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Extracts of *Halenia elliptica* exhibit antioxidant properties *in vitro* and *in vivo*

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ABSTRACT

The antioxidant properties of different extracts of *Halenia elliptica* was investigated by employing various established *in vitro* systems. The results showed that various extracts possessed strong antioxidant activity *in vitro*, and the 70% methanol extract (ME) had the strongest antioxidant activity. Based on our *in vitro* results, ME was used for investigating the antioxidant properties of *H. elliptica in vivo*. The liver and kidney of CCl_4-intoxicated animals exhibited a significant decrease in superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) levels. Additionally, these organs exhibited a significant increase in the level of malondialdehyde (MDA). These changes were significantly reversed, in a dose-dependent manner, after treatment with ME and the standard treatment Vitamin E. Thus, it may be concluded that the ME possesses potent antioxidant properties, and might be valuable natural source of antioxidants that could be applicable to both the medical and food industries.

1. Introduction

The genus *Halenia* (family Gentianaceae) has approximately 100 species primarily distributed in America, with a few species in Asia. *Halenia elliptica* is one of two species distributed in the Qinghai–Tibet Plateau (Ji et al., 2004). It is a medicinal Tibetan herb in China, and is used to treat hepatitis (Luo, 1997). It contains several xanthones (Zhang et al., 1983, 1987; Dhasmana, 1989; Zhang et al., 2003; Gao et al., 2004; Liu et al., 2009; Wang et al., 2009), flavonoids (Yang et al., 2006) and sesquiterpenes (Dai et al., 2002). Previous reports have shown that this plant possesses other biological properties, such as inducing vasodilation (Wang et al., 2008), antioxidant activity (Gao et al., 2004), antibacterial, and antitumor activity (Dai et al., 2002).

According to folk medicine, *H. elliptica* is a Tibetan herb commonly used for its hepatoprotective effects (Luo, 1997). Additionally, research shows hepatoprotective effects are associated with plant extracts that are rich in antioxidants (Amani et al., 2006; Avijeet et al., 2008; Faremi et al., 2008; Anilakumar et al., 2009; Celik et al., 2009; Sreelatha et al., 2009; Huang et al., 2010). To the best of our knowledge, few studies have examined the antioxidant activity of *H. elliptica in vitro* using a lipid inhibition assay (Gao et al., 2004), and the antioxidant activity of *H. elliptica* largely remains an area to be studied. The aim of this work was to test the antioxidant properties of different fractions of *H. elliptica in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals

α,α-Diphenyl-β-picrylhydrazyl (DPPH) and α-tocopherols (Vitamin E) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) was purchased from Fluka (Menlo Park, CA, USA). Linoleic acid was purchased from Alfa Aesar (Ward Hill, MA, USA). Ascorbic acid (Vitamin C), gallic acid, rutin, and butylated hydroxytoluene (BHT) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals used for analysis were AnalaR grade and obtained from China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, China).

2.2. Plant materials

H. elliptica (whole plant) was purchased in Tibetan Traditional Medicine Pharmaceutical Factory, Lhasa city, Tibet, PR China, and authenticated by Prof. Qing Wang, Wuhan botanical garden, Chinese Academy of Sciences, Wuhan, China.

2.3. Determinations of total phenolic, flavonoid, and proanthocyanidin contents

Total phenolic content of various extracts was determined using the Folin–Ciocalteau assay according to the method previously described (Ksouri et al., 2009). Total phenolic content was calculated from the calibration curve of a gallic acid standard solution. Results were expressed as gallic acid equivalents, in mg/g dry extract.

Total flavonoid content was determined by colorimetric assay (Moreno et al., 2000) using a calibration curve generated using rutin as reference compound. Results were expressed as rutin equivalents, in mg/g of dry extract.

Proanthocyanidin determination was based on the procedure of Sun et al. (1998). Extract solution (0.5 ml) was mixed with 3 ml of 4% vanillin–methanol solution and 1.5 ml of hydrochloric acid, and the mixture was allowed to stand for 15 min. Absorbance was measured at 500 nm and used to calculate the final result expressed as mg catechin equivalents per g dry extract.

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2.4. Preparation of extracts and fractions

The powder (50 g, size was less than 0.25 mm) of *H. elliptica* was extracted with *n*-hexane, chloroform, ethyl acetate, *n*-butanol, or 70% methanol at room temperature for 24 h (Bajpai et al., 2008). Solvents were evaporated by rotary evaporation at 35 °C. The residues were lyophilized and yielded five extracts: *n*-hexane extract (HE), chloroform extract (CE), ethyl acetate extract (EE), *n*-butanol extract (BE) and 70% methanol extract (ME).

