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Food Chemistry 105 (2007) 107–115
Food Chemistry
www.elsevier.com/locate/foodchem

Free radical scavenging and antioxidative activities of caffeic acid phenethyl ester (CAPE) and its related compounds in solution and membranes: A structure–activity insight

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Received 14 February 2006; received in revised form 16 January 2007; accepted 20 March 2007

Abstract

Caffeic acid phenethyl ester (CAPE) together with caffeic acid, ferulic acid and ethyl ferulate are constituents of propolis and structurally related, which allowed us to gather important information regarding the structure–activity relationships underlying the biological activity of such compounds. In this work, we have investigated the direct scavenging effects of the antioxidants on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, galvinoxyl radicals and superoxide anions ($O_2^{\cdot-}$), as well as the anti-lipoperoxidative capacity on human erythrocytes hemolysis and rat liver microsomal membranes peroxidation induced in vitro by two different sources of free radicals: 2,2′-azobis(2-amidinopropane) (AAPH) and Fe^{2+}/ascorbate ($OH^{\cdot}$). The results established that the radical scavenging activity of the compounds increased with increasing numbers of hydroxyl groups or catechol moieties in the molecule, while in the biomembrane systems, the antioxidative activity of the test compounds depends not only on the hydroxyl groups or catechol rings but also on the polarity and hydrophobicity of the antioxidants. In addition, CAPE is the most effective antioxidant of the compounds we tested in our in vitro systems.
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Keywords: Caffeic acid phenethyl ester (CAPE); Caffeic acid; Ferulic acid; Ethyl ferulate; Antioxidant; Radical scavenging activity; Lipid peroxidation; Erythrocyte; Microsome

1. Introduction

Phenolic compounds are secondary plant metabolites and naturally present in almost all plant materials, including food products of plant origin. These compounds are thought to be an integral part of both human and animal diets (Psomiadou & Tsimidou, 2002). Phenolic acids are simple phenols because of their structure. Hydroxycinnamic acid is the major subgroup of phenolic compounds. Hydroxycinnamates are phenylpropanoid metabolites and occur widely in plants and plant products (Clifford, 1999). Hydroxycinnamates and their derivates are bioactive plant food ingredients. They exhibit in vitro antioxidant activity, which might have beneficial health impact in vivo (Kroon & Williamson, 1999).

Recently, it has been reported that caffeic acid phenethyl ester (CAPE), present in propolis extract, exhibits interesting pharmacological activities. It has been identified as one of the major biologically active principles in propolis with chemoprevention and antitumour properties (Borrelli et al., 2002; Lee et al., 1999; Nomura, Kaji, Ma, Miyamoto, & Dong, 2001). It has been further demonstrated that CAPE prevents lipid peroxidation induced by ischaemia – reperfusion injury in renal tissue, spinal cord and brain (Ilhan et al., 1999; Irmak et al., 2003). Moreover, previous studies have shown that ethanolic extract of propolis

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doi:10.1016/j.foodchem.2007.03.049

deprived of CAPE was less potent than the extract with CAPE with respect to antioxidant activity (Russo, Longo, & Vanella, 2002).

The importance of reactive oxygen species (ROS) and free radicals has attracted increasing attention over the past decade. ROS, which include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and non-free radical species such as H_2O_2 and singlet oxygen ($^1O_2$), are various forms of activated oxygen. These molecules exacerbate factors in cellular injury and the aging process (Albers & Beal, 2000; Keaney et al., 1995).

ROS are continuously produced during normal physiologic events and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. However, they are removed by antioxidant defence mechanisms. There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. Under pathological conditions, ROS are overproduced and result in oxidative stress. ROS are formed when endogenous antioxidant defence is inadequate. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification in the cellular membrane or intracellular molecules (Büyükokuroğlu, Gülçin, Oktay, & Küfrevioğlu, 2001).

There are many antioxidants that are introduced to minimize actions of ROS, for example, the phenolic compounds (Anselmi et al., 2004; Chen & Ho, 1997; Gülçin, 2006). According to recent research, CAPE was a potent antioxidant (Ilhan et al., 1999; Irmak et al., 2003; Sud'ina et al., 1993). Yet until now, few detailed studies have ever been done on its protective effect working with cellular models that make use of physiological membranes or physiological free radical promoters. In this work, we have investigated the direct scavenging effect of CAPE on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, galvinoxyl radicals and superoxide anions ($O_2^{\cdot-}$), which are general indexes of antioxidant activity. And using membrane models (human erythrocytes and rat liver microsomal membranes), we also have evaluated the antioxidant activity of CAPE in inhibiting lipid peroxidation induced in vitro by two different sources of free radicals: 2,2′-azobis(2-amidinopropane) (AAPH), which exogenously produces peroxyl radicals ($ROO^{\cdot}$) by thermal decomposition, and Fe^{2+}/ascorbate, which endogenously produces hydroxyl radicals ($OH^{\cdot}$) by Fenton reaction. In addition, we have compared the antioxidant efficiency of CAPE with caffeic acid, ferulic acid and ethyl ferulate, which have analogous chemical structures with CAPE (shown in Fig. 1).

