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The antioxidant activity and active component of *Gnaphalium affine* extract

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ABSTRACT

The antioxidant activity of *Gnaphalium affine* extract (GAE) against H_2O_2-induced oxidative injury in Caco-2 cells was evaluated, and the main antioxidant component was isolated and identified by column chromatography, high performance liquid chromatography, time-of-flight mass spectrometer and nuclear magnetic resonance. In vitro assays, GAE showed remarkable antioxidant activity to scavenge free radicals (ABTS, DPPH, superoxide and hydroxyl radicals), inhibit lipid peroxidation and show reducing power. In food system, GAE exhibited the obvious capacity to inhibit the oxidation of peanut oil and lard, which may be attributed to its high content of phenolic compounds. Moreover, GAE could effectively protect Caco-2 cell against H_2O_2-induced oxidative injury. With the isolation and purification by chromatography, quercetin was identified as the main antioxidant component of GAE, which was capable of scavenging ABTS, DPPH, superoxide and hydroxyl radicals. These results suggest that *G. affine* is a potential source for preparing functional foods and nutraceuticals in food industry.

1. Introduction

Gnaphalium affine, belonging to *Asteraceae*, is normally named Cudweed or 'Qing Ming Cao' in China. It is traditionally used as a wild vegetable to process a variety of foods, such as drink, bread and frozen vegetable, and is also commonly used as a medicinal plant in folk medicine (Hou et al., 2011). *G. affine* is considered nutritional because it is rich of essential amino acids, minerals, trace elements and vitamins (Wang et al., 2005). Besides, *G. affine* has been observed to possess many pharmacological activities. Recent studies report that *G. affine* exhibit protective effect against carbon tetrachloride-induced acute liver injury and show anticomplementary activity (Hou et al., 2011; Xi et al., 2012). It is also used to lower blood pressure, or used as diuretic, antipyretic, antimalarial and insect antifeedant agents (Morimoto et al., 2000). Furthermore, *G. affine* is useful for the treatment of swellings, lumbago, respiratory and cardiovascular diseases, having therefore a potential for the development of functional food (Yu et al., 2006).

Oxidative stress is a result of excess formation and/or incomplete removal of highly reactive molecules such as reactive oxygen species (ROS) including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$) and hydroperoxyl radical ($HOO^{\cdot}$). They may damage the key organic substrates such as DNA, lipids and proteins, disrupting cell physiological function (Dennery, 2010). A variety of studies shows that oxidative stress is associated with ageing process in general, and with the initiation and progression of many chronic diseases, such as cancer, cardiovascular disease, Alzheimer's disease, Parkinson's disease and muscular degeneration (Halliwell, 2009). Oxidation is not only harmful to human health, it also has negative influence on food. Oxidation is a common phenomena occurring in food processing, which may lead to the degradation of lipids and proteins, and contribute to the deterioration in flavor, texture and color of food products (Borneo et al., 2009; Karpińska et al., 2001).

To the best of our knowledge, there is limited information available on the antioxidant properties of *G. affine*. In a previous study, we found that the essential oil of *G. affine* had strong antimicrobial and antioxidant activities (Zeng et al., 2011a,b). Herein, as a serial research, effort was made to evaluate the antioxidant activity of *G. affine* extract in cells and in food system, and to investigate the main antioxidant component in the extract.

2. Materials and methods

2.1. Chemicals

The aerial parts (leaves and flowers) of *G. affine* were collected from Chengdu, China, in March 2011, and identified by morphological features in Department of Biology, Sichuan University, China. The specimen (voucher number: 2011-0159) was preserved at the Institute of Agricultural Products Processing Technology, Sichuan University, China.

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2,2′-Azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS), phenazine methosulfate (PMS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), butyl hydroxy anisd (BHA), ascorbic acid (Vc), dihydronicotineamidadenine dinucleotide (NADH), ethylene diamine tetraacetic acidwere (EDTA), nitro blue tetrazolium (NBT), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Penicillin–streptomycin solution, high glucose Dulbecco's modified Eagle medium (DMEM, containing 4.5 g/L glucose), nonessential amino acids, heat-inactivated fetal bovine serum (FBS), hank's balanced salt solution (HBSS), trypsin–EDTA and L-glutamine were purchased from Gibco-BRL Life Technology (Grand Island, NY, USA). Caco-2 cell lines were purchased from Shanghai Institute of Cell Biology (Shanghai, China). Solvents for high performance liquid chromatography (HPLC) were of chromatographic purity. All other reagents used were of analytical grade.

2.2. Preparation of G. affine extract

Air-dried *G. affine* (300 g, 25 °C, 15 d) was crushed into powder with a mixer (JYL-350, Jiuyang Co., Ltd., Jinan, China). The powder (100 g) was added to methanol (2000 mL, 50%, v/v) with continuously stirring at 25 °C for 24 h. Then, the mixture was filtered and the filtrate was condensed using a rotary evaporator (RE-3000A, Yarong, Co., Ltd., Shanghai, China) at 45 °C. The dried *G. affine* extract (GAE) was obtained by freeze-drying with a yield of 8.5%, and stored at 4 °C for further research.

