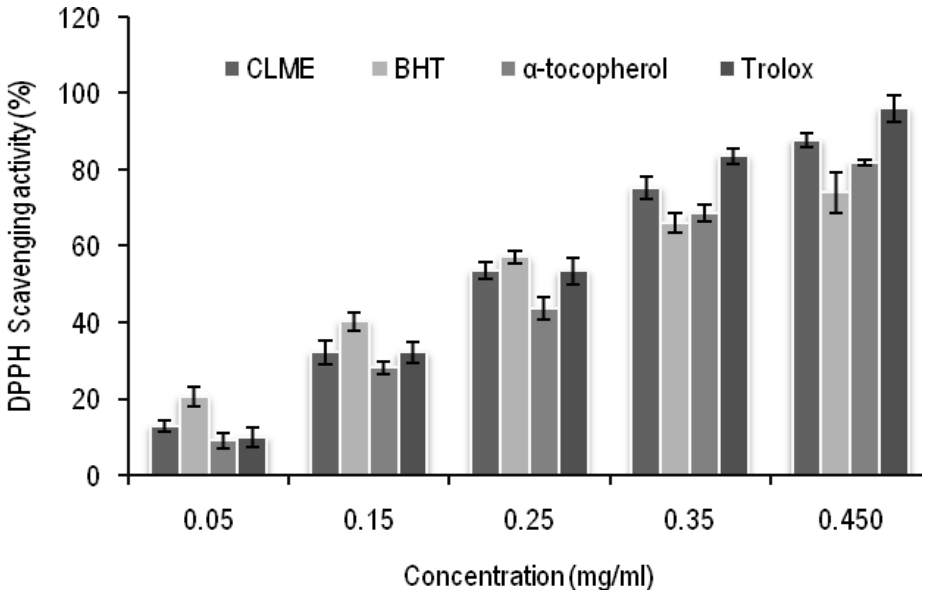

Figure 1. Scavenging effect of CLME on 1,1-diphenyl-2-picrylhydrazyl radicals. Each value is expressed as mean ± SD (n = 3).

DPPH radical (Saija et al., 1998). This assay provided information on the reactivity of crude extract with stable free radical. The effects of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). The reduction capability of DPPH radicals was determined by a decrease in absorbance at 517 nm induced by antioxidants. The extract exhibited DPPH free radical scavenging activity in a concentration-dependent manner (Figure 1).

CLME exhibited stronger DPPH scavenging activity. Scavenging activity of CLME and standard compounds followed the order: Trolox > CLME > α-tocopherol > BHT, and were 96.17 ± 3.51, 87.80 ± 1.73, 81.92 ± 0.58 and 74.0 ± 5.29% at 0.45 mg/ml concentration, respectively.

ABTS radical-scavenging activity assay

Another effective method to measure radical scavenging activity is the ABTS radical cation decolorization assay, which showed similar results to those obtained in the DPPH reaction. The ABTS radical scavenging by CLME and standard antioxidant decreased in the following order: Trolox (89.98%) and CLME (81.95%) at a concentration of 0.30 mg/ml test sample. Also, the percentages of ABTS cation radical scavenging activity of different concentrations (0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mg/ml) of CLME were found to be 25.37, 41.72, 51.27, 57.96, 77.81 and 81.95%, respectively. These results show that CLME has effective ABTS cation radical scavenging activity at higher concentration (Figure 2). However, these values are lower than those of standard antioxidant.

Reducing power

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and non-reactive species (Gülçin et al., 2007). The reducing power of CLME was investigated by FRAP. Antioxidant compounds reduce Fe^{3+}-ferricyanide complexes to the ferrous (Fe^{2+}) form. The Prussian blue colored complex is formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. Therefore, the amount of reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm (Chung et al., 2002). In this assay, the yellow color of the test solution changes to green or blue depending on the reducing power of the antioxidant. A higher absorbance indicates higher ferric reducing power. As shown in Figure 3, the ferric reducing power of CLME increased with increasing concentration, similar to the standard antioxidants. However, these differences were found as insignificant ($p > 0.05$). The reducing power of the CLME and standard antioxidants decreased in the following order: BHT > Trolox > α-tocopherol > CLME with 0.25 mg/ml test sample. CLME had a similar ferric reducing power to standard antioxidants (BHT, α-tocopherol and Trolox). Fe^{3+} reduction is often used as an