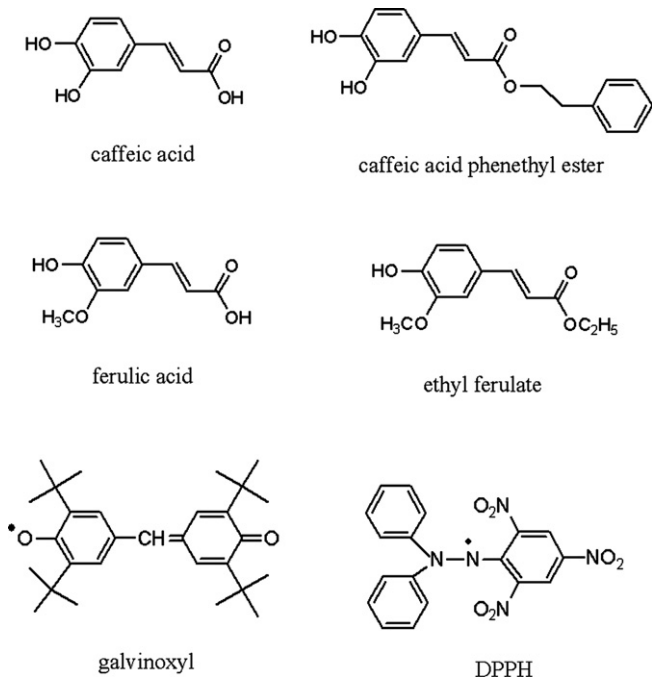

Fig. 1. Structural formulas of caffeic acid, caffeic acid phenethyl ester (CAPE), ferulic acid, ethyl ferulate, galvinoxyl radical and DPPH radical.

2. Materials and methods

2.1. Materials

Caffeic acid phenyl ester (phenethyl-3,4-dihydroxycinnamate) (CAPE), thiobarbituric acid (TBA) were purchased from Sigma Chemical (St. Louis, MO, USA). Ferulic acid (4-hydroxy-3-methoxycinnamic acid), ethyl ferulate (ethyl 4-hydroxy-3-methoxycinnamate) and butylated hydroxytoluene (BHT) were obtained from Aldrich (St. Louis, MO, USA). Caffeic acid (3,4-dihydroxycinnamic acid), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) and galvinoxyl were from Acros. All other chemicals were of the highest quality available.

2.2. Determination of scavenging activity on DPPH radicals

The compound to be tested was added into a 1 ml ethanol solution of DPPH (final concentration of 100 μM) to make final concentrations of 5, 10, 20, 40 μM. The mixture was shaken vigorously on a vortex mixer then incubated for 30 min in a water bath at 25 °C, after which the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. Ethanol was used as a blank solution. DPPH solution in ethanol without any tested compound served as the control. Special care was taken to minimize the loss of free radical activity of DPPH stock solution as recommended by Blois (1958). All analyses were carried out in triplicate.

2.3. Reaction with galvinoxyl radical

The reaction kinetics of galvinoxyl (5 μM) with the antioxidants to be tested (0.625–50 μM) in ethanol solution were monitored spectrophotometrically (Tsuchiya, Yamada, Niki, & Kamiya, 1985) with a HP 8453 UV–Visible System (Hewlett–Packard Development Company) at 25 °C. The change in maximum absorbance by galvinoxyl at 429 nm was followed continuously.

2.4. Determination of the inhibition of pyrogallol autoxidation

Scavenging of superoxide by antioxidants were estimated by the inhibition of pyrogallol autoxidation, which is superoxide dependent at pH < 9.5. Inhibition of pyrogallol autooxidation was performed as described previously (Marklund & Marklund, 1974). In brief, 1 ml reactions were monitored for increased absorbance at 420 nm from 0 to 5 min at 27 °C. Rates were determined from 3 to 5 min. Complete reactions contained 50 mM Tris–HCL, pH 8.0, 2 mM EDTA, 0.2 mM pyrogallol, and antioxidants. Every experiment was repeated three times and values represent mean ± SEM of the experiments.

