Antioxidative Activity of Essential Oil of Flowers of Tea (*Camellia sinensis L.*) Plants Extracted by Supercritical Carbon Dioxide

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Citation: Xia C, Tu Y, Yang Z, Jin Y, Xia H (2018) Antioxidative Activity of Essential Oil of Flowers of Tea (*Camellia sinensis L.*) Plants Extracted by Supercritical Carbon Dioxide. J Biomedical Sci Vol.7 No.3: 10.

Abstract

Background: Due to abundant and non-utilized resource of tea flowers (*Camellia sinensis*) world widely, many volatile compounds in tea flowers have been characterized. It is of interest to test the bioactivity of the natural essential oil of tea flowers.

Methods: Natural essential oil of tea flowers was prepared by using Supercritical Carbon dioxide Extraction (SFE). DPPH radical scavenging was used to evaluate the antioxidative activity of essential oil. All experiments were performed in triplicate and all data was expressed as a mean ± standard error of the mean. Comparisons among the means of various treatments were analyzed through Duncan's test. One Way Analysis Of Variance (ANOVA) was used to determine the differences among means of treatments and the control.

Results: The optimum extraction conditions were the pressure of 30 MPa, temperature of 50℃, static time of 10 min, and dynamic time of 90 min. Based on GC-MS analysis, 59 compounds including alkanes (45.4%), esters (10.5%), ketones (7.1%), aldehydes (3.7%), terpenes (3.7%), acids (2.1%), alcohols (1.6%), ethers (1.3%), and others (10.3%) were identified in the essential oil of tea flowers. The essential oil of tea flowers showed relatively stronger DPPH radical scavenging activity than essential oils of geranium and peppermint, although it was weaker than those of essential oil of clove, ascorbic acid, tertiary butylhydroquinone, and butylated hydroxyanisole.

Conclusions: Essential oil of tea flowers extracted by SFE contained many kinds of volatile compounds and showed considerable DPPH scavenging activity.

Keywords Antioxidative activity; Essential oil; Supercritical carbon dioxide extraction; Tea flower; Volatile

Abbreviations

ANOVA: One Way Analysis of Variance, BHA: Butylated Hydroxyanisole, DPPH: Di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium, GC-MS: Gas Chromatography-Mass Spectrometry, SC_{50}: 50% Scavenging Concentration, SDE: Simultaneous Distillation Extraction, SFE: Supercritical Carbon Dioxide Extraction, TBHQ: Tertiary Butylhydroquinone.

Introduction

Tea, made from leaves (*Camellia sinensis*), is one of the three most consumed beverages in the world [1]. There are a number of reports concerning human health benefits of tea leaves and their bioactive compounds, such as anti-bacterial, anti-viral, anti-histaminic, immune function, protective effects against many chronic diseases, and inflammation [2,3]. Recently, flowers of tea (*Camellia sinensis*) plants have also attracted increasing interest of researchers. This is due to that there are abundant and non-utilized resource of tea flowers, for example over 1.8 billion kilograms of tea flowers are available annually in China [4]. Moreover, many bioactive compounds including catechins, caffeine [5,6], flavonol glycosides [7], polysaccharides [8], amino acids [9], floratheasaponins [10-12], and spermidine derivatives [13] were identified in tea flowers. Besides these non-volatile metabolites, volatile compounds including fatty acid derived volatiles, volatile phenylpropanoids/ benzenoids, and volatile terpenes in tea flowers have been characterized [14-17]. However, the bioactivity of the essential oil of tea flowers has been rarely studied and reported so far. Occurrences of these volatile compounds in tea flowers provide basis for future application of essential oil of tea flowers in food flavor and cosmetic industries. Therefore, it is important to establish an

approach to prepare natural essential oil of tea flowers without organic solvent residue.