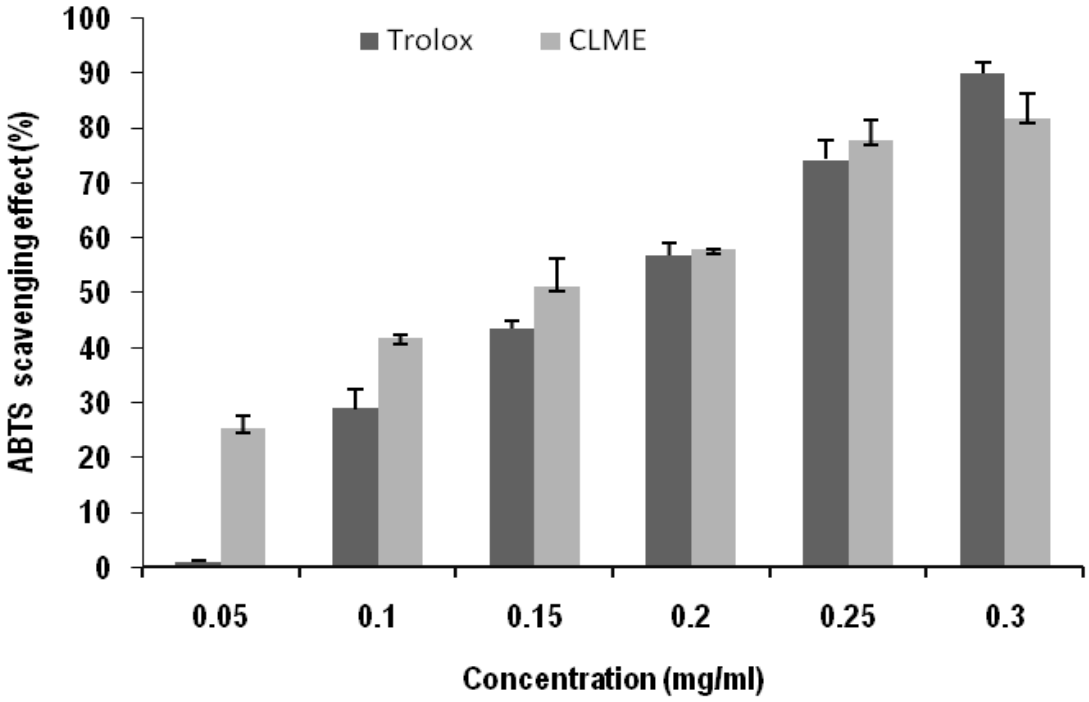

Figure 2. ABTS$^{\bullet+}$ radical scavenging activity of CLME.

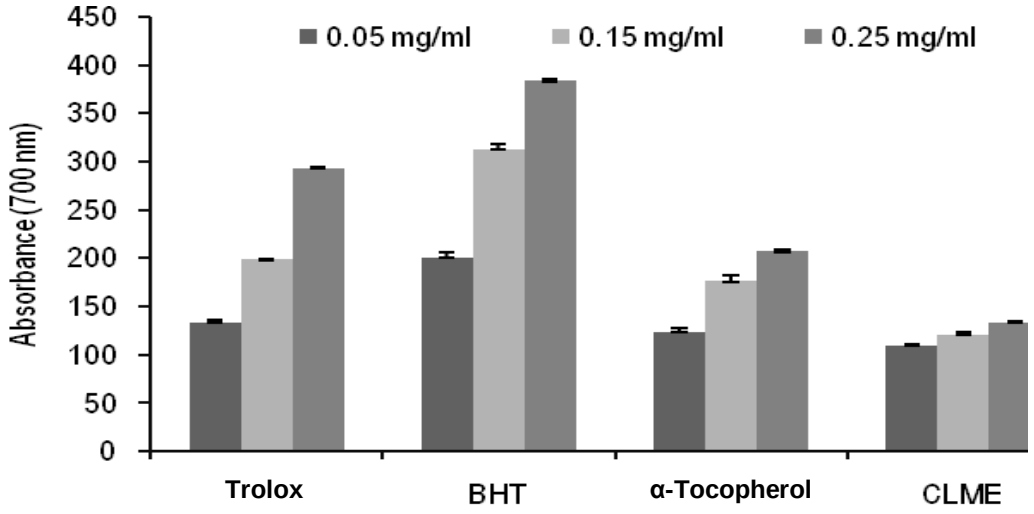

Figure 3. Reducing power of CLME.

indicator of electron-donating activity, which is an important mechanism of phenolic antioxidants (Dorman et al., 2003).