2.5. Assay for hemolysis of human erythrocytes

Human erthrocytes were separated from heparinized blood that had been drawn from a healthy donor. The blood was centrifuged at 3000 rpm for 10 min and the plasma and buffy coat were removed by aspiration. Then the erythrocytes were washed three times with PBS (pH 7.4). During the last washing, the cells were centrifuged to obtain a consistently packed cell preparation. A 5% suspension of human erythrocytes in PBS was preincubated with antioxidants (1.25–10 μM) at 37 °C for 10 min before adding AAPH (50 mM) to initiate hemolysis. The antioxidants to be tested were dissolved in ethanol before the experiment, and the volume of ethanol solution added to the erythrocyte suspension was less than 0.5% (v/v) of the reaction mixture. Controls received an equivalent volume of ethanol alone before adding AAPH. Samples were then incubated and gently shaken at 37 °C. Aliquots of the reaction mixture was taken out at specific intervals and diluted with 10 volumes of 0.15 M NaCl. The absorbance (A_1) of the supernatant at 540 nm was measured. Similarly, the reaction mixture was treated with distilled water to yield complete hemolysis, and the absorbance (A_2) at 540 nm of the supernatant after centrifugation at 3000 rpm for 10 min was measured. The percentage hemolysis was calculated from the ratio of the measurements as follows: $(A_1/A_2) \times 100$. The lag phase of the oxidative hemolysis was calculated as described by Palozza, Moualla, and Krinsky (1992) by drawing a straight line through the linear portion of the propagation phase until it intercepts the abscissa. Every experiment was repeated three times and the values represent mean ± SEM of the three experiments.

2.6. Preparation of rat liver microsomal membranes

Female Wister rats weighing 250 ± 20 g were starved overnight and sacrificed by cervical dislocation. Then the liver was rapidly removed, cut into small pieces and washed extensively with 0.15 M NaCl. Liver microsomes were prepared by tissue homogenization with ice-cold 0.25 M sucrose–0.01 M Tris buffer, pH 7.4, and with 1 mM EDTA (STE buffer), in a motor-driven glass homogenizer. Microsomal fractions were isolated by removal of the nuclear faction at 8000*g* for 10 min and removal of mitochondrial fraction at 18,000*g* for 10 min. The microsomal fraction was sedimented in a Hitachi 55P-72 ultracentrifuge at 105,000*g* for 60 min, washed two times with 0.15 M KCl at 105,000*g* for 30 min. The membranes, suspended in 0.1 M potassium phosphate buffer, pH 7.5, were stored in a deep freezer maintained at −80 °C. Microsomal protein was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.7. Microsomal membrane peroxidation as measured by TBA-reactive substance formation

The formation of thiobarbituric acid-reactive substances (TBARS) was used to monitor lipid peroxidation product malondialdehyde (MDA) (Buege & Aust, 1978). Rat liver microsomes were incubated at 37 °C in 0.1 M potassium phosphate buffer, pH 7.5, and made up to a final protein concentration of 0.5 mg/ml. The peroxidation was initiated by Fe^{2+}/ascorbate and inhibited by antioxidants (0–100 μM), which were added as an ethanol solution to the microsomal suspension. The final concentration of ethanol in the suspension was less than 0.5% (v/v) that did not show appreciable interference to the reaction as evidenced by control experiments. The reaction mixture was gently shaken at 37 °C and aliquots of the reaction mixture were taken out at specific intervals to which a TCA–TBA–HCl stock solution (15% (w/v) trichloroacetic acid; 0.375% (w/v) TBA; 0.25 N HCl) was added, together with 0.02% (w/v) BHT. This amount of BHT completely prevents the formation of any nonspecific TBARS. The solution was heated in a boiling water bath for 15 min. After cooling, the precipitate was removed by centrifugation. TBARS in the supernatant was determined at 532 nm using the extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.8. Statistical analysis

Values were expressed as mean ± SEM from three independent determinations. Statistical analyses were carried out by analysis of variance (ANOVA) followed by appropriate post hoc tests including multiple comparison tests (LSD). All analyses were made using the SPSS statistical software package, and a probability value less than 0.05 was accepted as statistically significant.

3. Results

3.1. Scavenging effect on DPPH radicals

The DPPH test is carried out in a homogeneous phase and is rather unspecific: it gives a rough index of the scavenging activity because it measures the H radical/electron-transferring ability of an antioxidant versus the stable free radical DPPH (Blois, 1958). Scavenging ability of all tested

compounds increased with concentration in the range of 5–40 μM (data not shown). As shown in Table 1, their scavenging activity of DPPH radicals decreased in the following order: CAPE > caffeic acid > ferulic acid > ethyl ferulate with all values significantly different at $P < 0.01$.

3.2. Scavenging activity of the antioxidants as studied by reaction with galvinoxyl

Galvinoxyl is a stable phenoxy radical that exhibits characteristic UV absorption at 429 nm in ethanol solution. This allows easy measurement of the depletion of galvinoxyl radicals in the presence of antioxidants (Tsuchiya et al., 1985). The galvinoxyl is stable in the absence of antioxidants. When the antioxidants were added to the ethanol solution of galvinoxyl, the UV spectrum of galvinoxyl diminished gradually with time. A set of representative experimental results with 2.5 μM CAPE is shown in Fig. 2. When two ethanol solutions containing 5 μM galvinoxyl and 50 μM CAPE, respectively, were mixed at room temperature, galvinoxyl disappeared instantaneously. On the other hand, when galvinoxyl was mixed with caffeic acid, ferulic acid or ethyl ferulate, galvinoxyl disappeared gradually with time and the first-order plot gave a good straight line. The pseudo-first-order rate constants obtained from this plot are summarized in Table 2.

