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Original Article

Characterization and determination of antioxidant components in the leaves of *Camellia chrysantha* (Hu) Tuyama based on composition–activity relationship approach

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ABSTRACT

Camellia chrysantha (Hu) Tuyama (CCT), an ornamental plant possessing antioxidant activity, has been infused as tea and drank for its health benefits. The antioxidant components in CCT, however, had not been clearly characterized. To quickly identify the antioxidant constituents of CCT, a composition–activity relationship strategy based on ultra high-pressure liquid chromatography coupled with linear ion trap hybrid orbitrap mass spectrometry and orthogonal partial least-squares method has been applied. As a result, 16 variables were found to make significant contributions to the 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity. Six of them were identified as catechin (**1**), epicatechin (**5**), vitexin (**8**), isovitexin (**10**), quercetin-7-*O*-β-D-glucopyranoside (**12**) and kaempferol (**16**). The strength of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity was found to be **12** > **1** > **5** > **16** > **8** > **10** by validation test. Meanwhile, a liquid chromatography-electrospray ionization-mass spectrometry method was established for quantitative determination of six marker compounds in CCT samples from different preparations. The validation of the method, including linearity, sensitivity (limitation of detection and limitation of quantification), repeatability, precision, stability, and recoveries, was carried out and demonstrated to meet the requirements of quantitative analysis. This is the first report on the comprehensive characterization and determination of chemical constituents in CCT by ultra high-pressure liquid chromatography coupled with linear ion trap hybrid orbitrap mass spectrometry. The results indicate

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that the composition–activity relationship approach may be a useful method for the discovery of active constituents in natural plants and the quality control of medicinal herbs.

1. Introduction

Camellia is the largest and economically most important genus in the family Theaceae. *Camellia chrysantha* (Hu) Tuyama (CCT), from a group of yellow-flowering species called golden camellias native to the south of China, is grown as an ornamental plant worldwide [1]. In south China, CCT are usually used by local people to make tea for its beneficial properties. Research has demonstrated that the extracts from the leaves of CCT have antioxidant activities in 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, superoxide anions, and hydroxyl free radicals scavenging assays [2]. A large body of evidence suggests important roles of oxygen free radicals in the expansion of tumor clones, acquisition of malignant properties [3], and development of hyperlipidemia [4]. Discerning the antioxidant constituents capable of scavenging free radicals may help reveal the active chemical basis of CCT. The chemical constituents related to the antioxidant effect of CCT are still unknown, however, and the complexity of the many constituents in herbal materials make identification of the chemical constituents related to bioactivity challenging.

Presently, bioassay-guided fractionation is one of the most commonly applied approaches to discover biologically-active compounds in natural plants [5,6]. This approach, however, takes a long time to produce a final result. Nowadays, increasing numbers of researchers are realizing that the benefits of natural plants used in medical care are generally a result of the synergistic effects of multiple compounds [7,8]. Inevitably, bioassay-guided fractionation ignores these synergistic effects, and thus may lead to incorrect conclusions. Novel approaches are in great demand to provide a deeper insight into bioactive constituents in herbal medicines more rapidly and accurately.

An approach using integral bioactivity tests based on the composition–activity relationship combined with chemometrics is increasingly being used in Traditional Chinese Medicines (TCMs) research to find the bioactive components [9–13]. The chemical constituents are identified by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) [10,11] or gas chromatography–mass spectrometry (GC-MS) [12,13] methods and the correlation between components and bioactivity is explored to identify significantly active constituents using orthogonal partial least squares (OPLS) [10], support vector regression [11], and a generalized regression neural network [12]. This approach can correlate the chemical constituents of TCMs with their bioactivity and consider the synergistic effects of the multiple components in herbal medicine. In a sense, it can overcome the shortcomings of the bioassay-guided fractionation approach. In the process of chemical constituent characterization, however, HPLC-MS/MS or GC-MS analysis without high-resolution MS made further precise elucidating of some constituents difficult. A quantitative method of active components screening was necessary for the quality control of medicinal herbs.

