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Comparison of in vitro antioxidant activities and bioactive components of green tea extracts by different extraction methods

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ABSTRACT

In this study, in vitro antioxidant activities and bioactive components of green tea extracts (GTE) by ultrahigh pressure extraction and conventional extraction methods (microwave extraction, ultrasonic extraction, Soxhlet extraction and heat reflux extraction) were investigated. DPPH radical-scavenging and FTC method were applied to test the antioxidant activities. The bioactive components were determined by chemical methods. The results indicated that the GTE by ultrahigh pressure extraction exhibited the strongest antioxidant activities. The contents of polyphenols and catechins in the GTE by ultrahigh pressure extraction were significantly higher than those by other extraction methods, which was possibly responsible for the higher antioxidant activities of the GTE by ultrahigh pressure extraction. From the results we can draw the conclusion that not only the more bioactive components are obtained but also the extract has better free radical and reactive oxygen species scavenging activities through ultrahigh pressure extraction method. These findings further illustrate that ultrahigh pressure extraction has a bright prospect for extracting active ingredients from plant materials.

1. Introduction

Tea is a widely popular beverage around the world for centuries. It is estimated that on an average, about 6 g of tea is consumed per day by a 60 kg human being (Chang et al., 2000). Green tea leaves contain polyphenols, caffeine, amino acids, saponins, tannins, etc. In general, dry green tea leaves (*Thea sinensis* L.) contain about 10–35% (w/w) polyphenols and 2–5% (w/w) caffeine (Xi et al., 2010). Polyphenols include catechines, flavanols, flavanones, phenolic acids, glycosides and the aglycons of plant pigments, etc., which are natural antioxidants that can be used as alternatives to synthetic antioxidants as they are typically less harmful than synthetic ones and appears to have an equivalent effect upon the inhibition of oxidation (Xi et al., 2009). These antioxidants, which inhibit the oxidation of organic molecules, are very important for living systems and their defence against oxidative stress (Chang et al., 2000). Phenolic antioxidants interrupt the propagation of the free radical autoxidation chain by contributing a hydrogen atom from a phenolic hydroxyl group, with the formation of a relatively stable free radical that does not initiate or propagate further oxidation processes (Xi et al., 2009).

Ultrahigh pressure extraction (UPE) as a novel technique is used for extraction of active ingredients from plant materials. High pressure ranging from 100 to 600 MPa is considered as an alternative extraction method, which is proven to be fast and more effective (Prasad et al., 2010). High pressure can improve the mass transfer rate and enhance solvent permeability in cells as well as secondary metabolite diffusion (Ahmed and Ramaswamy, 2006). High pressure can also cause some structural changes in vegetal materials, such as cellular deformation, cellular membrane damage and amino acid denaturation (Xi, 2006). Application of this technique for extraction of bioactive substances from plant materials yields some advantages, such as short extraction time, mild extraction condition, high extraction yield and less impurity (Prasad et al., 2010). Recently ultrahigh pressure technique had been successfully used for extraction of anthocyanins from grape skins (Corrales et al., 2009), ginsenosides from Korean panax ginseng powder (Shin et al., 2010), biologically active compounds from green tea leaves (Xi, 2009; Xi et al., 2009, 2010) and flavonoids and phenolic compounds from Litchi fruit pericarp (Prasad et al., 2009), corilagin from longan fruit pericarp (Prasad et al., 2010) and so on.

Studies have dealt with the methods of extraction of bioactive compounds from green tea, such as microwave-assisted extraction, supercritical carbon dioxide, heat reflux extraction and Soxhlet extraction, ultrasonic extraction and so on (Yuko et al., 1999; Chang et al., 2000; Park et al., 2008). UPE is a novel technique at present, which we had successfully used to extract major catechins and polyphenols from green tea leaves (Xi, 2009; Xi et al., 2009, 2010). However, there are no studies on the antioxidant activities of green tea extracts (GTE) by UPE. In the present study, we evaluated the GTE by UPE and conventional extraction methods (microwave extraction, ultrasonic extraction, Soxhlet extraction

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and heat reflux extraction) for their in vitro antioxidant activities by DPPH radical-scavenging and ferric thiocyanate (FTC) methods. In order to understand the relationship between bioactive properties and antioxidant activities, the bioactive constituents of the GTE by UPE and conventional extraction methods were analysed. Therefore, the present study is aimed at the investigation of the comparison of the antioxidant activity of GTE obtained by different extraction methods in relationship to their bioactive constituent contents.

2. Materials and methods

2.1. Materials

The fresh green tea leaves (*T. sinensis* L.) (place of origin: Hangzhou, China) were purchased from a local market, and dried at 50 °C in an oven until a constant weight was obtained before use.