Hydroxyl radical scavenging activity

The hydroxyl radical is the most reactive of the ROS, and it induces severe damage in adjacent biomolecules (Gutteridge, 1984). The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins (Spencer et al., 1994). The $^{\bullet}$OH scavenging activity of mushroom extracts was assessed by its ability to compete with salicylic acid for $^{\bullet}$OH radicals in the $^{\bullet}$OH generating/ detecting system. In the present study, the hydroxyl radical-scavenging effect of the CLME, in a concentration of 0.1 mg/ml, was found to be 45.33% and in a concentration of 0.2 mg/ml, was found to be 43.99% (Figure 4).

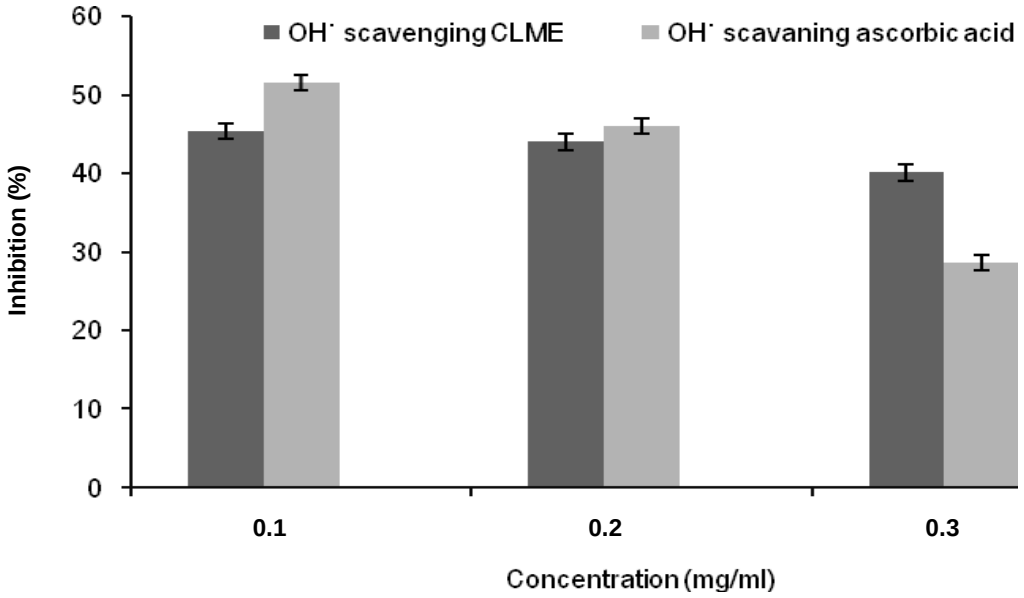

Figure 4. Hydroxyl radical scavenging activity of CLME.

