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RESEARCH ARTICLE

Antioxidant activity of bisbenzylisoquinoline alkaloids from Stephania rotunda: cepharanthine and fangchinoline

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Abstract

In the present study, we determined the antioxidant activity of cepharanthine and fangchinoline from *Stephania rotunda* by performing different *in vitro* antioxidant assays, including 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging, 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) radical scavenging, superoxide anion ($O_2^{\bullet-}$) radical scavenging, hydrogen peroxide scavenging, total antioxidant activity, reducing power, and ferrous ion (Fe^{2+}) chelating activities. Cepharanthine and fangchinoline showed 94.6 and 93.3% inhibition on lipid peroxidation of linoleic acid emulsion at 30 µg/mL concentration, respectively. On the other hand, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol, and trolox indicated inhibitions of 83.3, 92.2, 72.4, and 81.3% on peroxidation of linoleic acid emulsion at the same concentration (30 µg/mL), respectively. According to the results, cepharanthine and fangchinoline have effective antioxidant and radical scavenging activity.

Keywords: *Antioxidant activity; cepharanthine; fangchinoline; Stephania rotunda; alkaloid; radical scavenging*

Introduction

Inherent oxygen consumption in cell growth leads to the generation of a series of reactive oxygen species (ROS). They are continuously produced by the body's normal use of oxygen, including respiration and some cell mediated immune functions. ROS include free radicals such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$)[1,2]. ROS are constantly formed during normal physiologic events, and can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. ROS may be required for normal cell function at physiological concentrations. They are also capable of damaging crucial biomolecules such as nucleic acids, lipids, proteins, polyunsaturated fatty acids, and carbohydrates, and may cause DNA damage, which can lead to mutations. If ROS are not effectively scavenged by cellular constituents, they lead to disease conditions[3,4]. ROS are implicated in more than 100[5].

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[6,7]. 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[8]. Antioxidants are often added to foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation step that leads to termination of the reaction and delay the oxidation process[9–11]. At the present time, the most commonly used antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate, and *tert*-butyl hydroquinone. However, BHA and BHT are restricted by legislative rules because of doubts over their toxic and carcinogenic effects[11]. Therefore, there is growing interest in natural and safer antioxidants in food applications, and a growing trend in consumer preferences for natural antioxidants, all of which encourage the exploration of natural sources of antioxidants[12–14].

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Stephania rotunda Lour. (Menispermaceae) is an indigenous Cambodian wild plant that is commonly used in traditional food and medicine such as the treatment of fever and as a tonic[15]. *Stephania rotunda* has been chemically investigated and found to contain alkaloids such as rotundine, tuduranine, cepharamine, tetrahydropalmatine, and cycleanine[16–20].

The aim of this study was to investigate the inhibition of lipid peroxidation in the linoleic acid system, the ferric ion (Fe^{3+}) reducing antioxidant power (FRAP), the cupric ion (Cu^{2+}) reducing antioxidant power (CUPRAC method), 1,1-diphenyl-2-picryl-hydrazyl (DPPH)·radical scavenging, 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) radical scavenging, superoxide anion radical scavenging in the riboflavin/methionine/illuminate system, hydrogen peroxide scavenging, and the ferros ions (Fe^{2+}) chelating activities of cepharanthine and fangchinoline from *Stephania rotunda*. In addition, an important goal of this study was to clarify the antioxidant and radical scavenging and metal chelating mechanisms of cepharanthine and fangchinoline.

Materials and methods

Chemicals

N,*N*-Dimethyl-*p*-phenylenediamine (DMPD), neocuproine (2,9-dimethyl-1,10-phenanthroline), 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), riboflavin, methionine, 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 analytical grade and obtained from either Sigma-Aldrich or Merck.

Collection of plant material

The ethnobotanical survey was carried out in nine regions of Cambodia during the period of October to September 2004. The freshly picked parts of the plants were air-dried at room temperature for 2 weeks, without direct sunlight. The voucher specimens were identified by Professor S. K. Cheng and deposited in the herbarium of the Faculty of Pharmacy, University of Health Sciences, Phnom Penh, Cambodia.

Plant extraction and isolation of alkaloids

Dried and powdered plant material (100 g) was extracted with boiling water (2 L) for 10 min and filtered. The aqueous extract was freeze-dried to afford 17.50 g of a brown extract powder. The aqueous extract (15 g) was dissolved in 0.5% aqueous HCl solution (100 mL) to form a suspension (pH 2) and partitioned with dichloromethane (4 × 100 mL). The combined dichloromethane layers were filtered and evaporated *in vacuo* to afford 1.75 g of dichloromethane extract. To the aqueous layer was added 6.5 mL of NaOH solution (1 N) to adjust the pH to 10 followed by partitioning with dichloromethane (4 × 100 mL). The combined dichloromethane layers were filtered and evaporated *in vacuo* to afford 1.05 g of dichloromethane extract.

Then, the dichloromethane extract (1 g) was first subjected to chromatography over silica gel 60 (Kieselgel 60, 0.040–0. 063 mm; Merck) and eluted by EtOAc–MeOH–NH_4OH (80:20:0.5) to furnish cepharanthine and five fractions f1, f2, f3, f4, f5. Fraction f3 was subsequently subjected to silica gel chromatography and eluted by CH_2Cl_2–MeOH–NH_4OH (90:10:0.5) to yield fangchinoline. The chemical structures of cepharanthine and fangchinoline from *Stephania rotunda* are given in Figure 1. Structure elucidation was carried out using spectroscopic methods: liquid chromatography-ultraviolet light (LC-UV), LC-mass spectroscopy (MS) (mass spectrophotometer with negative ion detection), ^{1}H-nuclear magnetic resonance (NMR) (Bruker DRX 500 spectrophotometer operating at 500 MHz), and ^{13}C-NMR (Bruker DRX 500 spectrophotometer operating at 125 MHz). NMR measurements were performed in CD_3OD or in DMSO-d6.