The essential oil from plants was generally prepared through Simultaneous Distillation Extraction (SDE). SDE is an effective approach to prepare volatile concentrates, but thermal decompositions of volatile compounds, for example, hydrolysis of some glycosidically bound volatile compounds, may easily occur during the extraction process due to the comparatively high temperature of extraction [18,19]. In addition, residue of chemical solvents in the final essential oil product prepared by SDE is unavoidable. Therefore, Supercritical Fluid Extraction (SFE), a technique without use of organic solvents, has been developed and applied to extraction of natural products from vegetable matrices [20]. The non-toxic and volatile fluids, such as carbon dioxide, are used in SFE during the acquirement of several types of substances, so there is no doubt that it will protect extracts against thermal degradation and solvent toxicity [20]. Carbon dioxide is relatively an inert, non-flammable, non-explosive, inexpensive, odorless, and safe solvent and is easily removed from the extracts [21]. In addition, carbon dioxide has a low surface tension and viscosity but high diffusivity which is one or two orders of magnitude higher than other fluid. The critical condition (31.8℃ and 73 atm) of the carbon dioxide makes it attractive for the extraction of heat-sensitive compounds [22].

The aim of this study was to apply SFE to extract natural volatile compounds from tea flowers without thermal degradation and residue of organic solvents. The important parameters such as pressure, temperature, static and dynamic time affecting the SFE of essential oil of tea flowers were optimized. In addition, the Di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) radical scavenging activity of the essential oil of tea flowers was evaluated.

Methods

Samples and reagents

The dried tea flowers (moisture content of 100 g kg^{-1}, *Camellia sinensis* cv Longjing 43) were provided by Kaihua Jinmao Tea Plantation (Zhejiang, China). Carbon dioxide (99%, purity) was purchased from Zhejiang gas Co., Ltd. (Zhejiang, China). Tertiary Butylhydroquinone (TBHQ), Butylated Hydroxyanisole (BHA), ethyl ether, and ethanol were analytically pure and supplied by Huadong Medicine Hangzhou Co., Ltd. (Zhejiang, China). DPPH and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other plant essential oils including geranium, peppermint, and clove were obtained from Hangzhou Liyuan Fragrance Technology Co., Ltd. (Zhejiang, China).

Sample preparation

The samples were stored at room temperature (about 27℃) under the dark condition. Prior to SFE, the samples were weighed with electronic balance (Mettler Toledo Co. Ltd., Shanghai,China) and milled in a grinder (IKA-Werke GmbH & Co.

KG) to produce powder which was passed through a 20-mesh sieve. The particle size was 0.8 mm.

SFE

A supercritical fluid extractor Spe-edTM SFE2 (Applied Separation, USA) was used for all the extractions. The extraction vessel was a 50 mL thick-walled stainless cylindrical steel vessel (diameter: 2 cm, length: 33 cm). The prepared tea flower powder (15.0 g) was embedded into the extractor vessel with defatted cotton in the both ends. Then the extractions were carried out using different pressure, temperature, static and dynamic time as shown in **Table 1**. The flow rate of carbon dioxide was 2 L/min and the temperature of outvalve was 70℃. The extracts were collected in a volumetric flask at the ambient temperature and atmospheric pressure and their weights were measured. Finally, the extraction yield was calculated. The final volume of the extracts was diluted to 5 mL with ethyl ether and subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis. On the basis of the single-factor experiment (**Table 1**), a four-factors (pressure, temperature, static extract time, and dynamic extract time) at three-level orthogonal array design was used to examine the effects of parameters on the extraction yield (**Table 2**). There were 9 runs in the design and all the experiments were conducted in triplicate.

Table 1: Factors and levels of the single factor experiment.

Levels	Pressure (MPa)	Temperature (℃)	Static time (min)	Dynamic time (min)
1	10	40	10	50
2	15	45	20	60
3	20	50	30	70
4	25	55	40	80
5	30	60	50	90

Table 2: Orthogonal factors for SFE of essential oil of tea flowers.