Table 1
Scavenging activity of antioxidants for DPPH radical; data are shown as IC_{50} (μM) and % inhibition at 10 μM of antioxidants

Compound	IC_{50} (μM)	Inhibition (%)
Ferulic acid	30.07 ± 0.54	25.05 ± 1.49[a]
Caffeic acid	6.46 ± 0.17	60.57 ± 0.44[b]
Ethyl ferulate	47.48 ± 0.17	15.05 ± 1.01[c]
CAPE	4.27 ± 0.10	75.93 ± 1.45[d]

Note: Values are means ± SEM of three determinations. Values with different superscripts are significantly different ($P < 0.01$).

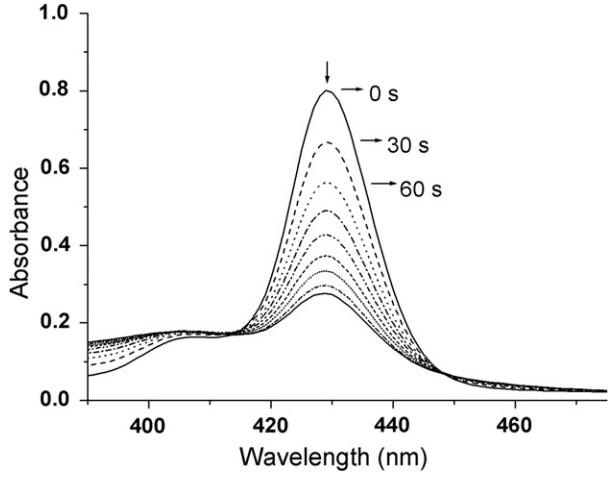

Fig. 2. UV spectra of galvinoxyl in the presence of CAPE in ethanol solution. The spectra were recorded every 30 s after mixing 5 μM galvinoxyl and 2.5 μM CAPE under air at 25 °C. The arrow at the spectrum peak shows the time-dependent decrease of the maximum absorbance.

Table 2
Pseudo-first-order rate constants (k_{obs}) for the interaction of galvinoxyl radicals with antioxidants in ethanol solution

Antioxidant	k_{obs} (10^{-3} s^{-1})
CAPE	Too fast
Caffeic acid	10.29
Ferulic acid	0.565
Ethyl ferulate	0.208

Note: The initial concentrations of galvinoxyl and the antioxidants were 5 and 50 μM, respectively.

3.3. Inhibiting effect on pyrogallol autoxidation

To quantify the extent of superoxide ($O_2^{\cdot-}$) scavenging by the antioxidants in our system, we determined the inhibition of pyrogallol autoxidation, which is superoxide-dependent at pH < 9.5 (Marklund & Marklund, 1974). As presented in Fig. 3A, a linear relationship was observed

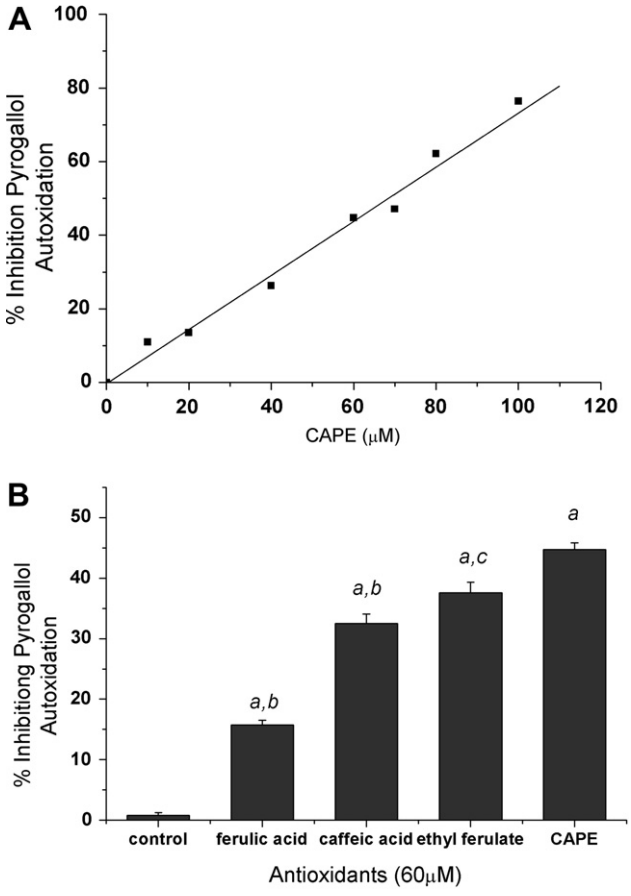

Fig. 3. The inhibition of pyrogallol autoxidation by antioxidants. The autooxidation of pyrogallol was monitored spectrophotometrically at 420 nm as described under Section 2. (A) Inhibition of pyrogallol autooxidation as a function of CAPE concentration. (B) Additions included antioxidants (60 μM). Results are the mean of three separate determinations ± SEM (error bars). (a) $P < 0.01$ vs control; (b) $P < 0.01$ vs CAPE; (c) $P < 0.05$ vs CAPE.

between the inhibition of pyrogallol autoxidation and the CAPE concentration. Fig. 3B shows the different inhibition of the antioxidants (60 μM) on the autoxidation of pyrogallol. All of them effectively scavenged $O_2^{\cdot-}$ at 60 μM (all $P < 0.01$ versus control, $n = 3$ by one-way ANOVA). CAPE is the most effective compound among them, which inhibited the autoxidation of pyrogallol by 44.8 ± 3.8% ($P < 0.01$ versus caffeic acid and ferulic acid, $P < 0.05$ versus ethyl ferulate, $n = 3$).

