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Full Length Research Paper

Evaluation of the antioxidant activity of Chinese Hickory (*Carya cathayensis*) kernel ethanol extraction

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The kernel of *Carya cathayensis* Sarg. is a special local product and is widely used as a kind of food in China. In the present study, the antioxidant properties of the kernel including the ability to inhibit the autooxidation of linoleic acid, the reducing capacity, and the scavenging activity on the free radicals were evaluated. The results indicated that a kernel of *C. cathayensis* has effective antioxidant function, especially in superoxide anion scavenging activity.

Key words: *Carya cathayensis,* antioxidant activity, reducing capacity, scavenging activity.

INTRODUCTION

The fruit of Chinese Hickory (*Carya cathayensis* Sarg.) is a highly prized nut crop, especially in South East Asia. *C. cathayensis* is the most important species of the six Carya species indigenous to this region. Cultivation of *C. cathayensis* is localised in Zhejiang Province, with 19 000 hectares under cultivation and yields of 600 kg to 2 400 kg per hectare (Yong-Ling et al., 1992). The kernel of *C. cathayensis* is a special local product of China and is widely used as a kind of food for Chinese people. In addition, the kernel of *C. cathayensis* is widely known for its health care function. It is not only a good tonic, but also frequently used in Chinese traditional medicine as therapeutic component and has been claimed to have beneficial effects on the consenescence, sex capacity, prevention of cancer, atherosclerosis and cardiovascular disease, but there is no epidemiological evidence.

Over the last decade, considerable experimental evidence has supported the view that reactive oxygen species (ROS), including free radicals such as superoxide anion radicals, hydroxyl radicals and nonfree-radical species such as H_2O_2 and singled oxygen, play a key role in the oxidation process which is viewed as one of the initial development steps of many chronic diseases, such as cancer, cardiovascular disease, atherosclerosis, diabetes and so on (Abdi and Ali, 1999; Abe and Berk 1998; Lefer and Granger, 2000).

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The objective of this study was to evaluate the antioxidant properties of kernel with the measurements including the ability to inhibit the autooxidation of linoleic acid, the reducing capacity, and the scavenging activity on the free radicals. This work will enable us to learn more about the influence of kernel of *C. cathayensis* on human beings and provides an important background for further study.

MATIARIALS AND METHODS

Chemicals

Nitroblue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonicacid)-1, 2,4-triazine (ferrozine), a-tocopherol, butylated hydroxyanisole (BHA), xanthine, xanthine oxidase were purchased from Sigma. All other chemicals used were of analytical grade.

Extraction and purification of phenolics from Chinese Hickory kernel

The antioxidant compounds of Chinese Hickory were extracted according to Zhang et al. (Zhang et al., 2000) with some modifycations. A fresh kernel of Chinese Hickory (10 g) was shattered into slurry, then extracted for 30 min by stirring at 4°C with cold aqueous ethanol (65%, 200 ml) containing 0.5% sodium metabisulphite. The homogenate was filtered through four layers of cheesecloth, and the residue was then extracted with two additional portions (100 mL each) of the same extraction solution as described above. The combined filtrate was centrifuged at 7,000 × g for 15 min at 4°C and residue was discarded. Ethanol was removed from the supernatant under vacuum on a rotary evaporator at 35°C, and the pigments

were eliminated by two successive extractions with petroleum ether. After addition of 20% ammonium sulphate and 2% metaphosphoric acid to the aqueous phase, the compounds were extracted three times with ethyl acetate. The extracts were combined, evaporated and then dried under vacuum at 35°C. The residue was redissolved in methanol (1g/L) for analyses of antioxidant properties and named Chinese Hickory ethanol extraction (CHEE).