Levels	Factors			
	Pressure (MPa)	Temperature (℃)	Static time (min)	Dynamic time (min)
1	10	40	10	50
2	20	50	30	70
3	30	60	50	90

GC-MS analysis

GC-MS analysis of the essential oil was carried out using an Agilent HP-6890 GC (Agilent Technologies Co. Ltd., USA) equipped with a HP-5 quartz capillary column (30 m × 0.25 mm, 0.25 μm film thickness; Restek, Bellefonte, PA) and an Agilent HP-5973 (N) mass selective detector in the electronic impact mode (Ionization energy: 70 eV), as described by Cuevas-Glory, et al (2012) with a modification [23]. Oven temperature was programmed to 50℃ for 5 min and increased to 230℃ at a rate of 3℃/min. Injector and the detector temperatures were set as

260 and 280℃, respectively. The carrier gas, helium, was adjusted to a linear velocity of 1 mL/min. 1 µL of sample was injected into GC using a splitless mode.

Determination of DPPH radical scavenging activity

The DPPH radical scavenging activities of the essential oil of tea flowers was evaluated using the method reported by Lee and Yen (2006) [24]. The DPPH radical was dissolved in ethanol with the final concentration of 0.1 mM. Firstly, the absorbance of the disposable cuvette with 2.7 mL DPPH radical solution and 0.3 mL ethanol was measured as negative control. Sample solution in ethanol (0.3 mL) with different concentrations was pipetted in a cuvette with 2.7 mL DPPH solution. Then the resultant solution was incubated for 30 min at 27℃ in the dark and then monitored at 517 nm. The DPPH scavenging activity was expressed as following equation: $S(\%) = ((A_{C(0)} - A_{A(t)})/A_{C(0)}) \times 100\%$. Where $A_{C(0)}$ is the absorbance of the negative control reaction, and $A_{A(t)}$ is the absorbance at 517 nm containing 0.3 mL sample and 2.7 mL DPPH solution. All samples were analyzed in triplicate. In this study, scavenging activity of the sample was expressed as a 50% scavenging concentration (SC_{50}), which represented the sample concentration (mg/ mL) scavenging 50% of the DPPH radical activity.

Statistical analysis

All experiments were performed in triplicate and all data was expressed as the mean ± standard error of the mean (S.E). Comparisons among the means of various treatments were analyzed through Duncan's test. One way Analysis Of Variance (ANOVA) was used to determine the differences among means of treatments and the control. A probability level of 5% ($p \leq 0.05$) was considered as significant level. The data were processed by using the SPSS statistical package (version 16.0). Relationship between the essential oil content and antioxidative activity was evaluated through correlation and regression by using the Microsoft Office Excel programme.

Results

SFE showed high extraction yield of the essential oil with optimized conditions

Figure 1 shows the effect of temperature, static and dynamic time on the extraction yield. The extraction yield of the essential oil increased with the pressure increasing from 10 Mpa to 20 Mpa (**Figure 1a**), the temperature increasing from 40℃ to 45℃

(**Figure 1b**), the static time increasing from 10 min to 30 min (**Figure 1c**), and the dynamic time from 50 min to 70 min (**Figure 1d**), respectively. It tended to decline when the pressure increased to over 20 Mpa, the temperature over 45℃, the static time over 30 min, and the dynamic time over 70 min, respectively.