Ethanol used in the experimental work was analytical reagent grade chemicals (Beijing Chemical Reagents Company, Beijing, China). Deionized water was prepared using a Milli-Q Plus system (Millipore, USA). Epigallocatechin (EGC), (+)-catechin ((+)-C), epicatechin (EC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), α, α-diphenyl-b-picrylhydrazyl (DPPH), α-tocopherol, acetic acid and acetonitrile of chromatographic grade were purchased from Sigma–Aldrich Chemical Co. (Sigma, USA). Linoleic acid (99%) and 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were of analytical grade and purchased from Chengdu Chemical Industry (Chengdu, China).

The HPLC system used was a LC-10AT vp HPLC system with a SPD-M10A vp diode-array detector (Shimadzu, Japan) and a Diamonsil™ C18 column (250 mm × 4.6 mm, 5 μm, Dikma, Beijing, China) at 25 °C.

The ultrahigh pressure apparatus was purchased from Chengdu Suohaipu Super-high Pressure Machine Co., Ltd. (Chengdu, China). Effective volume of vessel: 5 L; maximal working pressure: 600 MPa; inner diameter: 200 mm; pressure transmitting media: water and glycol (20:80, v/v).

2.2. Preparation of GTE

2.2.1. Ultrahigh pressure extraction (UPE)

The fresh green tea leaves were pulverized and sieved. Ten grams samples of green tea leaves powder through 40 mesh screen were mixed with 200 mL of 50% (v/v) ethanol solvent and then placed into a sterile polyethylene bag. The bag was sealed after eliminating air from the inside and placed into the ultrahigh pressure vessel. After processed in the ultrahigh pressure apparatus for 5 min (high pressure level: 400 MPa) at ambient temperature, the mixture was filtered through a filter paper (Xi, 2009; Xi et al., 2009, 2010). The extracts were centrifuged at 4000 × *g* for 10 min, and the supernatants were pooled. The extracts were again centrifuged under the same conditions and the supernatants were pooled. The supernatants obtained were combined and concentrated in a rotary evaporator under reduced pressure at 40 °C and then the supernatant was lyophilized. In this manner, the GTE by UPE were prepared.

2.2.2. Microwave extraction (ME)

A microwave extractor (MSP-100D, Beijing Rayme Science & Technology Co., Ltd., Beijing, China) equipped with a pressure control and a second time-base design. Sample was weighed exactly (10 g), then placed in a 300 mL quartz extraction vessel equipped with reflux system. All the microwave extractions were performed under a set microwave irradiation power (1000 W) for a certain period of time (10 min) in 200 mL of 50% ethanol (Yuko et al., 1999). After extraction, the vessels were allowed to cool to room temperature before opening. The extracts were prepared as UPE procedure.

2.2.3. Ultrasonic extraction (UE)

Sample was weighed exactly (10 g) and mixed with 200 mL of the extraction 50% ethanol in a reagent bottle. The bottle was then closed and placed in the ultrasonic bath with temperature maintained at 60 °C for all extraction for 40 min (Park et al., 2008) using ultrasonicator (42 kHz, heat power 250 W, KQ-500DE, MTH, China). Afterwards, the extracts were prepared as UPE procedure.

2.2.4. Soxhlet extraction (SE)

Sample was weighed exactly (10 g) and placed in Soxhlet extractor and 200 mL of 50% (v/v) ethanol solution was added into flask. Then, refluxed 6 h in a water bath to allow solvent boiled continuously (Corrales et al., 2009). The extracts were prepared as UPE procedure.

2.2.5. Heat reflux extraction (HRE)

Sample was weighed exactly (10 g) in a three-neck flask, and 200 mL 50% ethanol extraction solvent was added. A stirring apparatus and a reflux condenser were fixed. Extraction was carried out at boiling state for 4 h (Shin et al., 2010). Then the extracts were prepared as UPE procedure.

2.3. Determination of antioxidant activities with the DPPH radical scavenging activities method

The radical-scavenging capacity of α-tocopherol, GTE by UPE and conventional extraction methods were determined using the DPPH radical method (Sheng et al., 2007). A 2 ml aliquot of each solution (50 μg/ml) was added to 2 ml of 2×10^{-4} mol/L ethanolic DPPH solution in a cuvette. The mixture was shaken vigorously and the absorbance was measured at 517 nm immediately. The decrease in absorbance was determined at 15 and 30 min until the absorbance reached a steady state (after nearly 30 min). The mixture with the addition of α-tocopherol served as a positive control. All the tests were performed in triplicate, and the inhibition rate was calculated according to the formula of Yen and Duh (1994).