Total antioxidant activity determination by ferric thiocyanate method

The ferric thiocyanate method was used to evaluate the effect of cepharanthine and fangchinoline and reference antioxidants on the prevention of peroxidation of linoleic acid as described previously[14,21]. A stock solution contained 10 mg of cepharanthine and fangchinoline dissolved in 10 mL ethanol. Cepharanthine and fangchinoline (30 μg/mL) were prepared by diluting the stock solution in 2.5 mL of sodium phosphate buffer (0.04 M, pH 7.0) and these were added to 2.5 mL of linoleic acid emulsion

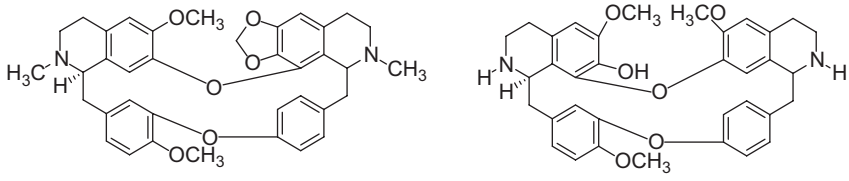

Figure 1. Chemical structures of cepharanthine and fangchinoline purified from *Stephania rotunda* Lour. (Menispermaceae).

in sodium phosphate buffer (0.04 M, pH 7.0). The linoleic acid emulsion was prepared by homogenizing 15.5 μL of linoleic acid, 17.5 mg of Tween-20 as emulsifier, and 5 mL phosphate buffer (pH 7.0). The control was composed of 2.5 mL of linoleic acid emulsion and 2.5 mL 0.04 M sodium phosphate buffer (pH 7.0). The reaction mixtures were incubated at 37°C in polyethylene flasks. The peroxide levels were determined by reading the absorbance at 500 nm in a spectrophotometer (UV-1208 UV-VIS; Shimadzu, Japan) after reaction with $FeCl_2$ and thiocyanate with intervals during incubation. The peroxides formed during linoleic acid peroxidation oxidize Fe^{2+} to Fe^{3+}, which forms a complex with thiocyanate that has a maximum absorbance at 500 nm. The assay step was repeated every 5 h until reaching a maximum. The percentage of inhibition was calculated at this point (30 h). Solutions without cepharanthine and fangchinoline were used as blank samples. The linoleic acid mixture without the addition of sample was used as a control. The percentage inhibition of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

$$\text{Inhibition of lipid peroxidation}\,(\%)=\left(1-\frac{\lambda_{500-S}}{\lambda_{500-C}}\right)\times 100$$

where $\lambda_{500\text{-}C}$ is the absorbance of the control reaction, which contains only linoleic acid emulsion and sodium phosphate buffer, and $\lambda_{500\text{-}S}$ is the absorbance of sample in the presence of cepharanthine, fangchinoline, or other test compound[21,22].

Ferric cyanide (Fe^{3+}) reducing antioxidant power assay (FRAP)

Reducing power was measured by the direct reduction of $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$ and was determined by absorbance measurement of the formation of Perl's Prussian blue complex following the addition of excess Fe^{3+}, as described by Oyaizu[23]. The FRAP method is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess relative to the antioxidant[24]. Different concentrations of cepharanthine and fangchinoline (10–30 μg/mL) in 0.75 mL of distilled water were mixed with 1.25 mL of 0.2 M, pH 6.6 sodium phosphate buffer, and 1.25 mL of potassium ferricyanide ($K_3Fe(CN)_6$) (1%); the mixture was incubated at 50°C for 20 min. After 20 min of incubation, the reaction mixture was acidified with 1.25 mL of trichloroacetic acid (10%). Finally, 0.5 mL of $FeCl_3$ (0.1%) was added to this solution and the absorbance was measured at 700 nm in a spectrophotometer. An increase in the absorbance of the reaction mixture indicates greater reduction capability[25,26].

Cupric ion (Cu^{2+}) reducing power: CUPRAC assay

To investigate the reducing ability of cepharanthine and fangchinoline, the cupric ion (Cu^{2+}) reducing power method was also used[27] with slight modification[28]. Hence, 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neocuproine solution (7.5×10^{-3} M), and 0.25 mL NH_4Ac buffer solution (1 M) were added to a test tube, followed by mixing with different concentrations of cepharanthine and fangchinoline (10–30 μg/mL). The total volume was adjusted to 2 mL with distilled water and mixed well. After 30 min, absorbance was measured at 450 nm against a reagent blank. An increase in the absorbance of the reaction mixture indicates increased reduction capability.

Chelating activity on ferrous ions (Fe^{2+})

Ferrous ion (Fe^{2+}) chelating activity was measured by inhibiting the formation of the Fe^{2+}–ferrozine complex after treatment of the test material with Fe^{2+}, following the method of Dinis *et al.*[29]. The Fe^{2+}-chelating ability of cepharanthine and fangchinoline was monitored by measuring the absorbance of the ferrous iron–ferrozine complex at 562 nm. Briefly, different concentrations of cepharanthine and fangchinoline (15–45 μg/mL) in 0.4 mL methanol were added to a solution of 0.6 mM $FeCl_2$ (0.1 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.1 mL) dissolved in methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated using the formula given bellow:

$$\text{Bounded ferrous ions}\,(\%)=\left(1-\frac{\lambda_{562-S}}{\lambda_{562-C}}\right)\times 100$$

where $\lambda_{562\text{-}C}$ is the absorbance of the control and $\lambda_{562\text{-}S}$ is the absorbance in the presence of cepharanthine and fangchinoline or standard. The control contained only $FeCl_2$ and ferrozine[21,30,31].