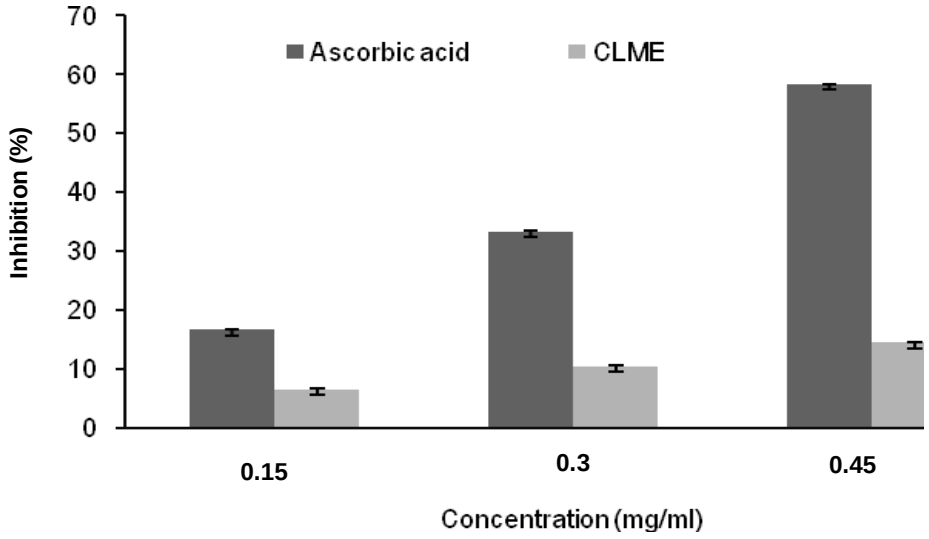

Figure 5. Hydrogen peroxide radical scavenging of CLME compared to that of ascorbic acid (Vitamin C).
Each value is expressed as mean ± standard deviation (n=3).

Hydrogen peroxide radical scavenging activity

The free radical scavenging activity of CLME was evaluated by hydrogen peroxide (H_2O_2) scavenging method. From the results, CLME showed concentration dependent activity and the H_2O_2 scavenging effect was 14.47% at a concentration of 0.45 mg/ml. This was comparable to the scavenging effect of ascorbic acid (58.33%) (Figure 5).

Deoxyribose assay

The deoxyribose method is a simple assay to determine the rate constants for reactions of hydroxyl radicals. When the mixture of $FeCl_3$-EDTA, H_2O_2 and ascorbate were incubated with deoxyribose in phosphate buffer (pH7.4), the generated hydroxyl radicals attack the deoxyribose and result in a series of reactions that cause the formation of malondialdehyde (MDA). Any hydroxyl

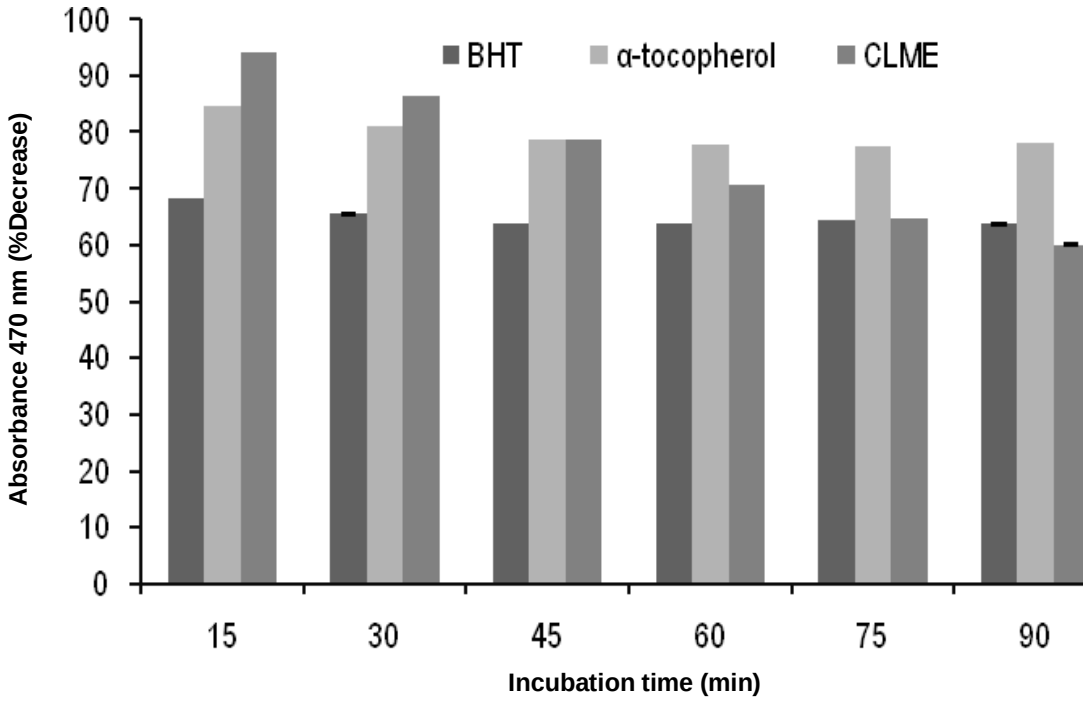

Figure 6. Relative changes in absorbance of beta carotene emulsions containing BHT, α-tocopherol and CLME.

radical scavenger added to reaction would compete with deoxyribose for the availability of hydroxyl radicals, thus reducing the amount of MDA formation (Wang et al., 2003). It has been found that the CLME showed concentration-independent scavenging activity on hydroxyl radicals (Figure 7). CLME exhibited 97% scavenging capacity at the 0.02 mg/ml concentration.