2.4. Determination of antioxidant activities with ferric thiocyanate (FTC) method

The antioxidant activities of α-tocopherol, GTE by UPE and conventional extraction methods were assayed using a linoleic acid system. One millilitre of 50 μg/ml sample, 2 ml of 2.5% (w/v) linoleic acid in ethanol, 4 ml of 0.05 M phosphate buffer (pH 7.0), and 2 ml of distilled water were mixed in a tube of 10 ml with a screw cap. The oxidation was initiated by the addition of 0.4 ml of 0.1 M 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and then kept in a 37 °C water bath in the dark. The above mixture (0.1 ml) was added to 10 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. After 5 min, 0.1 ml of 0.02 M ferrous chloride in 3.5% (v/v) hydrochloric acid was added to the above mixture and then mixed. The absorbance of the mixture was recorded at 50 min, 100 min and 200 min at 500 nm. The degree of oxidation was measured according to the FTC method, described in detail by Kikuzaki and Nakatani (1993). Linoleic acid mixture without the addition of sample was used as the control and α-tocopherol at the same concentration served as the reference antioxidant.

2.5. Analysis of constituents of GTE

Contents of polyphenol, saccharide and amino acid of green tea extracts were determined according to the method of Yu et al.

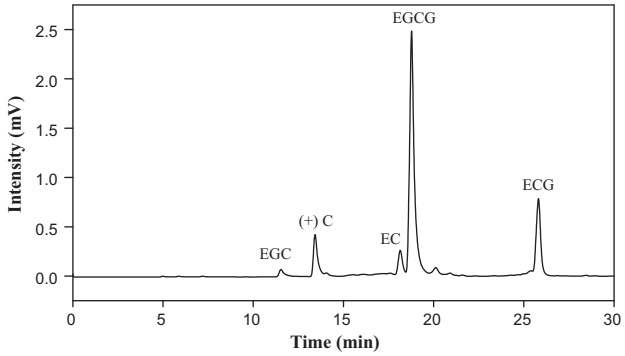

Fig. 1. The chromatogram of polyphenols components of GTE by UPE.

(2007) and Lowry et al. (1951). Polyphenol contents were measured by ferrous tartrate colorimetry. Saccharide contents were determined by phenol–sulfuric acid methods, using glucose as the standard. The amino acid content was analysed by the Lowry–Folin method.

2.6. Components analysis of polyphenol

The components of polyphenols were determined by high performance liquid chromatography. For the detection, pure EGC, (+)-C, EC, EGCG and ECG were used as standards. 0.25 g GTE was made to 10 ml with ultrapure water, filtered and detected. The detection wavelength was 280 nm, mobile phase was methanol (with 0.1% formic acid) and water (with 0.1% formic acid), injection volume was 20 μl, and flow speed was 1.0 ml/min. The five individual catechins were quantified in both extracts, by comparison with the generated standard curves. The retention time of ECG, (+)-C, EC, EGCG and EGC were 11.45 min, 13.30 min, 18.21 min, 18.76 min and 25.82 min, respectively. Fig. 1 shows the chromatogram of polyphenols components of GTE by UPE.

2.7. Statistical analysis

All computations were performed by SAS (version 8.0). The data were presented as means ± standard deviations of three replicates. Means for significant differences were compared using the Student's *t*-test. Multiple comparisons of means were done by LSD (least significant difference) test. A probability value of $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Antioxidant activities of α-tocopherol and GTE collected by UPE and conventional extraction methods assessed by DPPH radical-scavenging method

DPPH is a free radical compound and has been used widely to test the free radical-scavenging ability of various samples. Antioxidants react with DPPH, reducing a number of DPPH molecules equal to the number of available hydroxyl groups. Therefore, the absorption at 517 nm was proportional to the amount of residual DPPH. The results of the radical-scavenging capacity of α-tocopherol and GTE by UPE and conventional extraction methods are shown in Table 1. The higher the inhibition rate is, the greater the hydrogen-donating ability, thus the higher antioxidant activities. In this study, inhibition percentages were determined at both 15 min and 30 min. The α-tocopherol and GTE collected by UPE and conventional extraction methods exhibited high antiradical activities with all inhibition rates exceeding 60%. The GTE by UPE manifested the higher inhibition rates (80.4% at 15 min and 82.2% at 30 min) than other extraction technology, whereas GTE by ME and HRE showed the lowest inhibition (64.8%, 65.5% at 15 min and 66.6%, 67.5% at 30 min) at a concentration of 50 μg/ml. The antioxidant activities decreased following the same sequence: α-tocopherol > UPE > SE > UE > ME = HRE.