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was carried out according to the procedure of Ruch and co-workers[31,32]. A solution of 40 mM H_2O_2 was prepared in 0.1 M phosphate buffer (pH 7.4). Then, 30 μg/mL cepharanthine or fangchinoline in 3.4 mL phosphate buffer was added to 0.6 mL of H_2O_2 solution (40 mM) and the absorbance of the reaction mixture was recorded at 230 nm. The blank solution contained sodium phosphate buffer without H_2O_2. The concentration of hydrogen peroxide (mM) in the assay medium was determined using a standard curve ($r^2=0.9956$):

$$\text{Absorbance}\ (\lambda_{230})=0.505\times[H_2O_2]$$

The percentage of H_2O_2 scavenging by cepharanthine and fangchinoline and standard compounds was calculated using the following equation:

$$\text{Scavenged}\ \ H_2O_2\ (\%)\ =\ \left(1-\frac{\lambda_{230-S}}{\lambda_{230-C}}\right)\times 100$$

where $\lambda_{230\text{-}C}$ is the absorbance of the control and $\lambda_{230\text{-}S}$ is the absorbance in the presence of cepharanthine and fangchinoline or other scavenger[22].

DPPH free radical scavenging activity

The method of Blois[33] as previously described by Gülçin[1] was used with slight modifications in order to assess the DPPH free radical scavenging capacity of cepharanthine and fangchinoline. Briefly, 0.1 mM DPPH solution was prepared in ethanol, and 0.5 mL of this solution was added to 1.5 mL of cepharanthine and fangchinoline solution in ethanol at different concentrations (10–30 μg/mL). These solutions were vortexed thoroughly and incubated in the dark for 30 min. Then, the absorbance was measured at 517 nm against blank samples lacking scavenger. A standard curve was prepared using different concentrations of DPPH•. The DPPH• scavenging capacity was expressed as mM in the reaction medium and calculated from the calibration curve determined by linear regression ($r^2 = 0.9845$):

$$\text{Absorbance}\ (\lambda_{517}) = 0.5869 \times [\text{DPPH}^\bullet] + 0.0134$$

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

$$\text{Scavenged}\ \ \text{DPPH}^\bullet(\%) = \left(1 - \frac{\lambda_{517-S}}{\lambda_{517-C}}\right) \times 100$$

where $\lambda_{517\text{-}C}$ is the absorbance at 517 nm of the control reaction (containing all reagents except the test compound) and $\lambda_{517\text{-}S}$ is the absorbance at 517 nm with the test compounds cepharanthine and fangchinoline. The concentration of cepharanthine and fangchinoline providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against cepharanthine and fangchinoline concentration[33,34]. DPPH• decreases significantly upon exposure to radical scavengers[28].

ABTS radical cation decolorization assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ scavenging activity was determined according to the method of Re *et al.*[35]. In this method, an antioxidant is added to a preformed ABTS radical solution and after a fixed time period, the remaining $ABTS^{\bullet+}$ is quantified spectrophotometrically at 734 nm[22]. The $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 6 h. The $ABTS^{\bullet+}$ solution was diluted in 0.1 M sodium phosphate buffer (pH 7.4) to give an absorbance of 0.750 ± 0.025 at 734 nm. Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of cepharanthine or fangchinoline solution in ethanol at different concentrations (10–30 μg/mL). The absorbance was recorded 30 min after mixing, and the percentage of radical scavenging was calculated for each concentration relative to a blank without scavenger. The extent of decolorization was calculated, measuring the reduction percentage of absorbance. To prepare a standard curve, different concentrations of $ABTS^{\bullet+}$ (0.033–0.33 mM) were used. The $ABTS^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression ($r^2 = 0.9899$):

$$\text{Absorbance}\,(\lambda_{734}) = 2.5905 \times [\text{ABTS}^{\bullet+}]$$

The scavenging capability of test compounds was calculated using the following equation:

$$\text{Scavenged ABTS}^{\bullet+}(\%) = \left(1 - \frac{\lambda_{734-S}}{\lambda_{734-C}}\right) \times 100$$

where $\lambda_{734\text{-}C}$ is the absorbance of a control (blank) lacking any radical scavenger and $\lambda_{734\text{-}S}$ is the absorbance of the remaining $ABTS^{\bullet+}$ in the presence of scavenger[21].

Superoxide anion radical scavenging activity

Superoxide radicals were generated by the method of Zhishen and co-workers[36] with slight modification. Superoxide radicals were generated in riboflavin, methionine, and illuminate and assayed by the reduction of NBT to form blue formazan. 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 cepharanthine and fangchinoline in the reaction mixture was 30 μg/mL. The total volume of the reaction mixture was 3 mL and the concentrations of riboflavin, methionine, and NBT were 1.33×10^{-5}, 4.46×10^{-5}, and 8.15×10^{-8} M, respectively. The reaction mixture was illuminated at 25°C for 40 min. The photochemically reduced riboflavin generated $O_2^{\bullet-}$ which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as blank. The absorbance was measured at 560 nm. Cepharanthine and fangchinoline were added to the reaction mixture in which $O_2^{\bullet-}$ was scavenged, thereby inhibiting NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage of superoxide anion scavenged was calculated by using the following formula:

$$\text{Scavenged}\,O_2^{\bullet-}(\%) = \left(1 - \frac{\lambda_{560-S}}{\lambda_{560-C}}\right) \times 100$$

where $\lambda_{560\text{-}C}$ is the absorbance of the control and $\lambda_{560\text{-}S}$ is the absorbance in the presence of cepharanthine and fangchinoline or standard[4,37].