Antioxidant assay using the β-carotene bleaching method

The mechanism of beta-carotene bleaching is a free radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid by air oxidation. The antioxidant activity of carotenoids is based on the radical adducts of carotenoids with free radicals formed from linoleic acid. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated beta-carotene molecules. As beta-carotene molecules lose their double bonds by oxidation in the absence of an antioxidant, the compound loses its chromophore and characteristic orange color, which the anti-bleaching activity of sample of β-carotene was studied by monitoring the color intensity of emulsion at 470 nm for every 15 min for 90 min. The concentration taken was 0.05 mg/ml for the sample as well as standard (BHT and α-tocopherol). The initial concentration was considered to be 100%. As shown in Figure 6, in the first 15 min, the CLME showed 93.96% bleaching as compared to 68.27 and 84.50% to that of standard (BHT and α-tocopherol). In 60 min of incubation, percentage decrease was found to be 70.75 and 63.84%; 77.81% for CLME and standard (BHT; α-tocopherol), respectively. During the 90 min, it came to 60.06 and 63.69%; 78.17% for CLME and standard (BHT; α-tocopherol), respectively.

DNA damage protective activity of CLME

ROS-induced DNA damage can be described both chemically and structurally and shows a characteristic pattern of modification. It is well known that in various cancer tissues free radical-mediated DNA damage was found (Valko et al., 2001). The majority of these changes can be reproduced by ROS experimentally including the following: modification of all bases, production of base-free sites, deletions, frame shifts, strand breaks, DNA-protein cross-links, and chromosomal rearrangement. An important reaction involved in DNA damage involves generation of hyrodxyl radical, e.g., through Fenton chemistry (Brezova et al., 2003). Hydroxyl radical is known to react with all components of the DNA molecule: the purine and primidine bases as well as the deoxyribose backbone (Valko et al., 2004). When DNA was exposed to H_2O_2 and irradiated with UV light, H_2O_2 will be generated to hydroxyl radicals, then the super

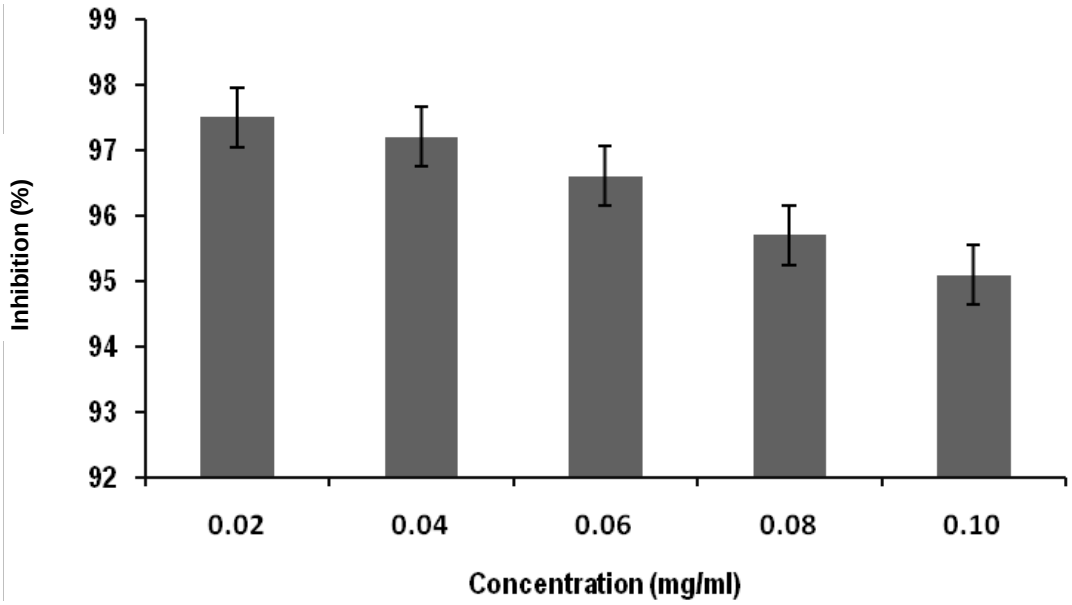

Figure 7. Effect of methanol extracts of CLME on deoxyribose degradation assay. Each value is expressed as mean of 3 replicates ± standard deviation.

coiled form of DNA would cleave.