Table 1
Radical-scavenging capacity of α-tocopherol and GTE collected by UPE and conventional extraction methods assessed by DPPH method.

Samples	Inhibition rate[a] (%)	
	At 15 min	At 30 min
GTE by UPE	80.4 ± 0.32 a	82.2 ± 0.25 f
GTE by ME	64.8 ± 0.23 b	66.6 ± 0.14 g
GTE by UE	70.4 ± 0.03 c	71.6 ± 0.31 h
GTE by SE	74.8 ± 0.43 d	75.8 ± 0.22 i
GTE by HRE	65.5 ± 0.14 b	67.5 ± 0.35 g
α-tocopherol	84.3 ± 0.23 e	86.6 ± 0.06 j

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$; Student's *t*-test).

[a] Inhibition rate was examined at a concentration of 50 μg/ml.

Table 2
Components analysis of GTE collected by UPE and conventional extraction methods.

Samples	Constituents (mg/g)		
	Polyphenols	Amino acid	Saccharide
GTE by UPE	572 ± 4.02 a	78 ± 3.71 e	146 ± 2.34 i
GTE by ME	398 ± 6.42 b	49 ± 5.12 f	132 ± 4.48 j
GTE by UE	486 ± 5.42 c	136 ± 2.13 g	175 ± 4.67 k
GTE by SE	560 ± 7.03 d	240 ± 3.34 h	189 ± 7.12 l
GTE by HRE	394 ± 3.32 b	50 ± 4.34 f	129 ± 3.96 j

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$; Student's *t*-test).

3.2. Antioxidant activities of α-tocopherol and GTE collected by UPE and conventional extraction methods assessed by FTC method

The FTC assay was used to measure the amount of peroxide during the initial stages of lipid oxidation. A low absorbance is an indication of a low concentration of the formed peroxides and a high level of antioxidant activities. As shown in Fig. 2, α-tocopherol and GTE collected by UPE and conventional extraction

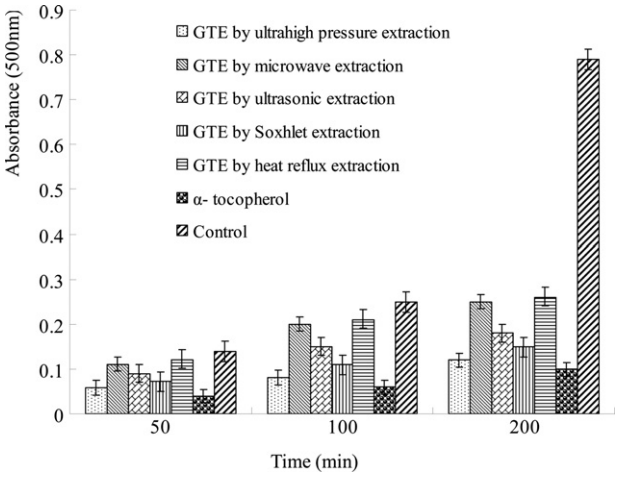

Fig. 2. Antioxidant activities of α-tocopherol and GTE collected by UPE and conventional extraction methods assessed by FTC method at 50 μg/ml. Bars are means ± standard deviations of triplicate measurement ($P < 0.05$; Student's *t*-test).

Table 3
Constituents analysis of polyphenols in GTE collected by UPE and conventional extraction methods.

Samples	Constituents (mg/g)				
	EGC	(+)-C	EC	EGCG	ECG
GTE by UPE	16.56 ± 0.06 a	54.8 ± 0.06 e	23.5 ± 0.23 i	296 ± 0.28 m	67.5 ± 0.23 q
GTE by ME	6.86 ± 0.057 b	23.6 ± 0.14 f	11.3 ± 0.14 j	172 ± 0.98 n	38.9 ± 0.34 r
GTE by UE	8.16 ± 0.034 c	32.8 ± 0.23 g	15.3 ± 0.02 k	210 ± 0.26 o	48.6 ± 0.25 s
GTE by SE	14.03 ± 0.09 d	49.6 ± 0.09 h	19.8 ± 0.21 l	284 ± 0.56 p	60.9 ± 0.53 t
GTE by HRE	6.67 ± 0.058 b	24.1 ± 0.05 f	10.8 ± 0.06 j	169 ± 1.45 n	39.3 ± 0.36 r

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$; Student's *t*-test).

methods delayed the oxidation of linoleic acid, on the basis of low absorbance values, and exhibited higher antioxidant activities than control. In particular, the GTE by UPE showed significantly stronger antioxidant activities than other extraction technology. The same patterns of activities were revealed as in the DPPH method: α-tocopherol > UPE > SE > UE > ME = HRE. Thus, the GTE by UPE had the higher inhibition activities of lipid oxidation. Altogether, these results suggested that the GTE by UPE possessed excellent antioxidant property.