Measurement of $DMPD^{\bullet+}$ scavenging ability

The DMPD radical scavenging ability of cepharanthine and fangchinoline was determined according to Fogliano and co-workers[38]. DMPD solution (100 mM) was prepared by dissolving 209 mg of DMPD in 10 mL of deionized water; 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.25) and the colored radical cation ($DMPD^{\bullet+}$) was obtained by adding 0.2 mL of a solution of 0.05 M ferric chloride ($FeCl_3$). The absorbance of this solution, which was freshly prepared daily, was constant for up to 12 h at room temperature. Different concentrations of standard antioxidants or cepharanthine and fangchinoline (10–30 μg/mL) were added to

the test tubes and the total volume was adjusted to 0.5 mL with distilled water. Ten minutes later, the absorbance was measured at 505 nm. One milliliter of $DMPD^{\bullet+}$ solution was directly added to the reaction mixture and its absorbance at 505 nm was measured. The buffer solution was used as a blank sample. The $DMPD^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression ($r^2 = 0.9993$):

$$\text{Absorbance}\,(\lambda_{505}) = 0.0088 \times [\text{DMPD}^{\bullet+}]$$

The scavenging capability of $DMPD^{\bullet+}$ radical was calculated using the following equation:

$$\text{Scavenged}\ \ \text{DMPD}^{\bullet+}(\%) = \left(1 - \frac{\lambda_{505-S}}{\lambda_{505-C}}\right) \times 100$$

where $\lambda_{505\text{-}C}$ is the absorbance of the initial concentration of $DMPD^{\bullet+}$ and $\lambda_{505\text{-}S}$ is the absorbance of the remaining concentration of $DMPD^{\bullet+}$ in the presence of cepharanthine and fangchinoline[28,39].

Statistical analysis

The experiments were performed in triplicate. The data were recorded as mean ± standard deviation and analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). One-way analysis of variance (ANOVA) was performed. Significant differences between means were determined by Duncan's multiple range test, and $p < 0.05$ was regarded as significant and $p < 0.01$ as very significant.

Results

The ferric thiocyanate method measures the amount of peroxides produced during the initial stages of oxidation, i.e. the primary products of oxidation. Cepharanthine and fangchinoline exhibited effective antioxidant activity in the linoleic acid emulsion system. The effect of cepharanthine and fangchinoline on lipid peroxidation of linoleic acid emulsion at the same concentration (30 μg/mL) is shown in Figure 2, and was found to be 94.6 and 93.3%. Their activities were greater than those of the same concentration of BHA (83.3%), BHT (92.2%), α-tocopherol (72.4%), and trolox (81.3%). The autoxidation of linoleic acid emulsion without cepharanthine and fangchinoline or standard compound was accompanied by a rapid increase of peroxides. Consequently, these results clearly indicated that cepharanthine and fangchinoline had an effective and powerful antioxidant activity.

As can be seen from Figure 3A, cepharanthine and fangchinoline had effective reducing activity when compared to the standards (BHA, BHT, α-tocopherol, and trolox). For measurement of the reductive ability of cepharanthine

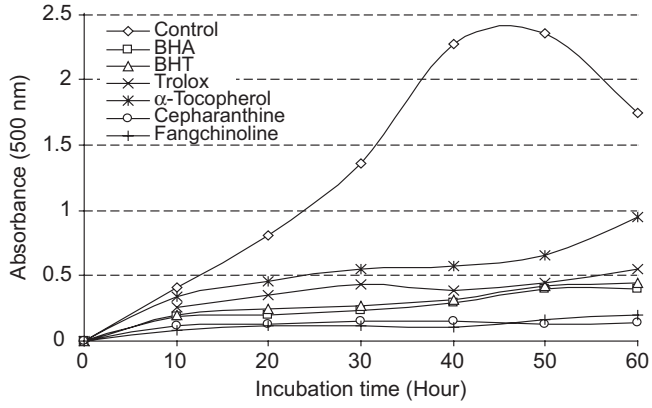

Figure 2. Total antioxidant activities of cepharanthine, fangchinoline, and standard antioxidant compounds such as BHA, BHT, α-tocopherol, and trolox at the same concentration (30 μg/mL). BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