The result of the four-factors (pressure, temperature, static extract time, and dynamic extract time) at three-level orthogonal array design indicated that the conditions to acquire the highest yield of the essential oil extracted with SFE was at 30 MPa of pressure, 50℃ of temperature, 10 min of static time, and 90 min of dynamic time (**Table 3**). The order of primary and secondary factors affecting the extraction yield were the pressure, the static time, the dynamic time, and the temperature (**Table 3**). The ANOVA for the orthogonal array revealed that each selected parameter played an important role in the SFE of the essential oil of tea flowers and the influence was appeared to be significant ($p < 0.05$) for all the analytes (**Table 4**). The F value indicated that the importance of the parameters influencing the extract yield was in order as pressure > static time > dynamic time>temperature (**Table 4**), which was in accordance with the results of orthogonal design (**Table 3**).
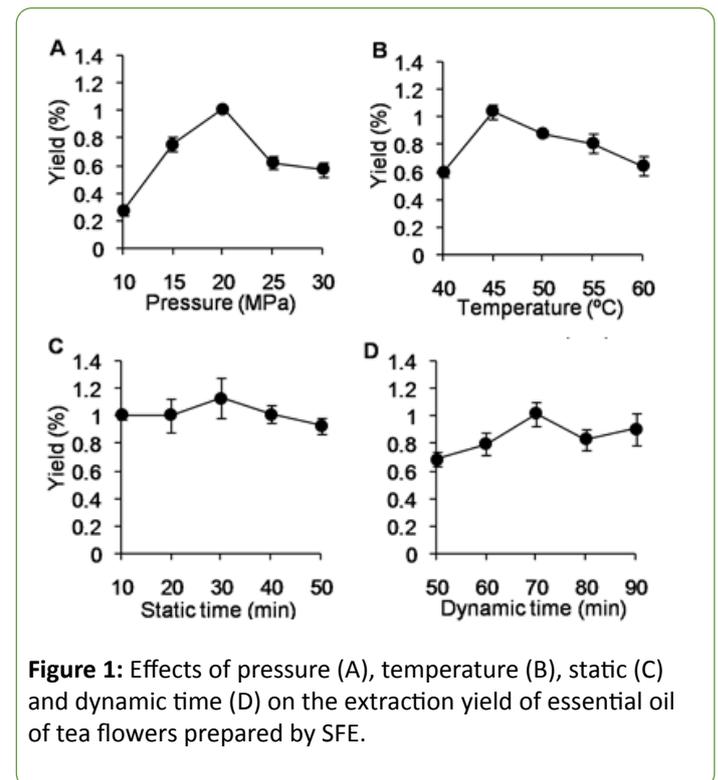
Figure 1: Effects of pressure (A), temperature (B), static (C) and dynamic time (D) on the extraction yield of essential oil of tea flowers prepared by SFE.

Table 3: Orthogonal design for different conditions of SFE and their extraction yields.

No.	Pressure (MPa) (A)	Temperature (℃) (B)	Static time (min) (C)	Dynamic time (min) (D)	Yield (%)
1	1(10)	1(40)	1(10)	1(50)	0.742 ± 0.002
2	1(10)	2(50)	2(30)	2(70)	0.350 ± 0.018
3	1(10)	3(60)	3(50)	3(90)	0.524 ± 0.001

4	2(20)	1(40)	2(30)	3(90)	0.867 ± 0.017
5	2(20)	2(50)	3(50)	1(50)	0.543 ± 0.003
6	2(20)	3(60)	1(10)	2(70)	0.793± 0.019
7	3(30)	1(40)	3(50)	2(70)	0.732 ± 0.004
8	3(30)	2(50)	1(10)	3(90)	0.976 ± 0.030
9	3(30)	3(60)	2(30)	1(50)	0.755 ± 0.024
K1	0.538	0.78	0.837	0.68	
K2	0.734	0.623	0.657	0.625	
K3	0.821	0.69	0.6	0.789	
k1	0.179	0.26	0.279	0.227	
k2	0.245	0.208	0.219	0.208	
k3	0.274	0.23	0.2	0.263	
R	0.282	0.157	0.237	0.164	
Primary and secondary factors	RA > RC > RD > RB				
Optimum conditions	$A_3B_2C_1D_3$				

Table 4: AVONA of SFE of the essential oil of tea flowers.