3.4. Inhibition of AAPH-induced erythrocyte hemolysis by the antioxidants

The choice of the erythrocyte as a model is due to the fact that it is a complete cellular system that presents structural characteristics (high content of polyunsaturated fatty acids). Moreover, the membrane contains cytoskeleton proteins that, intercalating with lipids and phospholipids, create the typical flexible structure of the erythrocyte (Anselmi et al., 2004). AAPH is a water-soluble azo compound that could decompose at physiological temperatures to generate alkyl radicals via an oxygen-dependent iron-independent mechanism at constant rates (Niki, 1990). Once inside the membrane, it generates a lipid peroxidation burst, which propagates to cytoskeleton proteins. They lose stability and the erythrocyte membrane undergoes the hemolytic shock.

When human erythrocytes were incubated in air at 37 °C as a 5% suspension in PBS, they were stable and little hemolysis was observed for 270 min (Fig. 4). When AAPH (final concentration 50 mM) was added to the erythrocyte suspension, it induced hemolysis in a time-dependent manner. Under these experimental conditions, the onset of oxidative hemolysis occurred within 1–2 h. The addition of antioxidants and lack of incubation with AAPH did not cause significant hemolysis in the erythrocyte suspensions after 2–5 h of incubation (data not shown). Figs. 4 and 5 show the antihemolysis effect of the antioxidants. It is clearly seen from Fig. 4 that addition of CAPE to the human erythrocyte suspension significantly increased the inhibition period of the native erythrocytes, and the inhibition period depended on the concentration of CAPE. The other antioxidants also exhibited apparent antihemolysis activities, as exemplified in Fig. 5. The effects of antioxidants (10 μM) on the lag phase of hemolysis are listed in Table 3. It is seen from Table 3 that the onset of AAPH-induced haemolysis was delayed greatly in the presence of the antioxidants. On basis of the inhibition period, the inhibitory activity against AAPH-induced erythrocyte hemolysis follows the sequence of CAPE > ethyl ferulate > caffeic acid > ferulic acid.

3.5. Inhibition of hydroxyl radical induced peroxidation of rat liver microsomes by the antioxidants

Microsomes, especially smooth surfaced endoplasmic reticulum, are particularly susceptible to oxidative stress because of the high content of polyunsaturated fatty acids, hence have been widely used as a model for oxidative stress and antioxidant studies (Cos et al., 2001). Iron (Fe^{2+} plus a reducing reagent) is an extensively used system for generating hydroxyl radicals to induce lipid peroxidation. In the present study, we used Fe^{2+} and ascorbate to induce peroxidation of rat liver microsomes to investigate the antioxidant effect of the four compounds in our system. The concentration of cellular membrane malondialdehyde (MDA) was used as an index of induced oxidative membrane damage, which can be measured by the TBA method (Bird & Draper, 1984).

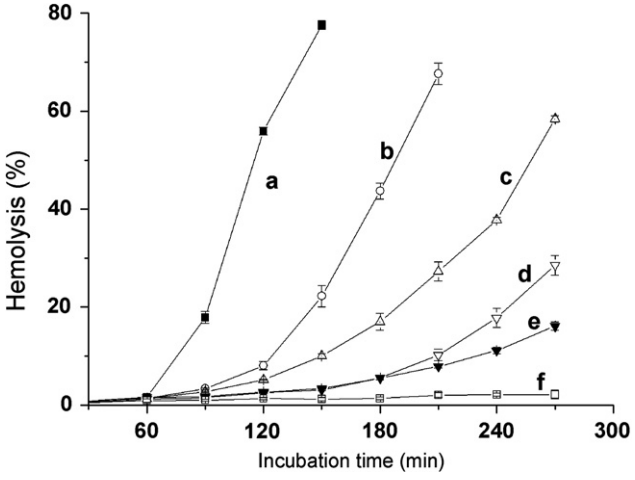

Fig. 4. Time course of hemolysis of human erythrocytes by AAPH. RBC suspensions (5%) were incubated without AAPH or with 50 mM AAPH alone or plus the presence of CAPE at 37 °C. Values represent means ± SEM of three experiments. (a) In the absence of CAPE; (b) in the presence of 1.25 μM CAPE; (c) 2.5 μM CAPE; (d) 5 μM CAPE; (e) 10 μM CAPE; (f) control without AAPH and CAPE.