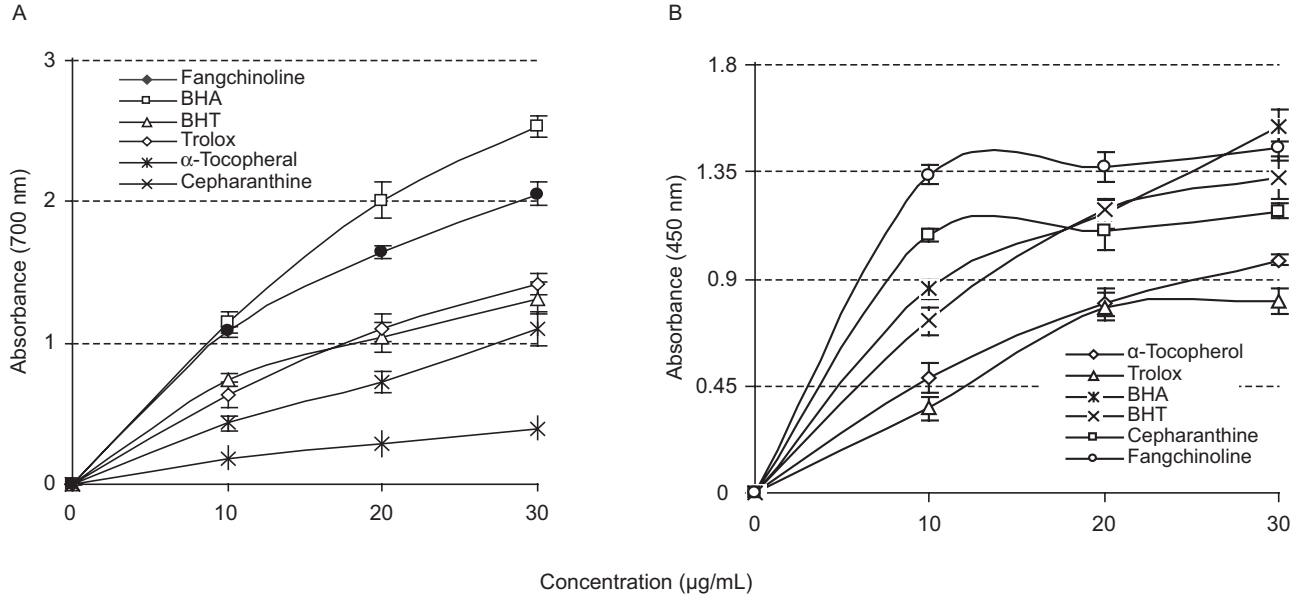

Figure 3. Reducing activity of different concentrations (10–30 μg/mL) of cepharanthine, fangchinoline, and reference antioxidants: BHA, BHT, α-tocopherol, and trolox. (A) Fe^{3+}–Fe^{2+} reductive potential of cepharanthine ($r^2 = 0.9773$) and fangchinoline ($r^2 = 0.9507$) and reference antioxidants. (B) Cupric ion (Cu^{2+}) reducing ability (CUPRAC method) of cepharanthine, fangchinoline, BHA, BHT, α-tocopherol, and trolox.

and fangchinoline, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of cepharanthine and fangchinoline using the method of Oyaizu[23]. At different concentrations (10–30 μg/mL), cepharanthine ($r^2 = 0.9773$) and fangchinoline ($r^2 = 0.9507$) demonstrated powerful Fe^{3+} reducing ability, and these differences were statistically very significant ($p < 0.01$). The reducing power of cepharanthine, fangchinoline, BHA, BHT, α-tocopherol, and trolox increased steadily with increasing concentration of sample. The reducing power of cepharanthine, fangchinoline, and standard compounds is as follows: BHA > fangchinoline > trolox ≈ BHT > α-tocopherol > cepharanthine. The results demonstrated that fangchinoline had marked ferric ion (Fe^{3+}) reducing ability. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging.

The cupric ion (Cu^{2+}) reducing ability (CUPRAC method) of cepharanthine and fangchinoline is shown in Figure 3B. A correlation was found between the cupric ion (Cu^{2+}) reducing ability and cepharanthine or fangchinoline concentration ($r^2 = 0.9913$). The cupric ion (Cu^{2+}) reducing capability of cepharanthine and fangchinoline by the CUPRAC method was found to be concentration dependent (10–30 μg/mL). The cupric ion (Cu^{2+}) reducing power of cepharanthine, fangchinoline, and standard compounds is as follows at the same concentration (30 μg/mL): BHA > fangchinoline > BHT > cepharanthine > α-tocopherol > trolox.

Cepharanthine and fangchinoline had effective ferrous ion (Fe^{2+}) chelating capacity. The difference between the various concentrations of cepharanthine or fangchinoline (15–45 μg/mL) and control values was statistically significant ($p < 0.01$). In addition, cepharanthine and fangchinoline exhibited 97.1 and 93.5% chelation of ferrous ions at 45 μg/mL concentration. As can be seen in Figure 4, the ferrous ion chelating effect of cepharanthine and fangchinoline was compared to that of BHA, BHT, α-tocopherol, trolox, and ethylenediaminetetraacetic acid (EDTA). The ferrous ion chelating capacity of the same concentration of EDTA, BHA, BHT, α-tocopherol, and trolox was found to be 94.8, 92.3, 87.3, 93.3, and 56.1%, respectively. These results show that the ferrous ion chelating effect of cepharanthine and fangchinoline was statistically similar to that of EDTA, BHA, BHT, and α-tocopherol ($p > 0.05$) but higher than that of trolox ($p < 0.05$).

The ability of cepharanthine and fangchinoline to scavenge hydrogen peroxide is shown in Figure 5 and was compared with that of BHA, BHT, α-tocopherol, and trolox as reference compounds. The hydrogen peroxide scavenging activity of cepharanthine and fangchinoline at 30 μg/mL was found to be 31.8 and 41.9%. On the other hand, BHA, BHT, α-tocopherol, and trolox exhibited 46.8, 82.5, 39.1, and 37.7% hydrogen peroxide scavenging activity at the same

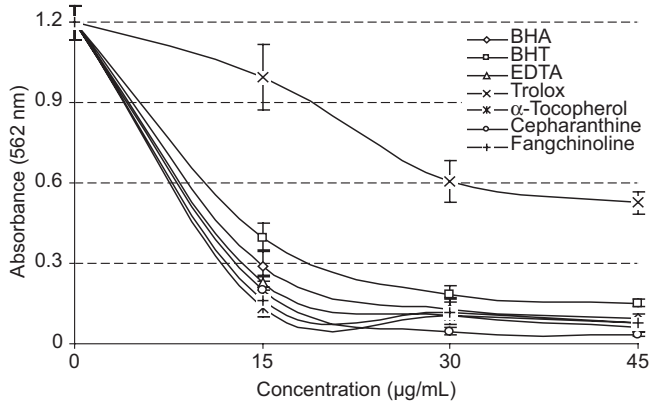

Figure 4. Comparison of ferrous ion (Fe^{2+}) chelating activity of cepharanthine, fangchinoline, and standard antioxidant compounds such as BHA, BHT, α-tocopherol, and trolox at different concentrations (15–45 μg/mL).