DNA damage protective activity of CLME was investigated with pBR322 super coiled plasmid DNA (Vivantis). Figure 8A shows the quantified band intensity for the super coiled-DNA (form I), circular relaxed -DNA (form II) and lineer-DNA (form III). Figure 8B shows the electrophoretic pattern of DNA after UV-photolysis of H_2O_2 (2.5 mM) in the absence and presence of CLME (0.20 and 0.40 mg/ml). DNA derived from pBR322 super coiled plasmid DNA showed two bands on agarose gel electrophoresis (lane 1), the faster moving band corresponded to the native form of super coiled circular DNA and the slower moving band was the circular relaxed DNA form. The UV irradiation of DNA in the presence of H_2O_2 (lane 3) resulted in the cleavage of super coiled DNA to linear DNA form, indicating that OH radical generated from UV photolysis of H_2O_2 produced DNA strand scission. The addition of extract (lanes 5 to 7) to the reaction mixture suppressed the formation of lineer DNA and induced a partial recovery of super coiled DNA.

In fact, the intensity of super coiled DNA bands scanned from the agarose gel electrophoretic patterns was 95.7 and 96.2% for plasmid DNA treated with H_2O_2 in the presence of 0.20 and 0.40 mg/ml extract, respectively, as compared with the untreated plasmid DNA.

Thus, the identification of natural products able to provide protection against UV radiation-induced inflammatory responses and the generation of oxidative stress may have important human health implications. The DNA cleavage analysis demonstrated the strong antioxidant properties of CLME. In fact, this extract suppressed the formation of linear DNA, generated by exposure of plasmid DNA to OH radical generated by H_2O_2 UV-photolysis, and induced a partial recovery of super coiled DNA. DNA damage protecting activity of CLME is corresponding to its antioxidant potential. It is known that metal-induced generation of oxygen radicals results in the attack of not only DNA in cell nucleus, but also other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Esterbaurer et al., 1991; Marnett, 1999). The initial products of unsaturated fatty acid oxidation are short-lived lipid hydroperoxides. When they react with metals they produce a number of products (e.g., aldehydes and epoxides) which are themselves reactive. MDA is one of the major aldehyde products of lipid peroxidation. It is mutagenic in mammalian cells and carcinogenic in rats (Valko et al., 2004).

Conclusion

This study is the first to evaluate the antioxidant activity of CLME in a comprehensive manner employing a variety of *in vitro* methods. It was reported in this study that an extract from CLME was active in scavenging OH radicals in a deoxyribose assay, as well as quenching the stable free radical DPPH. The antioxidant potential of CLME was also further demonstrated through its reducing activity and total polyphenol content. The results of the present study would certainly help to ascertain the potency of the crude extract of CLME as a potential source of natural antioxidants. This work has gathered experimental evidence on the commonly used CLME as natural antioxidant for its capacity to protect organisms