3.3. Analysis of bioactive components of GTE collected by UPE and conventional extraction methods

Table 2 shows polyphenol, amino acid and saccharide contents of different GTE. The GTE collected by UPE showed the maximum polyphenol contents and the GTE obtained by ME and HRE showed the lowest value for polyphenol contents. Some impurities could not or had not been fully dissolved in ethanol solution at ambient temperature, but were largely dissolved at high temperature. Thus, the impurity contents of GTE collected by ME and HRE would increase, which would lead to a decrease of total polyphenol contents. Therefore, the polyphenol contents of GTE obtained by ME and HRE were low. The amino acid content in GTE collected by SE and UE was significantly higher than those by UPE, ME and HRE ($P < 0.05$). It is well-known that high temperature and high pressure would result in proteins denaturation. Gómez-Guillén et al. (2005) have indicated that high pressure at above 150 MPa could induce protein denaturation by disturbing the balance of non-covalent interactions within or between proteins. Therefore the minima of amino acid were in GTE prepared with UPE, ME and HRE. Moreover, the saccharide content in GTE collected by UPE, ME and HRE was significantly lower than by SE and UE, for the reason that high temperature and high pressure would induce various saccharides degradation reaction (Saito et al., 2008).

The results of the constituent analysis of polyphenols in GTE collected by UPE and conventional extraction methods are shown in Table 3. The contents of EGC, (+)-C, EC, EGCG and ECG in the GTE collected by UPE were significantly higher than those by SE, ME, UE and HRE ($P < 0.05$). The maxima of catechins appeared in GTE prepared with UPE, while the minima of catechins were prepared in GTE with ME and HRE.

The antioxidant activities shown in Table 1 and Fig. 2 seemed to correlate with total polyphenol (especially catechins content) of the GTE (Tables 2 and 3). Positive correlations were found between total polyphenol content in the GTE and their antioxidant activities. Rusak et al. (2008) investigated the antioxidant activities of green tea samples by different solvents extraction and reported that the correlation between total polyphenol content and antioxidant activities was significant.

The DPPH radical-scavenging assay and the FTC method were performed to determine the abilities of the extracts to inhibit oxidation (Table 1 and Fig. 2). The GTE by UPE exhibited the strongest antioxidant activities. The antioxidant activities of extracts by UPE and conventional extraction methods are in accordance with the amount of polyphenol compounds. Tea polyphenol is the leading functional component and also the important parameter of tea quality. It is mainly composed of catechins with a proportion up to 70–80%. The polyphenol compounds exhibit extensive free radical scavenging activities through their reactivities as hydrogen or electron-donating agents, and metal ion chelating properties (Rice-Evans et al., 1997). Green tea leaves contain a high amount of polar compounds like catechines, flavanols, flavanones, phenolic acids, glycosides and the aglycons which are good antioxidant compounds (Tanaka et al., 1998).

Ultrahigh pressure can cause deprotonation of charged groups and disruption of salt bridges and hydrophobic bonds, resulting in conformational changes and denaturation of protein (US FDA, 2000), which lead to the cellular wall, membrane and organelles to be collapsed, and enhanced the mass transfer of the solvents into the leaves materials and the soluble constituents into the solvents. Butz et al. (1994) reported that pressures of 100 MPa were enough to cause rupture of intracellular vacuoles and plant cell walls in onions. During this rupture process, the chemical substances within the cell are rapidly released into the surrounding extraction solvents. Thereby the compounds are more accessible to extraction up to equilibrium (Barbosa-Canovas et al., 1998). In addition, ultrahigh pressure processing can cause enhancement of chemical and biochemical reactions in the cells by both desired and undesired modification (Oey et al., 2008). Moreover, ultrahigh pressure treatment provides the possibility of inactivating degrading enzymes which may account for higher extraction yield and antioxidant activities compared to other methods.

4. Conclusion

In this study, in vitro antioxidant activities and bioactive components of GTE by UPE and conventional extraction methods have been evaluated. The results indicated that GTE by UPE exhibited strongest antioxidant activities. The contents of polyphenols and catechins in the GTE by UPE were significantly higher than those by other extraction methods, which were possibly responsible for higher antioxidant activities of GTE by UPE. From the results we can draw the conclusion that not only the more bioactive components are obtained but also the extract has better free radical and reactive oxygen species scavenging activities through UPE method. These findings further illustrate that UPE has a bright prospect for extracting active ingredients from plant materials.

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