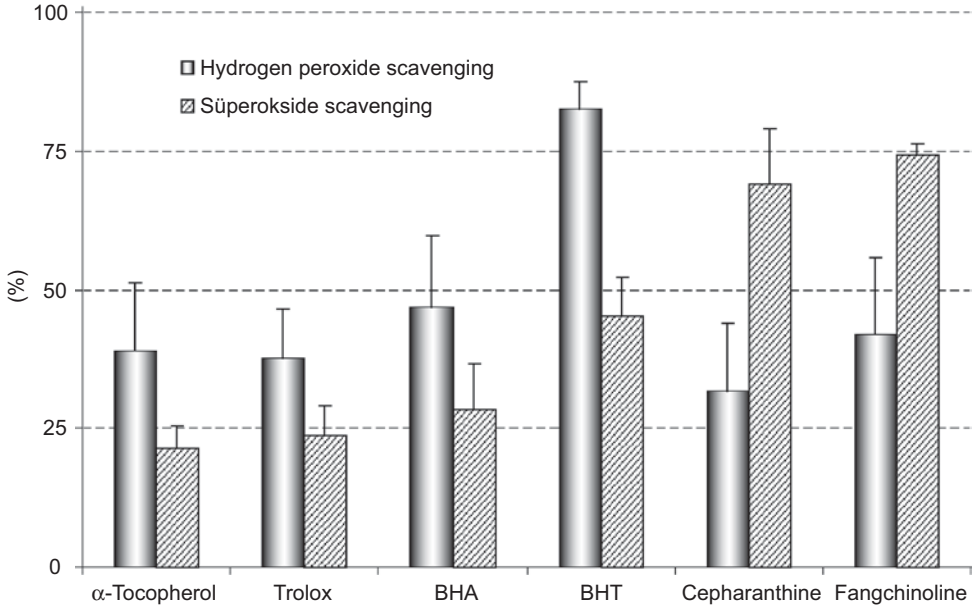

Figure 5. Comparison of hydrogen peroxide (H_2O_2) scavenging activity and superoxide anion radical ($O_2^{\bullet-}$) scavenging activity of cepharanthine and fangchinoline and standard antioxidant compounds such as BHA, BHT, α-tocopherol, and trolox at the concentration of 30 μg/mL.

concentration, respectively. These results show that cepharanthine and fangchinoline had effective hydrogen peroxide scavenging activity. At the concentration above, the hydrogen peroxide scavenging effect of cepharanthine and fangchinoline and four standard compounds decreased in the order of: BHT > BHA> fangchinoline > α-tocopherol > trolox >cepharanthine.

Figure 6A illustrates a significant decrease ($p < 0.01$) in the concentration of DPPH radical due to the scavenging ability of cepharanthine and fangchinoline and the reference compounds. BHA, BHT, α-tocopherol, and trolox were used as references for radical scavenging activity. IC_{50} values for cepharanthine, fangchinoline, BHA, BHT, α-tocopherol, and trolox on the DPPH radical were found to be 22.2, 6.4, 6.1, 10.3, 27.1, and 24.7 μg/mL, and decreased in the order of: BHA ≈ fangchinoline > BHT > cepharanthine > trolox > α-tocopherol. A lower IC_{50} value indicates a higher DPPH free radical scavenging activity.

All the tested compounds exhibited effective radical cation scavenging activity. As seen in Figure 6B, cepharanthine and fangchinoline were effective $ABTS^{\bullet+}$ radical scavengers in a concentration-dependent manner (10–30 μg/mL, $r^2 = 0.9961$, $r^2 = 0.9698$). IC_{50} values for cepharanthine and fangchinoline in this assay were 7.26 and 3.90 μg/mL. There was a significant decrease ($p < 0.01$) in the concentration of $ABTS^{\bullet+}$ due to the scavenging capacity at all cepharanthine and fangchinoline concentrations. On the other hand, IC_{50} values for BHA, BHT, α-tocopherol, and trolox were found to be 7.5, 8.4, 18.6, and 4.2 μg/mL, respectively. The scavenging effect of cepharanthine and fangchinoline and standards on $ABTS^{\bullet+}$ decreased in the order: BHA ≈ fangchinoline ≈ BHT > trolox > cepharanthine > α-tocopherol, at the concentration of 30 μg/mL.

The inhibition of superoxide radical generation by cepharanthine and fangchinoline was higher than those for α-tocopherol and trolox but lower than for BHA and BHT. As seen in Figure 5, the inhibition of superoxide anion radical