2.3. Measurement of total phenol and total flavonoids contents of GAE

The concentration of phenols and flavonoids in GAE was measured by the method described in our previous study (Zeng et al., 2011a,b). The Folin–Ciocalteu assay and $AlCl_3$ colormetric assay were applied for the determination of phenols and flavonoids, respectively. GAE was dissolved in pure water at the concentration of 1 mg/mL for test.

2.4. Evaluation of in vitro antioxidant activity of GAE

2.4.1. Assay of ABTS radical scavenging

ABTS (7 mM) and potassium persulphate (2.45 mM) were dissolved in pure water at 23 °C for 16 h in dark. Then, pure water was used to thin the reaction solution to obtain an absorbance of 0.700 ± 0.005 at 734 nm. Diluted solution (3.9 mL) and GAE solution (0.1 mL, 50–250 μg/mL) were mixed and then stored at 23 °C for 6 min, and the absorbance was recorded at 734 nm (Re et al., 1999). The ability of GAE to scavenge ABTS radical in percent was calculated as $(1 - A_{\text{sample } 734}/A_{\text{control } 734}) \times 100\%$. BHA was used as the positive control.

2.4.2. Assay of DPPH radical scavenging

GAE solution (2 mL, 50–250 μg/mL) and DPPH solution (2 mL, 0.1 mM, dissolved in 95% ethanol freshly) were mixed and kept at 25 °C for 30 min in dark. Then, the absorbance of mixture was measured at 517 nm (Mendes et al., 2011). The ability of GAE to scavenge DPPH in percent was calculated as $(1 - A_{\text{sample } 517}/A_{\text{control } 517}) \times 100\%$. BHA was used as the positive control.

2.4.3. Assay of superoxide radical scavenging

Tris–HCl (16 mM, pH 8.0) was used as solvent in this assay. GAE solution (0.1 mL, 50–250 μg/mL) was mixed with NADH solution (1 mL, 557 μM), PMS solution (1 mL, 45 μM) and NBT solution (1 mL, 108 μM), and incubated at 25 °C for 5 min. Then, the absorbance of mixture was measured at 560 nm (Zhang et al., 2003). The scavenging activity was calculated as $(1 - A_{\text{sample } 560}/A_{\text{control } 560}) \times 100\%$. BHA was used as the positive control.

2.4.4. Assay of hydroxyl radical scavenging

Phosphate buffered saline (PBS, 0.2 M, pH 7.4) was used as solvent in this assay. GAE solution (0.1 mL, 50–250 μg/mL) was mixed with ferrous ammonium sulfate solution (0.2 mL, 0.4 mM), Vc solution (0.05 mL, 2.0 mM), H_2O_2 solution (0.05 mL, 20 mM) and mixed solution (0.6 mL, contenting 2.67 mM of deoxyribose and 0.13 mM of EDTA), and incubated at 37 °C for 15 min. Then, thiobarbituaric acid solution (1 mL, 1%, w/v) and trichloroacetic acid solution (1 mL, 2%, w/v) were added. The mixture was boiled in water bath for 15 min and cooled in ice, and its absorbance was measured at 532 nm (Zhang et al., 2003). The scavenging activity was calculated as $(1 - A_{\text{sample } 532}/A_{\text{control } 532}) \times 100\%$. BHA was used as the positive control.

2.4.5. Assay of lipid peroxidation inhibition

GAE solution (0.1 mL, 50–250 μg/mL) was mixed with egg yolk homogenate solution (0.5 mL, 10%, v/v) and pure water (0.4 mL). Then, ferrous sulfate solution (50 μL, 70 mM) was added, and the mixture was incubated at 37.5 °C for 30 min. Subsequently, acetic acid solution (1.5 mL, 20%, v/v, pH 3.5) and thiobarbituric acid solution (1.5 mL, 0.8%, w/v, dissolved in 1.1% sodium dodecyl sulfate solution) were added and the mixture was heated at 95 °C in water bath for 60 min. When the reaction solution cooled down, 1-butanol (5 mL) was added and the mixture was centrifuged at 5000 rpm for 15 min. The upper layer was collected and its absorbance was measured at 532 nm (Dasgupta and De, 2004). The inhibition of lipid peroxidation was calculated as $(1 - A_{\text{sample } 532}/A_{\text{control } 532}) \times 100\%$. BHA was used as the positive control.

2.4.6. Assay of reducing power

The mixture of GAE solution (1 mL, 10–50 μg/mL), PBS solution (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide solution (2.5 mL, 1%, w/v) was incubated at 50 °C for 20 min. Then, trichloroacetic acid solution (2 mL, 10%, w/v) was added and the mixture was centrifuged at 2000 rpm for 15 min. Subsequently, the upper layer (2.5 mL) was mixed with pure water (2.5 mL) and ferric chloride solution (0.5 mL, 0.1%, w/v). The absorbance of mixture was observed at 700 nm (Zeng et al. 2011a,b). BHA was used as the positive control.