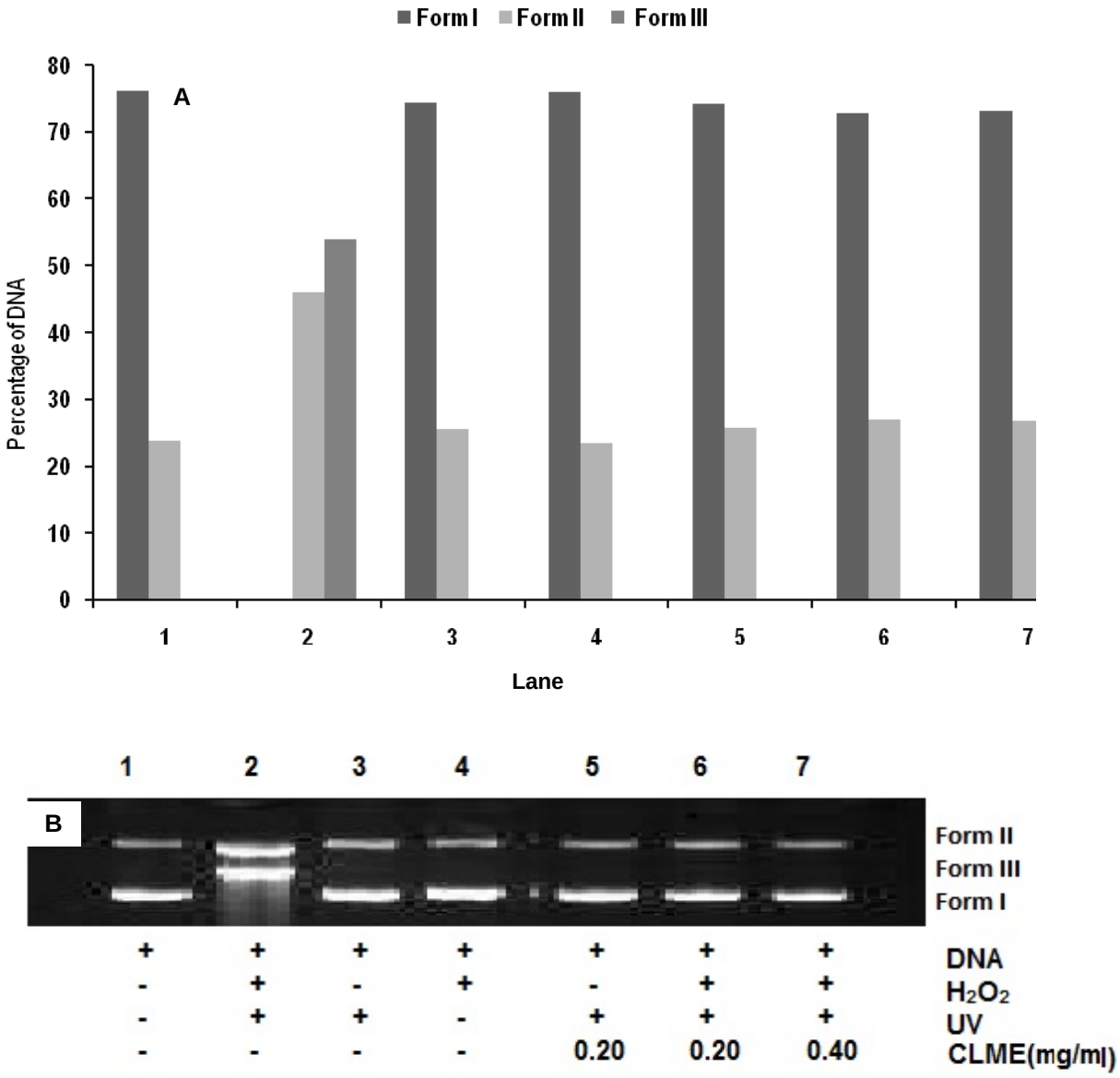

Figure 8. (A) The quantified band intensity for the sc-DNA (form I), oc-DNA (form II) and l-DNA (form III) with Quantity One 4.5.2. version software. (B) Electrophoretic pattern of pBR322 super coiled plasmid DNA after UV-photolysis of H_2O_2 in the presence or absence of CLME. Reaction vials contained 200 ng of super coiled DNA (31.53 nM) in distilled water, pH 7. Electrophoresis was performed using 1% agarose at 40 V for 3 h in the presence of ethidium bromide (10 mg/ml). Electrophoresis running buffer: TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.2).
Lane 1, control DNA; Lane 2, DNA + H_2O_2 (2.5 mM)+UV; Lane 3, DNA + UV; Lane 4, DNA + H_2O_2 (2.5 mM); Lane 5, DNA + CLME (0.20 mg/ml) + UV; Lane 6, DNA + CLME (0.20 mg/ml) + H_2O_2 (2.5 mM) + UV; Lane 7, DNA + CLME (0.40 mg/ml) + H_2O_2.

and cells from oxidative DNA damage associated with aging, cancer and degenerative diseases. This profound protective effect of CLME against oxidative DNA damage, free radicals scavenging, and inhibition of lipid peroxidation may explain its extensive use in daily life and possible health benefits.

Thus, CLME may serve as an ideal candidate for a cost-effective, readily exploitable natural polyphenolic phytochemical. However, further research is needed to identify individual components forming the antioxidative system and develop their application for food and pharmaceutical industries.

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Conflict of Interest

Authors declare no conflict of interest.

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