| Source	Type III Sum of Squares	Degree of freedom	Mean Square	F value	Sig.
Corrected Model	0.89	8	0.11	137.24	**
Intercept	13.15	1	13.15	16238.62	**
Pressure	0.38	2	0.19	232.58	**
Temperature	0.11	2	0.06	69.22	**
Static time	0.27	2	0.14	169.82	**
dynamic time	0.13	2	0.06	77.36	**
Error	0.01	18	0		
Total	14.05	27			
Corrected Total	0.9	26			
R Squared=0.984 (Adjusted R Squared=0.977). $^*p<0.05$, $^{**}p<0.01$					

## SFE of tea flowers had many kinds of volatiles and considerable DPPH scavenging activity

The 59 kinds of compounds identified by GC-MS contributed to 86.6% of the essential oil of tea flowers prepared by SFE (**Figure 2**). The main constituents were nonadecane (18.7%), heneicosane (12.2%), dibutyl phthalate (5.0%), tricosane (4.9%), 2-propenoic acid, 2-methyl-, 1, 1'-(1, 2-ethanediyl) ester (3.1%), 6, 10, 14-trimethyl-2-pentadecanone (3.0%), caffeine (1.7%), and phytol (1.6%) (**Table 5**). The identified compounds included 7 families of compounds including alkanes (45.4%), esters (10.5%), ketones (7.1%), aldehydes (3.7%), terpenes (3.7%), acids (2.1%), and ethers (1.3%) (**Figure 3**).

In view of potential application of the essential oil of tea flowers in food flavor and cosmetic industries, we evaluated its antioxidative activity using the DPPH radical system and also determined DPPH radical scavenging activities of essential oils of geranium, peppermint, clove, ascorbic acid, TBHQ, and BHA (**Table 6**). DPPH radical scavenging activity of the essential oil of tea flowers was assessed to be in a dose-dependent manner. Based on the SC_{50} value, the essential oil of tea flowers showed relatively stronger DPPH radical scavenging activity than essential oils of geranium and peppermint, and weaker than that of essential oil of clove, ascorbic acid, TBHQ, and BHA (**Table 6**).
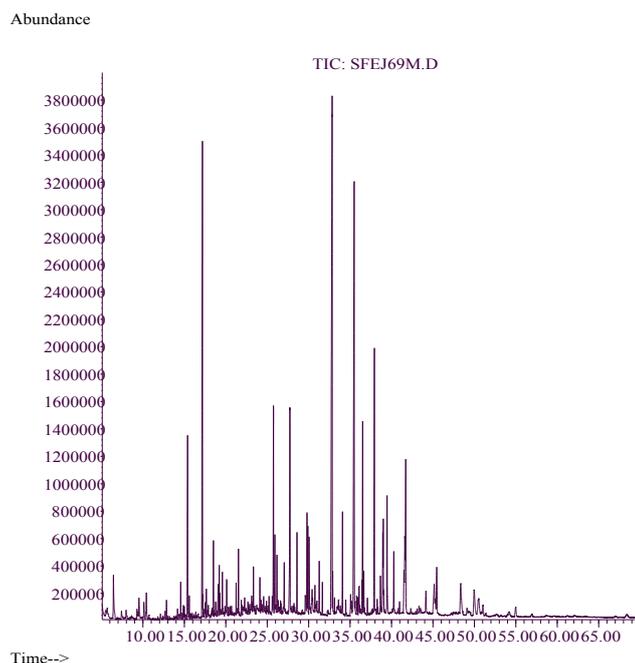
Figure 2: Total ion current chromatogram(TIC) of the essential oil from tea flower.
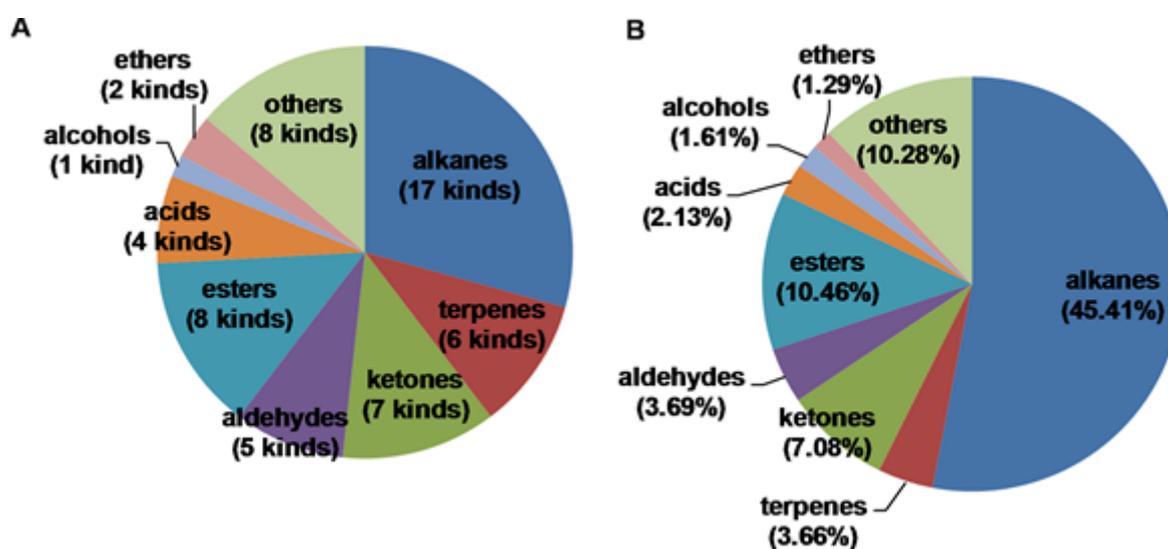
Figure 3: Categories (A) and relative proportions (% total peak areas) (B) of identified volatile compounds in the essential oil of tea flowers prepared by SFE.
(A) Total categories of the identified volatile compounds were 59 kinds. (B) Total relative proportions of the identified volatile compounds were 86.61%.