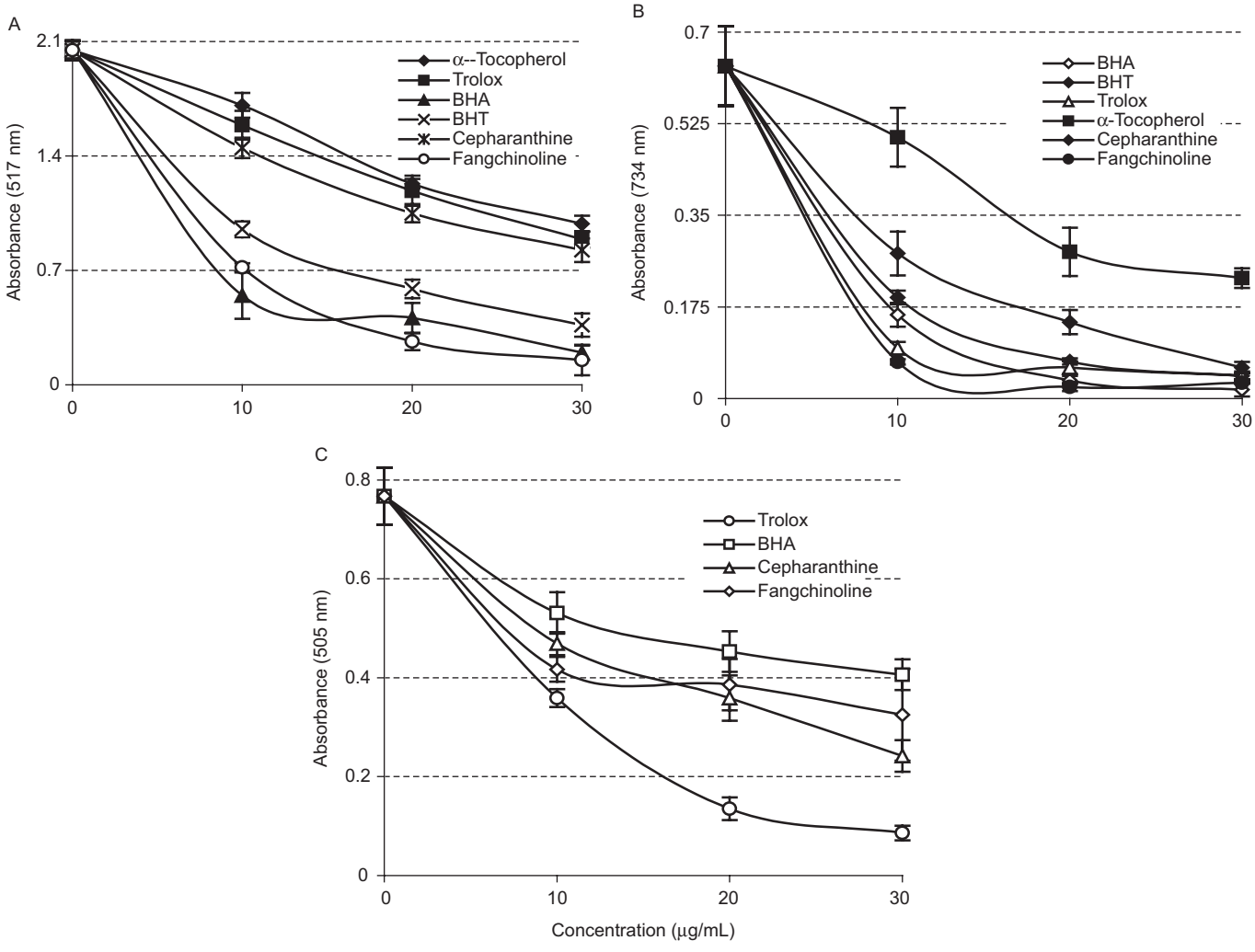

Figure 6. Radical scavenging activity of cepharanthine and fangchinoline. (A) DPPH free radical scavenging activity at different concentrations (10–30 μg/mL) of cepharanthine ($r^2 = 0.9940$) and fangchinoline ($r^2 = 0.9827$) and reference antioxidants (DPPH•: 1,1-diphenyl-2-picryl-hydrazyl free radical). (B) ABTS radical scavenging activity of different concentrations (15–45 μg/mL) of cepharanthine and fangchinoline ($r^2 = 0.9250$) and reference antioxidants ($ABTS^{\bullet+}$: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). (C) DMPD radical scavenging activity of different concentrations (10–30 μg/mL) of cepharanthine and fangchinoline ($r^2 = 0.9974$) ($DMPD^{\bullet+}$: *N*,*N*-dimethyl-*p*-phenylenediamine radical).

generation by 30 μg/mL concentration of cepharanthine and fangchinoline was found to be 68.9 and 74.2%. On the other hand, at the same concentration, BHA, BHT, α-tocopherol, and trolox exhibited 28.3, 45.2, 21.3, and 23.9% superoxide anion radical scavenging activity, respectively. According to these results, cepharanthine and fangchinoline had higher superoxide anion radical scavenging activity than all of the tested reference compounds, and these differences were found to be statically significant.

As shown in Figure 6C, cepharanthine and fangchinoline were effective $DMPD^{\bullet+}$ radical scavengers in a concentration-dependent manner (10–30 μg/mL, $r^2 = 0.988$, $r^2 = 0.839$). IC_{50} values for cepharanthine and fangchinoline were 19.4 and 21.6 μg/mL. This value was found to be 12.9 μg/mL for BHA and 28.3 μg/mL for trolox. There was a significant decrease ($p < 0.05$) in the concentration of $DMPD^{\bullet+}$ due to the scavenging capacity at all cepharanthine and fangchinoline concentrations. No significant differences in $ABTS^{\bullet+}$ scavenging potential could be determined between cepharanthine and fangchinoline.

Discussion

Lipid peroxidation consists of a series of free radical-mediated chain reaction processes and is associated with several types of biological damage. The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation, i.e. the primary product of lipid oxidation. In this assay, hydroperoxides produced from linoleic acid added to the reaction mixture, which has oxidized in air during the experimental period, are indirectly measured. Ferrous chloride and thiocyanate react with each other to produce ferrous thiocyanate by means of hydroperoxides[39].

It was indicated that the electron donating capacity, reflecting the reducing power of bioactive compounds, is associated with antioxidant activity[40]. The reducing capacity of a compound or crude extract can be measured by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian blue complex, $Fe_4[Fe(CN^-)_6]_3$, which has a strong absorbance at 700 nm. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of the antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[40].