Total antioxidant activity

The antioxidant activity of CHEE was determined based on thiocyanate method (Mitsuda et al., 1966) with some modifications. The solution, which contained different volumes of (50,100,and 200 µl) in 2.5 ml of potassium phosphate buffer (0.04 mol/L, pH 7.0), was added to 2.5 ml of linoleic acid emulsion in potassium phosphate buffer (0.04 mol/L, pH 7.0). On the other hand, 5.0 ml control was composed of 2.5 ml of linoleic acid emulsion and 2.5 ml of 0.04 mol/L potassium phosphate buffer (pH 7.0). Fifty millilitres of linoleic acid emulsion was prepared with 350 mg of Tween-20 and 310 ml of linoleic acid, and 0.04 mol/l potassium phosphate buffer (pH 7.0) was added up to the volume. The mixed solution (5 ml) was incubated at 37°C in the dark. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer (UV-120-02, Shimadzu, Japan) after reaction with $FeCl_2$ (20 mmol/L in 3.5 g/100 ml HCl) and thiocyanate (30 g/100 ml) at intervals (every 12 h) during incubation. During the linoleic acid oxidation, peroxides are formed, which oxidize Fe^{2+} to Fe^{3+}. Fe^{3+}form a complex with SCN^- and this complex has a maximum absorbance at 500 nm. Therefore, high absorbance indicated high linoleic acid oxidation. The solutions without wines were used as blank samples. All data on total antioxidant activity were the average of triplicate analyses. The inhibition of lipid peroxidation in percentage was calculated by the following equation:

$$\text{Inhibition (\%)} = (1- A_1/A_0)\ \%$$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of CHEE, A 50, 100, 200 µl of 1 g/l BHA and 1 g/l a-tocopherol were used as positive controls.

Reducing capacity

The reducing capacities of CHEE were determined by the method of Oyaizu (Oyaizu, 1986). Different volumes (50, 100, 200 ml) of CHEE in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 mol/L, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1 g/100 ml). The mixture was incubated at 50°C for 20 min. Aliquots (2.5 ml) of trichloroacetic acid (10 g/100 ml) were added to the mixture, which was then centrifuged for 10 min at 1000 g (HITACHI SCR20BC, Japan). The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 g/100 ml), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing capacity. A 50, 100, 200 ml of 1 g/l BHA and 1 g/l a-tocopherol were used as positive controls.

DPPH radical scavenging activity

Free radical scavenging is one of the known mechanisms whereby antioxidants inhibit lipid peroxidation. The free radical scavenging activity of CHEE was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Shimada et al. (Shimada et al., 1992) with slight modifications. The 0.1 mmol/l solution of DPPH radical in ethanol was prepared and 2 ml of this solution was added to 2 ml of water solution containing different volumes of CHEE (50, 100, 200 µl). After 30 min absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The scavenging of DPPH radical in percentage was calculated by the following equation:

$$\text{Scavenging activity (\%)} = (1- A_1/A_0)\ \%,$$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of CHEE. A 50,100,200 µl of 1 g/L BHA and 1 g/L a-tocopherol were used as positive controls.

Superoxide anion scavenging activity

The superoxide anion scavenging activity of CHEE was measured using the xanthine/xanthine oxidase method (Lu and Foo, 2001) with some modifications. 0.5 ml aqueous solution containing different volumes of CHEE extract (50, 100, 200 µl) were separately added to a 1.0 ml mixture of 0.4 mmol/L xanthine and 0.24 mmol/L NBT in 0.1 mol/L phosphate buffer (pH 8.0). A 1.0 ml solution of xanthine oxidase (0.049 units/ml), diluted in 0.1 mol/L phosphate buffer (pH 8.0), was added and the resulting mixture incubated in a water bath at 37°C for 40 min. The reaction was terminated by adding 2.0 ml of an aqueous solution of 69 mmol/l sodium dodecylsulfate and the absorbance of NBT was measured at 560 nm. The scavenging of superoxide anion in percentage was calculated by the following equation: Scavenging activitye (%)=(1- A_1/A_0) %, where A_0 was the absorbance of the control (without CHEE) reaction and A_1 was the absorbance in the presence of CHEE.