Table 5: Identified components of the essential oil of tea flowers prepared by SFE
[a] RT, retention time. [b] RP, relative proportions (% total peak areas). [c] Compounds listed in order of elution from HP-5 quartz capillary column.

No.	RT[a] (min)	RP[b] (%)	Compounds[c]	CAS#
1	6.44	1.34	1S-.alpha.-Pinene	7785-26-4

2	9.52	0.58	Acetophenone	98-86-2
3	10.11	0.41	2-Nonanone	821-55-6
4	10.41	0.66	Nonanal	124-19-6
5	12.84	0.29	Decanal	112-31-2
6	14.55	0.53	Heptadecane, 8-methyl-	13287-23-5
7	15.39	3.07	2-Propenoic acid, 2-methyl-, 1, 1'-(1, 2-ethanediyl) ester	97-90-5
8	15.59	0.29	Decane, 2,3,6-trimethyl-	62238-12-4
9	17.22	0.23	Tetradecane	629-59-4
10	17.65	0.47	1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-	469-61-4
11	18.51	0.98	Hexadecane	544-76-3
12	18.77	0.25	3H-Benz[e]indene, 2-methyl-	1000164-77-1
13	19.08	0.82	8-Quinolinol, 4-methyl-	3846-73-9
14	19.21	0.66	Octadecane	593-45-3
15	19.27	0.29	Pentadecane	629-62-9
16	19.58	0.62	Phenol, 2,4-bis(1,1-dimethylethly)	96-76-4
17	21.24	0.39	Diethyl Phthalate	84-66-2
18	21.53	0.96	Cedryl propyl ether	1000131-90-6
19	23.12	0.32	Heptadecane	629-78-7
20	23.34	0.05	Eicosane	112-95-8
21	24.11	0.49	Hexacosane	630-01-3
22	24.57	0.47	5-Ethylcyclopent-1-enecarboxaldehyde	36258-07-8
23	25.22	0.2	Tetradecanal	124-25-4
24	25.75	2.99	2-Pentadecanone, 6,10,14-trimethyl	502-69-2
25	25.92	1.68	Caffeine	58-08-2
26	26.17	0.75	1,2-Benzenedicarboxylic acid	84-69-5
27	27.05	0.98	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1000143-92-4
28	27.73	4.97	Dibutyl phthalate	84-74-2
29	28.59	1.07	Octadecanal	638-66-4
30	29.59	0.59	1-Hexadecene	629-73-2
31	29.91	1.13	2-Nonadecanone	629-66-3
32	30.05	1.61	Phytol	150-86-7
33	30.41	0.39	9-Octadecenoic acid, (E)-	112-79-8
34	30.76	0.39	Octadecanoic acid	57-11-4
35	31.27	0.71	Hexadecane, 2,6,10,14-tetramethyl	638-36-8
36	31.66	0.42	Bicyclo[10.8.0]eicosane, (Z)-	1000155-82-2
37	32.83	18.67	Nonadecane	629-92-5
38	32.9	0.52	2-Undecanone, 6,10-dimethyl-	1604-34-8
39	33.12	0.22	Eicosanoic acid, methyl ester	1120-28-1
40	34.09	1.46	Tetracosane	646-31-1