The CUPRAC method was developed as a reducing power assay. This method is simultaneously cost-effective, rapid, stable, selective, and suitable for a variety of antioxidants regardless of chemical type or hydrophilicity. Moreover, it was reported that the results obtained from *in vitro* cupric ion (Cu^{2+}) reducing measurements may be more efficiently extended to possible *in vivo* reactions of antioxidants. The CUPRAC chromogenic redox reaction is carried out at a pH (7.0) that is close to the physiological pH[41], and the method is capable of measuring thiol-type antioxidants such as glutathione and non-protein thiols, unlike the widely applied FRAP test, which is non-responsive to -SH group antioxidants[42].

One measurement of the metal-chelating activity of an antioxidant is based on absorbance of the Fe^{2+}–ferrozine complex after prior treatment of a ferrous ion solution with test material. Ferrozine forms a complex with free Fe^{2+} but not with Fe^{2+} bound to other chelators; thus, a decrease in the amount of ferrozine–Fe^{2+} complex formed after treatment indicates the presence of antioxidant chelators. The ferrozine–Fe^{2+} complex produces a red chromophore with absorbance that can be measured at λ_{562} nm. A significant drawback of this complexation reaction, in measuring the presence of antioxidant chelator, is that the reaction is affected by both the antioxidant–Fe^{2+} and the ferrozine–Fe^{2+} complex formation constants and the competition between the two chelators for binding to iron. Thus, a weak antioxidant iron chelator would be seriously underestimated in quantitative determination. From a nutritional point of view, it is not yet possible to assess the role of a weak antioxidant iron chelator in preventing the Fenton reaction *in vivo*. Nonetheless, this reaction serves as a convenient assay to assess the iron chelating activity of an antioxidant.

The data obtained from Figure 4 reveal that cepharanthine and fangchinoline demonstrate a marked capacity for iron binding, suggesting that the main action as a peroxidation inhibitor may be related to the iron binding capacity. In this assay, cepharanthine and fangchinoline interfere with the formation of the ferrous–ferrozine complex.

Biological systems can produce hydrogen peroxide, which can cross membranes and may slowly oxidize a number of compounds. It is used in the respiratory burst of activated phagocytes. The hydrogen peroxide scavenging capacity of cepharanthine and fangchinoline was determined according to the method of Ruch and co-workers[32], as shown in Figure 5. Cepharanthine and fangchinoline had effective hydrogen peroxide scavenging activity. It is known that H_2O_2 is toxic and induces cell death *in vitro*. Hydrogen peroxide can attack many cellular energy-producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase[43].

The structures of cepharanthine and fangchinoline provide a chromophoric system, which leads to interference in the current $DPPH^{\bullet}$·method using the 517 nm wavelength, as described above. The absorbance decreases when the $DPPH^{\bullet}$ is scavenged. Cepharanthine and fangchinoline donate hydrogen to form a stable DPPH radical molecule. In the radical form, this molecule has an absorbance at 517 nm, which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule[44].

$ABTS^{\bullet+}$ has a characteristic long-wavelength absorption spectrum showing absorption at 734 nm. Bleaching of a preformed solution of the blue-green radical cation $ABTS^{\bullet+}$ 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[45].

The principle of the $DMPD^{\bullet+}$ assay is that DMPD can form a stable and colored radical cation at acidic pH and in the presence of a suitable oxidant solution ($DMPD^{\bullet+}$). The UV-visible spectrum of $DMPD^{\bullet+}$ shows a maximum absorbance at 505 nm. Antioxidant compounds which are able to transfer a hydrogen atom to $DMPD^{\bullet+}$ quench the color and produce a decoloration of the solution. This reaction is rapid, and the end point, which is stable, is taken as a measure of the antioxidative efficiency. Therefore, this assay reflects the ability of radical hydrogen-donors to scavenge the single electron from $DMPD^{\bullet+}$[28].

In contrast to the ABTS procedure, the $DMPD^{\bullet+}$ method guarantees a very stable end point. This is particularly important when a large-scale screening is required. It was reported that the main drawback of the $DMPD^{\bullet+}$ method is that its sensitivity and reproducibility decrease dramatically when hydrophobic antioxidants such as α-tocopherol or BHT are used. Hence, these standard antioxidant compounds are not used in this antiradical assay[46].

Superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of free radicals and oxidizing agents[47]. Superoxide anions derived from dissolved oxygen by the riboflavin/methionine/illuminate system 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 blue NBT formation[48]. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture[49]. Figure 5 shows the inhibition of superoxide radical generation by 15 μg/mL concentration of cepharanthine and fangchinoline and standards.

Conclusion

Cepharanthine and fangchinoline were found to be effective antioxidants in different *in vitro* assays including: reducing power, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$, and $O_2^{\bullet-}$ radical scavenging, hydrogen peroxide scavenging, and metal chelating activities, when compared to standard antioxidant compounds such as BHA, BHT, α-tocopherol, a natural antioxidant, and trolox, which is a water-soluble analog of α-tocopherol. As can be seen from the results, fangchinoline has more antiradical activity than cepharanthine. The reason for this is the hydroxyl group (-OH) of the phenolic ring of fangchinoline. The phenolic ring gives extra antioxidant and radical scavenging properties to fangchinoline. Based on the discussion above, cepharanthine and fangchinoline can be used for minimizing or preventing lipid oxidation in pharmaceutical products, retarding the formation of toxic oxidation products, maintaining nutritional quality, and prolonging the shelf life of pharmaceuticals.

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