Hydroxyl free radical scavenging activity

The reaction mixture contained calf thymus DNA (200 µg), ascorbic acid (10 mM), Cu (II) (100 µM), and CHEE samples to be tested at different volumes (50, 100, and 200 µL). The mixture was incubated in a shaking water bath at 37°C for 1 h. Electrophoresis of DNA was performed after incubation of DNA samples in 1.0% agarose gels. The DNA bands were viewed under UV light and photographed with a digital camera (Rivero et al., 2005).

Statistical and analysis

Experimental results were means ± SD of three parallel measurements. Analysis of variance was performed by ANOVA procedures (DPS 3.1 for Windows). Significant differences between means were determined by Duncan's Multiple Range tests. P values < 0.05 were regarded as significant and P values < 0.01, very significant.

RESULTS AND DISCUSSION

Total antioxidant activity of CHEE

The result for linoleic acid peroxidation after the addition of CHEE (50, 100, 200 µl), 100 µl BHA (1 g/l) and 100 µl α-tocopherol (1 g/l) in 5 ml system determined by measuring the absorbance at 500 nm were plotted in Figure 1. High inhibition was the indication of high concentration of formed peroxides. CHEE, BHA, and α-tocopherol showed effective antioxidant activity during the whole incubation time. BHA exhibited the highest antioxidant activity, the antioxidant activity of CHEE and

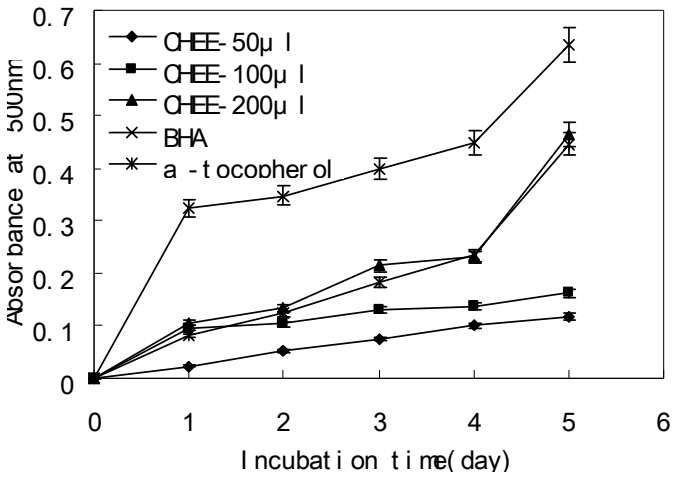

Figure 1. Antioxidant activity of 1 g/l CHEE, 1 g/l BHA and 1 g/l α-tocopherol in the 2.5 ml of linoleic acid emulsion during 120 h of incubation. Each value represents mean ± standard deviation of three replicates. *P* value <0.01 when compared to control.

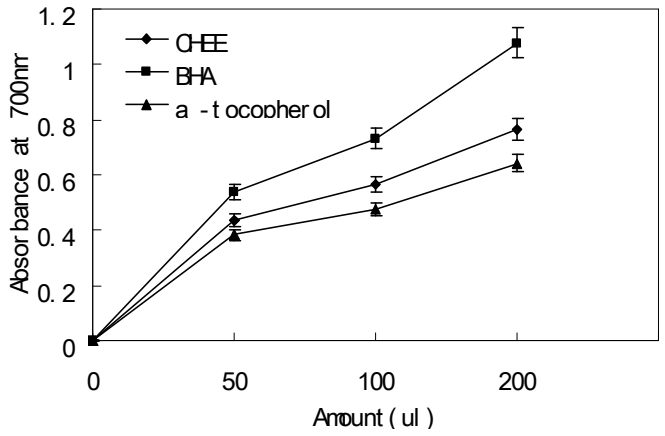

Figure 2. Reducing capacity of different amounts of CHEE, α-tocopherol, BHA using spectrophotometric detection of the Fe^{3+}–Fe^{2+} transformations. Each value represents mean ± standard deviation of three replicates. *P* value <0.01 when compared to control.