2.5. Test animals

Sprague–Dawley rats (220 ± 20 g) and KM mice (20 ± 2 g) of both sexes were purchased from the Laboratory Animal Center of Wuhan University (Wuhan, China). They were housed on a 12 h light–dark cycle at 25 ± 2 °C, and in a relative humidity of 30–60%. Animals were fed *ad libitum* with a standard diet of pellets and water. Animals were allowed to acclimate to housing conditions for 3 days prior to experimentation. The study received clearance from the Institutional Animal Ethical Committee (IAEC) of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Wuhan University, Wuhan, China.

2.6. In vitro antioxidant properties

2.6.1. DPPH radical scavenging assay

The free-radical-scavenging activity of the different extracts was measured using DPPH according to the procedure described by our previously published method (Huang et al., 2010). Extract solution at a range of concentrations (0.3 mL) was mixed with a solution of 0.2 mM DPPH in methanol (2.7 mL). The mixture was shaken vigorously and allowed to stand for 1 h before the absorbance was measured at 517 nm. Radical-scavenging activity was calculated as the following percentage: $[(As - Ai)/As] \times 100$ (As = absorbance of DPPH alone, Ai = absorbance of DPPH in the presence of various extracts). A concentration of BHT that was identical to the experimental samples was used as reference.

2.6.2. ABTS radical scavenging assay

The ability of various extracts to scavenge the ABTS radical cation was determined by our previously published method (Huang et al., 2010). A solution of ABTS radical cation ($ABTS^+$) was prepared by the reaction of 7 mM ABTS and 2.45 mM potassium persulfate at room temperature in the dark for 16 h. The $ABTS^+$ solution was then diluted with 80% ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. Extract solution at a range of concentrations (0.3 mL) was mixed with ABTSt solution (2.7 mL), the reaction mixture was allowed to stand at 30 °C for exactly 30 min, and absorbance at 734 nm was recorded. BHT at the same concentration as the samples was used as reference. The level of radical scavenging was calculated using the equation described above for DPPH.

2.6.3. Scavenging of ·OH radicals

The ability of the test extracts to scavenge ·OH was determined as described previously (Kaur et al., 2008) with slight modifications. The reaction mixture contained 500 mL of 2-deoxyribose (2.8 mM) in potassium phosphate buffer (50 mM, pH 7.4), 200 mL of premixed ferric chloride (100 mM) and EDTA (100 mM) solution (1:1, v/v), 100 mL of H_2O_2 (200 mM) without or with the extract solution (100 mL). The reaction was initiated by the addition 100 mL of 300 mM ascorbate and incubated for 1 h at 37 °C. A solution of thiobarbituric acid (TBA) in 1 mL (1%, w/v) of 50 mM NaOH and 1 mL of 2.8% (w/v, aqueous solution) trichloroacetic acid (TCA) was added. The mixture was heated for 15 min in a boiling water bath and then cooled. The absorbance was measured at 532 nm. The absorbance of the control was determined by replacing the sample with methanol. BHT at the same concentration as the samples was used as reference. Radical-scavenging activity was calculated using the equation described above for DPPH.

2.6.4. Superoxide radical scavenging assay

The capacity of each extract to scavenge superoxide radicals was examined by a pyrogallol auto-oxidation system (Xiang and Ning, 2008) with slight modifications. Briefly, reaction mixtures containing test extracts (0.2 mg/mL) in Tris–HCl buffer (4.50 mL, 50 mM, pH 8.2) were incubated for 10 min at 25 °C, and then 150 μL of pyrogallic acid (3 mM, prepared in 10 mM HCl) was added. The absorbance of the reaction mixture at 325 nm was measured immediately, and then at 30 s intervals thereafter. The auto-oxidation rate constant (*Kb*) of pyrogallic acid was calculated from the curve of A_{325nm} vs time. The control did not contain test extracts and a concentration of Vitamin C identical to the samples was used as a reference. The inhibitory actions of test extracts on the auto-oxidation rate of pyrogallic acid correlated with their ability to scavenge superoxide radicals.

2.6.5. Reducing power assay

The reducing power of various extracts was determined by the method of Oyaizu (1986). Various extracts were diluted to a range of concentrations in distilled water, and the solution of extracts was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and potassiumferricyanide (2.5 mL, 1%). After incubation at 50 °C for 20 min, trichloroacetic acid (2.5 mL, 10%) was added, and each mixture was centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%), and absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. A BHT reference was used as a comparison.