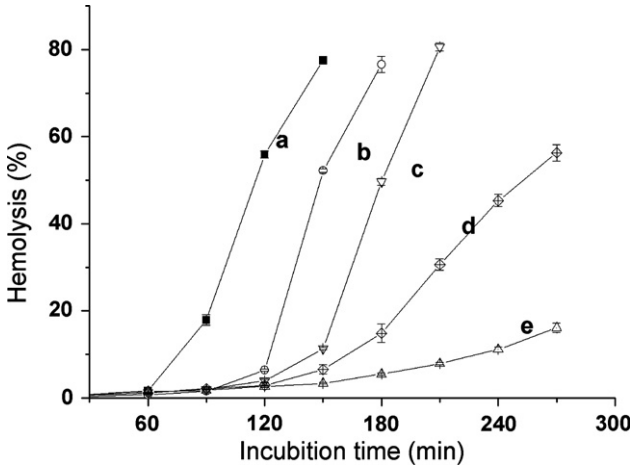

Fig. 5. Inhibition of AAPH-induced hemolysis of human erythrocytes by CAPE, caffeic acid, ferulic acid and ethyl ferulate in a 5% suspension of human erythrocytes in PBS (pH 7.4) under air at 37 °C. The reaction was inhibited with 50 mM AAPH and inhibited with 10 μM antioxidants. Values represent means ± SEM of three experiments. (a) AAPH alone; (b) ferulic acid; (c) caffeic acid; (d) ethyl ferulate; (e) CAPE.

Table 3
Effect of antioxidants (10 μM) on the lag phase of AAPH-induced hemolysis of human erythrocytes in vitro

Antioxidant	Inhibition period (min)
Control	76.73 ± 0.44
Ferulic acid (FA)	118.06 ± 0.32*
Caffeic acid (CA)	143.13 ± 0.77*
Ethyl ferulate (EF)	161.75 ± 1.07*
Caffeic acid phenethyl ester (CAPE)	253.17 ± 1.35*

Note: Values represent means ± SEM of three experiments. $^{*}P < 0.01$ vs control.

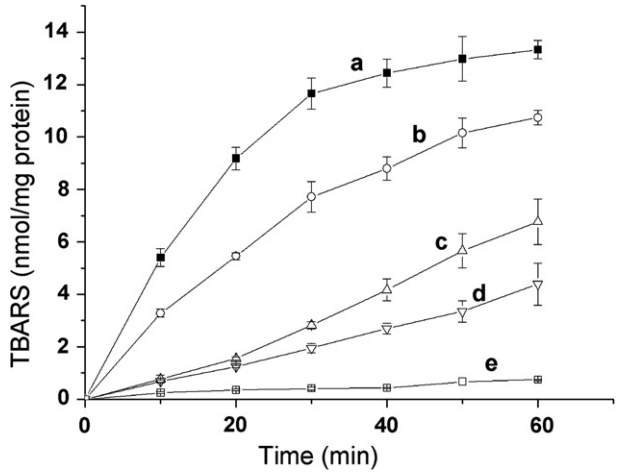

Fig. 6. Inhibition of TBARS formation during the Fe^{2+}/ascorbate-induced peroxidation of rat liver microsomes by CAPE at 37 °C. The rat liver microsomes were suspended in 0.1 M potassium phosphate buffer (pH 7.5) at a concentration of 0.5 mg/ml. [$FeSO_4$] = 5 μM; [ascorbate] = 50 μM; the initial concentration of CAPE were (a) 0, (b) 6.25, (c) 12.5, (d) 25 and (e) 50 μM, respectively. Values represent means ± SEM of three experiments.

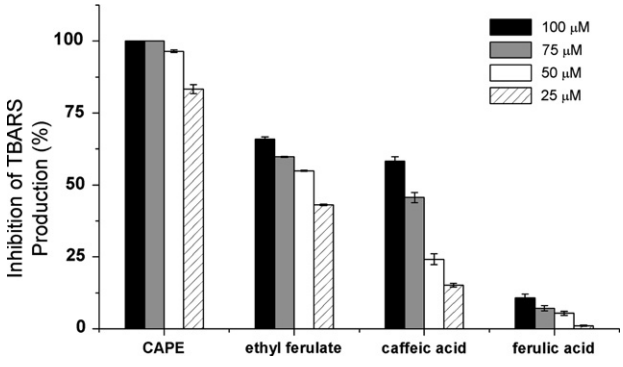

Fig. 7. Inhibition of TBARS formation during the Fe^{2+}/ascorbate-induced peroxidation of rat liver microsomes by antioxidants at 37 °C. The rat liver microsomes were incubated in 0.1 M potassium phosphate buffer (pH 7.5) at a concentration of 0.5 mg/ml for 30 min. [$FeSO_4$] = 5 μM; [ascorbate] = 50 μM. Antioxidants were added before the initiation with the initial concentration of 25, 50, 75 and 100 μM, respectively, as shown in the figure. Values represent mean ± SEM of three experiments.