α-tocopherol was much weaker. The antioxidant activity of CHEE (200 µl) was equal to α-tocopherol than that of 100 µl of 1 g/l α-tocopherol. After 120 h of incubation, the inhibiting percentages of CHEE (50, 100, 200 µl), 100 µl BHA (1 g/l) and 100 µl α-tocopherol (1 g/l) were 11.7, 16.3, 46.4, 63.4, 44.6%, respectively.

Reducing capacity

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities (Yildirim et al., 2001). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Gulcın and Oktay, 2003). In this study, the Fe^{3+}–Fe^{2+} transformation was investigated. CHEE exhibited effective reducing capacity at all amounts. The reducing capacity of CHEE increased with increasing amount, and the reducing capacity of CHEE was slightly higher than that of α-tocopherol, but was much lesser than BHA (Figure 2), indicating some compounds in CHEE were electron donors and could react with free radicals to convert them into more stable products and terminate the radical chain reactions. At the amount of 200 µL, the absorbance of CHEE, α-tocopherol, BHA at 700 nm was 0.76, 0.64, 1.08, respectively, which indicated that the reducing capability followed the order: BHA> CHEE > α-tocopherol.

DPPH radical scavenging activity

Alkyl free radicals are formed during the induction period of lipid auto-oxidation. Hydrogen peroxide and hydrogen peroxide free radicals, derived from alkyl free radicals, are the major products during the propagation period. The chain reaction of lipid auto-oxidation stops if two free radicals combine together (Hsu et al., 2003). One of the antioxidant mechanisms is to provide hydrogen atoms to free radicals and to stop the chain reaction (Diplock, 1997). The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. CHEE, α-tocopherol and BHA showed significant effects on the DPPH radical scavenging at all amounts (Figure 3). The percentage of DPPH radical scavenging activity at 200 µl volume of CHEE, α-tocopherol and BHA were 87.8, 82.4 and 87.8%, respectively.

Superoxide anion scavenging activity

Superoxide anion is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complex such as cytochrome c. The superoxide anion scavenging activity of CHEE was determined using the cellular xanthine/xanthine oxidase system as a superoxide source. In the present study, 50, 100 and 200 µL of CHEE, α-tocopherol and BHA was used to check the effect of superoxide anion scavenging activity (Figure 4). Just like the total antioxidant activity, reducing capacity, DPPH radical scavenging activity, the CHEE exhibited effective scavenging activity, increasing with the amounts, while α-tocopherol had little scavenging activity. The percentage of superoxide anion radical scavenging activity of 200 µL of CHEE, α-tocopherol and BHA was 76.3, 32.3 and 58.3%, respectively.

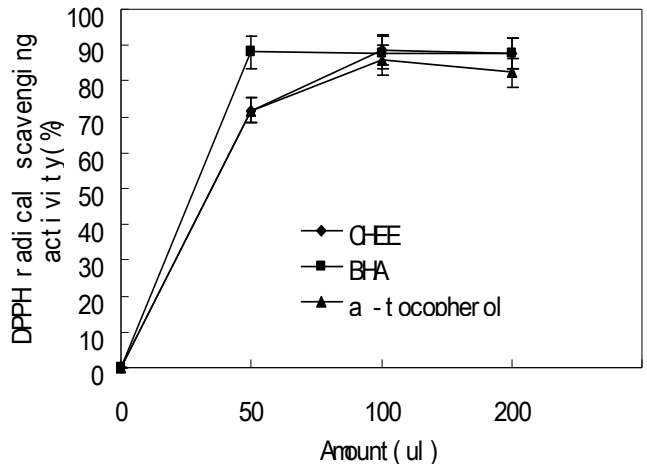

Figure 3. Free radical scavenging activity of different amounts of CHEE, α-tocopherol and BHA (butylated hydroxyanisole) by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals. Each value represents mean ± standard deviation of three replicates. *P* value <0.05 when compared to control.