41	34.48	0.19	1,19-Eicosadiene	14811-95-1
42	35.06	0.35	9-Tricosene, (Z)-	27519-02-4
43	35.49	12.2	Heneicosane	629-94-7
44	35.8	0.28	Octadecanoic acid, 10-methyl	2490-19-9
45	35	0.34	Benzene, 1-fluoro-2-methoxy-	321-28-8
46	36.08	0.46	Bis(2-ethylhexyl) phthalate	117-81-7
47	36.43	0.45	Octanoic acid, phenylmethyl ester	10276-85-4
48	37.93	4.91	Tricosane	638-67-5
49	38.27	0.15	Heneicosanoic acid, methyl ester	6064-90-0
50	38.66	0.73	Benzene, (1,3-dimethyl-3-butenyl)-	56851-51-5
51	39.46	2.11	2,6,10,14,18-Pentamethyleicosane,Eicosane, 2,6,10,14,18-pentamethyl-,	75581-03-2
52	40.28	1.08	Docosane	629-97-0
53	41.5	1.07	4-Pentenoic acid, 2-acetyl-, ethyl ester	610-89-9
54	41.6	2.02	9-Thiabicyclo[3.3.1]non-7-en-2-ol	1000189-39-0
55	44.15	0.56	Benzenamine, 3-methoxy-2,4,6-trifluoro	34874-88-9
56	45.16	1.37	Silane, diazidodimethyl-	4774-73-6
57	48.34	1.76	Temazepam	846-50-4
58	49.97	1.47	2-(4-Fluorophenyl)-1H-indole-5-carbaldehyde	1000163-57-9
59	50.51	1.16	2-(1-Hydroxynaphthyl-2)quinolin	24641-28-9
Total		86.61		

Table 6: DPPH radicals scavenging activity of the essential oils and compounds
Values are means of three determinations.

Sample	Regression equation	R^2	SC_{50} (mg/mL)	Linear range(mg/mL)
Tea flowers	y=1.354x +32.10	0.996	13.22	10~40
Geraniums	y=0.239x +17.21	0.967	137.2	10~200
Peppermints	y=0.33x+24.78	0.986	76.42	400~800
Cloves	y=0.982x +4.333	0.967	0.05	0.020~0.100
TBHQ	y=3.22x +6.06	0.967	0.01	0.005~0.030
BHA	y=0.151x +5.131	0.965	0.3	0.050~0.500
Ascorbic acid	y=1.698x +7.75	0.999	0.02	0.005~0.025

Discussion

The extraction yield increased with the pressure increasing from 10 Mpa to 20 Mpa and declined afterwards. This is due to that the increasing of carbon dioxide density reduces the distance between molecules, which means the analyte solubility of flower would be enhanced in carbon dioxide and the SFE system [25,26]. When the pressure continuously increased over 20 Mpa, the extraction yield decreased. This may result from the decreasing of diffusivity making it difficult for molecules to diffuse into the solid pores to dissolve the solute and the reduction of the free space owing to the condensation of the solid matrix [27]. Therefore, excess pressure was not recommended because of its poor effect on the yield and the presence of unexpected complex extracts [28].