2.6.6. Antioxidant activity in a linoleic acid system using ferrothiocyanate (FTC) and thiobarbituric acid (TBA)

The FTC method was adapted from Osawa and Namaki (1981). Extracts (400 μg) in ethanol (4 mL) were mixed with 2.5% linoleic acid in ethanol (4 mL), phosphate buffer (8 mL, 50 mM, pH 7.0), and distilled water (4 mL). The mixtures were incubated at 40 °C in screw-cap tubes in the dark. Aliquots (0.1 mL) were withdrawn and mixed with 75% ethanol (9.7 mL) and 30% ammonium thiocyanate (0.1 mL). Ferrous chloride (0.1 mL, 20 mM in 3.5% HCl) was added to each aliquot, and precisely 3 min later, absorbance at 500 nm was measured. Aliquots were withdrawn and assayed in an identical fashion at 24 h intervals until a constant maximum value was reached. Controls without extract and standard containing BHT in place of extract were subjected to the same procedure.

The method of Kikuzaki and Nakatani (1993) using TBA was also employed to determine the antioxidant activity of various extracts. Aqueous TBA (2 mL) and 20% trichloroacetic acid (2 mL) were added to 1 mL of extract solution that was prepared as described in the FTC method above. The mixture was placed in a boiling water bath for 10 min, cooled, and centrifuged at 3000 rpm for 20 min. Absorbance of the resulting supernatant was measured at 532 nm. The inhibition rate was calculated using the following equation: $[(A_c - A_s)/A_c] \times 100$ (A_c = absorbance of control; A_i = absorbance of sample).

2.7. In vivo antioxidant properties

2.7.1. Carbon tetrachloride-induced oxidative toxicity

Rats were divided into six groups consisting of six animals in each group. Rats in Group I received distilled water containing 0.3% sodium carboxymethylcellulose (CMC-Na) (1 mL/kg body weight, p.o.) for 5 days, and olive oil (1 mL/kg body weight, s.c.) on days 2 and 3 (Avijeet et al., 2008). Group II (CCl_4) received 0.3% CMC-Na (1 mL/kg body weight, p.o.) for 5 days, and a 1:1 mixture of CCl_4 and olive oil (2 mL/kg body weight, s.c.) on days 2 and 3. Group III was treated with the standard drug vitamin E (50 mg/kg body weight, p.o.) daily for 5 days, and also received the same CCl_4–olive oil mixture (1:1, 2 mL/kg body weight, s.c.) on days 2 and 3, 30 min after administration of vitamin E. Groups IV, V, and VI (test group animals) were administered an oral dose of 50, 100, and 200 mg/kg body weight of ME, respectively, for 5 days. Additionally, 30 min after administration of ME, these animals also received the CCl_4–olive oil mixture (1:1, 2 mL/kg, s.c.) on days 2 and 3. On day 6, animal were sacrificed by bleeding, the liver and kidney were dissected for biochemical characterization.

2.7.2. Biochemical determinations

Liver and kidney samples were dissected and immediately washed with ice-cold saline to remove blood. Liver and kidney homogenates (10% w/v) were prepared in cold 50 mM potassium phosphate buffer (pH 7.4), and the resulting suspension was centrifuged at 1000 rpm for 10 min at 4 °C. The clear supernatant was used for determination of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and glutathione (GSH), using assay kits obtained from the Institute of Biological Engineering of Nanjing Jianchen, (Nanjing, China), according to the manufacturer's protocol. SOD activity was estimated by the inhibition of spontaneous epinephrine oxidation (Kakkar et al., 1972). For calculation, the standard curve of SOD activity was used. The results were expressed in units per mg protein. Determination of CAT activity was based on the rate of H_2O_2 reduction (Beer and Seizer, 1952). One unit of CAT reduces 1 mM of H_2O_2. The results were expressed in units per mg protein. MDA in liver and kidney tissues was examined using the method of thiobarbituric acid-reactive substances (TBARS) as previously method described by Yagi and Rastogi (1979). The concentration of MDA was expressed as *n* moles per mg protein using 1,1,3,3-tetraethoxypropane (TEP) as a standard. GSH in liver and kidney tissues was determined according to the Ellman method (Ellaman, 1959), which measures the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) (Ellman's reagent) by sulfhydryl groups to 2-nitro-5-mercaptobenzoic acid, which has an intense yellow color. The results were expressed in mg per g protein.

2.8. Statistical analysis

All experiments were done in triplicate and results were reported as mean ± SD. The data were analyzed by one-way ANOVA; statistically significant effects were further analyzed and means were compared using Duncan's multiple range test. Statistical significance was determined at $p < 0.05$.

3. Results and discussion

The yields of various extracts from *H. elliptica* are shown in Table 1. Phenolic compounds have been reported to be responsible