Addition of Fe^{2+} and ascorbate to the microsomal membranes suspension increased TBARS time-dependently up to 60 min, as shown in Fig. 6. When the microsomes had been pre-treated with each compound tested, the production of TBARS was inhibited markedly. It is clearly seen in Fig. 6 that CAPE significantly suppressed rate of TBARS formation in a dose-dependent and time-dependent manner. Other antioxidants showed the same behavior and the results of different concentrations are shown in Fig. 7. The antioxidant activity also follows the sequence of CAPE > ethyl ferulate > caffeic acid > ferulic acid.

4. Discussion

Antioxidants are closely related to their biofunctionalities, such as the reduction of chronic diseases like DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacteria growth which is often associated with the termination of free radical propagation in biological systems (Zhu, Hackman, Ensunsa, Holt, & Keen, 2002). Thus, antioxidant capacity is widely used as a parameter for medicinal bioactive components.

It is proposed nowadays that the use of more than one marker of oxidation is required to evaluate the efficacy of an antioxidant (Frankel, 2001). The antioxidant activity of a molecule is currently investigated in "in vitro" systems which make use of acellular and cellular models: the first furnish preliminary evidence of the capacity to quench free radicals species ($OH^{\bullet}$, $ROO^{\bullet}$, $RO^{\bullet}$, $R^{\bullet}$, etc.), the second (phosphatidylcholine liposomes, liver microsomes, erythrocytes, cell cultures) give deeper insight into the protective action of the molecule (more close to an in vivo situation) since they provide biological information that takes into account also its interaction with specific receptor/s on the membrane surface (Anselmi et al., 2004). Hence, we evaluated the antioxidant effects of caffeic acid phenethyl ester (CAPE), as well as caffeic acid, ferulic acid and ethyl ferulate in inhibiting lipid peroxidation in both human erythrocytes and rat liver microsomal membranes together with their radical scavenging activities.

The direct scavenging effects of the four compounds toward DPPH radicals, galvinoxyl radicals were in the following order: CAPE > caffeic acid > ferulic acid > ethyl ferulate. The structural feature responsible for the better antioxidative and free radical scavenging activity of CAPE and caffeic acid is the *ortho*-dihydroxyl functionality in the catechol ring. The presence of the electron-donating hydroxyl group at the *ortho*-position would make the oxidation intermediate and increase the rate of H-atom transfer to peroxyl radicals (Lucarini, Mugnaini, & Pedulli, 2002), resulting in the formation of a phenoxy radical. The *ortho*-hydroxyl phenoxyl radical is more stable due to (1) the unpaired electron can delocalize across the entire molecule (Graf, 1992); (2) the intramolecular hydrogen bonding interaction as reported recently from both experiments (Chen & Ho, 1997) and theoretical calculations

(Wright, Johnson, & DiLabio, 2001). The theoretical calculation showed that the intramolecular hydrogen bond in *ortho*-OH phenoxyl radical is ca. 16.7 kJ/mol stronger than that in the parent molecule catechol and the bond dissociation energy (BDE) of catechol is 38 kJ/mol lower than that of phenol and 36.8 kJ/mol lower than that of resorcinol. In addition, *ortho*-OH phenoxyl radical and/or *ortho*-semiquinone radical anion shall be easier to further oxidize to form the final product *ortho*-quinone. Methoxylation of one hydroxyl group of caffeic acid to form ferulic acid decreases the efficiency of the scavenging reaction with these radicals. With regards to structure–activity relationship, ferulic acid and ethyl ferulate cannot form quinone oxidation products, so they are inefficient in quenching radicals in these systems.

In our work, inhibition of the autoxidation of pyrogallol was used as a measure of the ability of the antioxidants to scavenge superoxide anion ($O_2^{\cdot-}$). The data showed that CAPE could inhibit the autoxidation of pyrogallol efficiently. Sud'ina et al. (1993) demonstrated CAPE completely blocks production of ROS in human neutrophils and the xanthine/xanthine oxidase system. Their results are consistent with ours. So we can reach the conclusion that CAPE markedly scavenged $O_2^{\cdot-}$ produced in both enzymatic reaction and nonenzymatic reaction.

In the pyrogallol autoxidation assay, it is noticeable that ethyl ferulate has better activity compared to caffeic acid and ferulic acid. This assay is homogeneous as the assay for DPPH and galvinoxyl, while the ordering is reversed. The possible mechanism might be explained as follows: for CAPE and ethyl ferulate, the conversion of the acid group to the ester group decreases the polarity of the molecule. Their hydrophobic ester side chains might gather together in water (the aqueous phase) and form tiny droplets of the organic compound (the organic phase). The better solubility of pyrogallol in organic phase than in water (Dean, 1999) might facilitate the transport of this compound to the organic phase formed by the esters, which then could scavenge the superoxide at the true site where they were produced. On the contrary, caffeic acid and ferulic acid becomes inactive in this assay since they form a homogeneous phase when solvated in water, and the side chain ionization in water of pH 8.0 hinders access to pyrogallol and scavenging the superoxide.