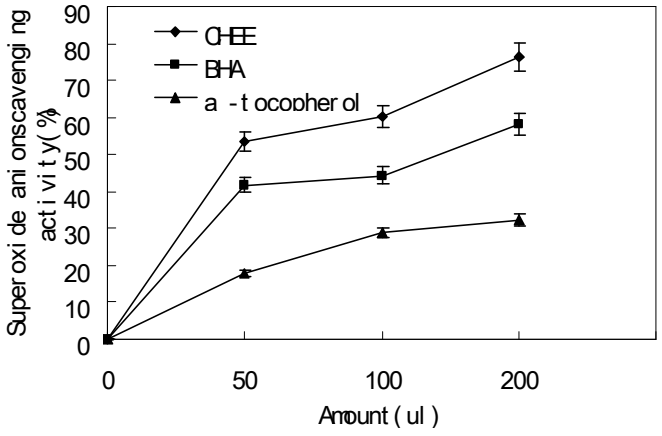

Figure 4. Superoxide anion radical scavenging activity of different amounts of CHEE, α-tocopherol and BHA by the xanthine/xanthine oxidase method. Each value represents mean ± standard deviation of three replicates. *P* value <0.05 when compared to control.

DNA oxidative damage inhibition

Some authors, Rivero and Perez-Magarino (2005) have shown the usefulness of using DNA scission as an effective method to assess the antioxidant activity against active oxygen species *in vitro.* Both ascorbic acid and copper may induce damage of DNA, because they produce highly reactive oxygen species (viz. hydroxyl radicals) which have been implicated in degradation of DNA (Halliwell and Aruoma, 1991), thus producing DNA strand breaks that can be adequately visualized by electrophoresis. The effects of CHEE and BHA on DNA degradation are depicted in Figure 5, which conveys electrophoretic separation of its fragments. The results

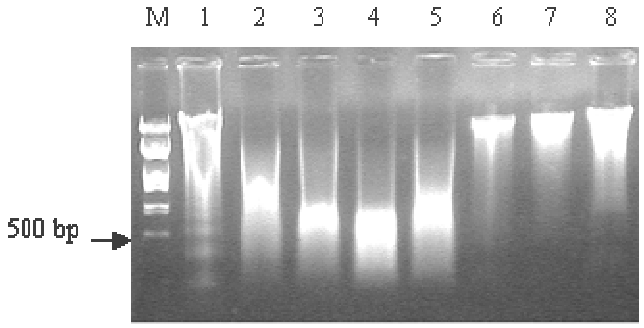

Figure 5. DNA oxidative damage inhibition test of CHEE. lane M: marker of lambda DNA digested with EcoR I and Hind III; lane 1: calf thymus DNA only; lane 2: DNA + Cu (II)-ascorbic acid; lane 3: DNA + Cu (II)-ascorbic acid +200 µL CHEE; lane 4: DNA + Cu (II)-ascorbic acid +100 µL CHEE; lane 5: DNA + Cu (II)-ascorbic acid +50 µL CHEE; lane 6: DNA + Cu (II)-ascorbic acid +200 µL BHA; lane 7, DNA + Cu (II)-ascorbic acid +50 µL BHA; lane 8, DNA + Cu(II)-ascorbic acid +50 µLBHA.

obtained pertaining to CHEE (lanes 4, 5, and 6) indicate that no protective effect took place at 50, 100, 200 µl. The results obtained pertaining to BHA (lanes 7, 8, and 9) indicate that protective effect took place at 50, 100, 200 µl.

Conclusion

The antioxidant property of kernel of *C.cathayensis* was screened in this study. Results showed that CHEE owned effective antioxidant activity. The total antioxidant of CHEE is lower than BHA and α-tocopherol, the reducing capacity is between the BHA and α-tocopherol. The CHEE, BHA and α-tocopherol have almost same DPPH radical scavenging capacity, but CHEE has the highest superoxide anion scavenging activity, and has no hydroxyl radicals scavenging activity determined by DNA oxidative damage inhibition test. The results indicated that a kernel of *C. cathayensis* has effective antioxidant function, especially in superoxide anion scavenging activity. It is of paramount interest to further identify the specific antioxidant components in kernel of *C. cathayensis* and may develop a new health care function food or new drug for special use in the future.

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