The extraction yield increased with the temperature increasing from 40℃ to 45℃, which most likely attributed to the strengthened mass transfer speed and the increase in vapor pressure of fluid bringing about the solubility of the essential oils in the solvent [29]. When the temperature was over 45℃, the extraction yield decreased, which might be due to the decrease of the carbon dioxide density and the increase of the solute vapor pressure. On the other hand, for the volatile solute, there is a competition between its solubility in carbon dioxide and its volatility [30].

Static time did not significantly influence the extraction yield, and relatively longer static time would lead to a little stoppage in the vessel thereby decrease of the extraction yield (**Figure 1**). The dynamic time is considered as another critical factor influencing the extraction yield. The maximum yield was obtained at the 70 min of dynamic time but even longer dynamic time did not significantly increase the extraction yield.

In our previous experiments, the extraction yield of essential oil of tea flowers prepared by SDE was less than 0.1%, and alcohols such as acetophenone, 1-phenylethanol, and 2-pentanol were the major components [19]. The extraction yield was 1.21% under the optimized condition in this test, indicating that SFE had much higher extraction yield of the essential oil and obtained more kinds of components in the essential oil. This implies that decomposition of some unstable volatile compounds may occur during the SDE process. In fact, decomposition and volatilization may take place during the drying process of fresh tea flowers, the extraction yield and component identification of the essential oil of fresh tea flowers extracted with SFE should be carried out in fure.

The DPPH radical is a stable free radical widely used in the determination of the antioxidative activity of different plant extracts as well as single compounds [31]. It is well-known that polyphenols, especially catechins, mostly contribute to the antioxidative activities of extracts of tea leaves, and volatile compounds can in part contribute to the antioxidative activities of tea leaves [32]. In this study, the essential oil of tea flowers prepared by SFE contained diversity of volatile compounds and showed higher DPPH scavenging activity than those essential oils of geranium and peppermint, implying that it would be of interest to further test the bioactivity and identify the detailed volatile compound of the essential oils of tea flowers due to the large amout of the unutilized tea flowers.

Conclusions

Under the optimized extraction condition (pressure of 30 MPa, temperature of 50℃, static time of 10 min, and dynamic time of 90 min). the extraction yield of the essential oil of tea flowers obtained with SFE was 1.21%, much higher than that of with SDE reported in our previous studies. Based on GC-MS analysis, up to 59 compounds including alkanes (45.4%), esters (10.5%), ketones (7.1%), aldehydes (3.7%), terpenes (3.7%), acids (2.1%), alcohols (1.6%), ethers (1.3%), and others (10.3%) were identified in the essential oil of tea flowers. The essential oil of tea flowers showed relatively stronger DPPH radical scavenging activity than those of geranium and peppermint, although it was weaker than those of essential oil of clove, ascorbic acid, tertiary butylhydroquinone, and butylated hydroxyanisole in this study. Which implyed that it would be of interest to further test the bioactivity and identify the detailed volatile compound of the essential oils of tea flowers due to the large amout of the unutilized tea flowers.

Studies and reports on the essential oil of tea flowers are rare, and the research results of this test might provide essential information for application of tea flowers in health-care food and food flavor industries.

Authors' Contributions

CX performed most of experiments and statistical analysis and wrote part of the manuscript. YT supervised the work and wrote part of the manuscript. ZY supervised part of the work. YJ performed part of experiments. HX wrote most of the manuscript and supervised part of the work. All authors read and approved the final manuscript.

Funding

Competing and Conflicting Interests

The authors declare that they have no competing interests.

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