Biological membranes are important targets for reactive species. They induce lipid peroxidation (LPO) of membrane phospholipids unsaturated fatty acids (PUFAs), accompanying pathological events such as chronic inflammatory processes, diabetes, carcinogenesis, aging, stroke, ischemia, and reperfusion injury (Yin, Smith, Eppley, Page, & Sphon, 1998). Hence there is an increasing interest in the investigation on the LPO levels induced by oxidants and the membrane protection by antioxidants. In our evaluation of lipid peroxidation, two different initiating assays, that is, AAPH which produces alkyl radicals followed by fast reaction with oxygen to give initiating alkyl peroxyl radicals and Fe^{2+}/ascorbate which produces hydrogen peroxide and the latter generates hydroxyl radicals by Fenton reaction, were used to initiate the erythrocyte hemolysis and the microsomal peroxidation.

In the biomembrane systems, factors such as compositional and interfacial phenomena relating to solubility and hydrophobicity of components, and partitioning properties of antioxidants between lipid and aqueous phases, can be included (Rice-Evans, Miller, & Paganga, 1996). Also it is recognized that peroxyl radicals produced by AAPH in the aqueous phase attack phospholipids at the membrane surface (Niki, 1990), suggesting that antioxidants need to be located near the surface of the membrane in order to act, so the lipophilicity of the antioxidants effects their activity in inhibiting the lipid peroxidation to a great extent. It is interesting to observe that the trend of anti-lipoperoxidant activity in liver microsomes perfectly matches that observed in human erythrocytes: CAPE > ethyl ferulate > caffeic acid > ferulic acid. This indicates that the CAPE acts with a similar mechanism in both the membrane models and that the side chain is primarily involved in the interaction with the phospholipid bilayer of the membrane. Compared with ferulic acid, caffeic acid is a more lipophilic phenolic acid and more efficient in these biomembrane systems. It has been demonstrated that caffeic acid prevents the chain initiation of lipid peroxidation by scavenging peroxy radical ($LOO^{\cdot}$), and is known to be more effective radical scavengers than Trolox C, α-tocopherol, and ascorbic acid (Vinson, Kabbagh, Serry, & Jang, 1995). Conversion of the acid group of caffeic acid to the phenethyl group enhanced the radical scavenging and antioxidant activity: this may be due to the esterification of caffeic acid increasing the lipophilicity of the compound and enhancing the antioxidant properties. Son and Lewis (2002) reported the $\log P$ value of CAPE (3.493 ± 0.127) is much higher than that of caffeic acid (1.303 ± 0.240), so CAPE has higher potential activity. While ferulic acid, more active in homogeneous phase than ethyl ferulate, becomes inactive in heterogeneous phase since ferulic acid itself does not associate with the lipid portion of microsomes and exerts its antioxidant properties from the aqueous phase (Rice-Evans et al., 1996). The ethyl esterification decreases the polarity and might facilitate the transport of this compound across the cell membrane, where it can scavenge free radicals (Burdette et al., 2002); hence the side chain ionization in physiological medium hampers binding to the biological membrane. The results show that the antioxidative activity in the membrane system depends not only on the number of hydroxyl groups in the catechol rings but also on the solubility, hydrophobicity (or partition coeffcient, $\log P$), and stability of the compounds. Our data indicate that esterification of phenolic acids increases lipophilicity of their esters and may enable a better incorporation in the membranes and the exertion of their antioxidant effect in the true site of lipoperoxidation.

In conclusion, in the current study, we successfully used complex, structurally ordered systems to determine the antioxidant behavior of CAPE, caffeic acid, ethyl ferulate,

ferulic acid and their related structure–activity relationships with three different oxidants, including DPPH, galvinoxyl (a phenoxyl radical), superoxide anion ($O_2^{\cdot-}$), AAPH (an ROO$^{\cdot}$ generator), and Fe^{2+}/ascorbate (mainly an OH$^{\cdot}$ generator). The results clearly indicate CAPE exhibits stronger antioxidant activity both in scavenging free radicals and in inhibition of lipid peroxidation, as compared to that of the structure-related compounds caffeic acid, ferulic acid and ethyl ferulate. The free radical scavenging activity of the compounds increased significantly with the number of hydrogen groups or catechol moieties in the molecule. The antioxidative activity of the compounds in the AAPH-induced hemolysis and Fe^{2+}/ascorbate-induced lipid peroxidation of microsome membranes, however, depends not only on the hydrogen groups or catechol rings but also on the polarity, hydrophobicity (or partitioning properties between lipid and aqueous phases) and stability of the antioxidants. These in vitro results also suggest the possibility that CAPE could be effectively employed as an ingredient in health or functional food, to alleviate oxidative stress.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Nos. 20372028, 20472026) and Chang Jiang Program of the Ministry of Education China. We thank the State Key Laboratory of Applied Organic Chemistry, Lanzhou University, for helpful suggestions and comments on the manuscript.

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