2.5. Protection of GAE against H_2O_2-induced oxidative injury in Caco-2 cell lines

Caco-2 cells, the chosen cell model, were cultured in DMEM (4.5 mg/L), and supplemented with nutritional solution which contained FBS (10%, v/v), L-glutamine (2 mM), nonessential amino acids (1%, v/v), penicillin G (100 U/mL) and streptomycin (50 U/mL). The cultured condition was kept at 37 °C, 5% CO_2 and 95% relative humidity. When reaching 80% confluence, cells were digested by trypsin (0.25%, containing 1 mM EDTA). The passage number of cell was kept at 30–40 throughout.

Antioxidant capacity of GAE in cell lines was determined by measuring the cell viability using MTT test. When the cells were cultured to a density of 1×10^5 cells/mL for 24 h, culture medium containing various concentrations of GAE (10–50 μg/mL) was used in cell incubation for 1 h. After that, medium was removed and washed three times with PBS. Then, fresh medium containing H_2O_2 (2 mM) was added and incubated for 2 h. Subsequently, the medium was removed and fresh medium containing MTT (0.5 mg/mL) was added and incubated for 4 h. With the removing of culture medium, cells were lysed by using DMSO. The absorbance of mixture was measured at 490 nm. Cells cultured without GAE and H_2O_2 treatment were used as control. All incubation was carried out at 37 °C, 5% CO_2 and 95% relative humidity in the cell incubator. The antioxidant activity of GAE was described using the cell viability which was calculated as $(A_{\text{sample } 490}/A_{\text{control } 490}) \times 100\%$.

2.6. Antioxidant capacity of GAE in a food system

The antioxidant capacity of GAE in food system was determined by the method described in our previous study (Zhang et al., 2011). Peanut oil and lard, purchased from a local market (Chengdu, China) were employed for antioxidant test. Peanut oil (or lard, 25 g) was added to brown bottles and incubated at 45 °C for 30 min. GAE was added to the oil to reach final concentrations of 0.01%, 0.05% and 0.1% (w/w). Then, the bottles without stopper were stirred and incubated at 70 °C. Sample without GAE was used as negative control and BHA (0.05%, w/w) was used as positive control.

The peroxide value (POV) of sample was determined to evaluate the antioxidant capacity of GAE. The sample (2 g) was mixed with 30 mL acetic acid-chloroform solution (acetic acid: chloroform = 3:2, v/v) and 1 mL saturated potassium iodide solution (140%, w/v), and was incubated at 25 °C for 3 min. Subsequently, 100 mL pure water and 1 mL starch indicator (1%, w/v) were added and the mixture was titrated with sodium thiosulfate solution (STS, 0.002 M) until blue violet color disappeared. Pure water was used as blank control. Analyses were carried out after regular intervals of 24 h. POV was calculated according to the equation: POV (meq/kg)=$N \times (V_1 - V_2) \times (1000/M)$, there, N is STS concentration (M), V_1 is the volume of STS consumed by sample (mL), V_2 is the volume of STS consumed by blank control (mL) and M is sample quality (g).

2.7. Isolation and identification of the main antioxidant component in GAE

GAE (3 g, dissolved in 50% methanol) was isolated using an MCI-gel column (5 × 75 cm, Sci-Bio-Chem Co., Ltd., Chengdu, China) and a Sephadex LH-20 column (3 × 50 cm, Sigma–Aldrich, St. Louis, MO, USA). The water–methanol gradient system (500 mL for each fraction in MCI-gel column; 100 mL for each fraction in Sephadex LH-20 column) was used as eluant. Then, the free radical (ABTS, DPPH, superoxide and hydroxyl radicals) scavenging activity of each fraction was determined. With the strongest antioxidant activity, the most active fraction was further purified by the HPLC system (Agilent 1100 Series Purification System, Agilent Technologies, Inc., CA, USA) with a reverse-phase column (Inertsil PREP-ODS-3, 4.6 × 250 mm i.d. with a particle size of 5 μm, GL-Science, Tokyo, Japan). The mobile phase was composed of solvent A (methanol) and B (0.1% formic acid in ultrapure water). A gradient program was used as follows: 0% A (0 min), 50% A (5 min), 60% A (10 min), 70% A (15 min), 80% A (20 min), 90% A (25 min), 100% A (30 min). The column temperature was 25 °C; the flow rate was 1 mL/min; the dual wavelength detector was recorded at 256 nm. Subsequently, the main antioxidant compound obtained was identified and characterized by time-of-flight mass (TOF-MS) and nuclear magnetic resonance (NMR) analysis, which was performed by using a Waters Q-TOF Premier (Waters, Co., Ltd., MA, USA) with an electrospray source operating in positive ion mode and a Bruker AV II-600 instrument (^1H, 600 MHz; ^{13}C, 125 MHz; D_2O was used as solvent, Bruker Daltonics Inc., MA, USA).