In this study, the composition–activity relationship was utilized to find the antioxidant components in CCT. The chemical profiles of different preparations of CCT were characterized using ultra high pressure liquid chromatography (UHPLC) coupled with linear ion trap hybrid orbitrap MS, which could perform a rapid separation taking advantage of the efficacy of UHPLC and provide accurate structural information about the constituents by means of the high-resolution of orbitrap MS. Meanwhile, a DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging test that is stable, commercially available, and does not have to be generated prior to the assay and which is utilized in almost 90% of antioxidant studies [14,15], was used to investigate the scavenging rate of free radicals. The correlation between chemical profiles and antioxidant activity was explored to identify significantly active constituents using OPLS and canonical correlation analysis. An analytical method of simultaneous determination of multi-active components would be established. This survey provides new insights into the rapid investigation of active constituents from natural plants and the bioactive basis of quality control.

2. Materials and methods

2.1. Solvents and chemicals

HPLC-grade acetonitrile and methanol from Fisher (Fair Lawn, NJ, USA) were used for chromatography. Analytical-grade ethanol was purchased from Beijing Reagent Company (Beijing, China). Water was purified by Milli-Q Academic Water Purification System (Millipore, France). Catechin, epicatechin, vitexin, isovitexin, quercetin-7-*O*-β-D-glucopyranoside and kaempferol were purchased from Chengdu MUST Biotechnology Co Ltd (Chengdu, Sichuan, China). Their purities were all >98%. DPPH was purchased from Sigma-Aldrich (Shanghai, China). CCT was purchased from Guangxi Guiren Tang Co Ltd (Fangchenggang, China) and identified as the leaves of CCT by Associate Professor Quanfang Hang of the First Affiliated Hospital of Guangxi University of Chinese Medicine (Nanning, China). The voucher specimens have been deposited in our laboratory of Guangxi Medical University (Nanning, China).

2.2. Standard solutions and preparations

Stock solutions of six reference substances (catechin, epicatechin, vitexin, isovitexin, quercetin-7-*O*-β-D-glucopyranoside, and kaempferol) were prepared in concentrations ranging from 1.82 mg/mL to 3.13 mg/mL in methanol and stored at 4°C until use. A standard working solution was obtained by diluting stock solutions to the desired concentrations. Aliquots

of this solution were further diluted with an initial mobile phase to a series of concentrations for quantification. The concentrations of six reference substances were processed in ranges from 0.0182 μg/mL to 62.6 μg/mL with repeatable calibration curves plotted from 0.182 μg/mL to 5.105 μg/mL, and a precision of between 0.091 μg/mL and 62.6 μg/mL (intra and inter day) with recoveries from 0.182 μg/mL to 51.05 μg/mL.

CCT extract was prepared based on orthogonal design. Ten grams of mixed crude herb was crushed into small pieces and placed in water (the extraction solvent) for various soaking (0 hours, 0.5 hours, and 1 hour) and extraction times (1 hour, 3 hours, and 5 hours) and amounts of water (100 mL, 150 mL, 200 mL). The details are given in Table S1 in the supplementary material online. The filtrates were concentrated under vacuum to give 0.5–0.8 g extracts respectively. The extracts were weighed and dissolved in 10 mL of deionized water (equivalent to 1 g of raw herbs in 1 mL solvent). Both of the solutions were filtered through a 0.22 μm filter. From the resulting solution, 5 μL was injected into the UHPLC system for LC coupled with multistage accurate MS analysis. All samples were analyzed in triplicate.

2.3. UHPLC-MS/MS analysis

2.3.1. Chromatography

The LC system consisted of a Thermo Scientific Accela UHPLC system with a built-in degasser and autosampler. LC analysis was performed on an Agilent SB-C_{18} (2.1 mm × 50 mm, 1.8 μm) column and the column temperature was set at 35°C. A mixture of water with (A) 0.1% formic acid and (B) acetonitrile was used as the mobile phase. Gradient elution was performed in linear gradient (2:98 at 0–3 minutes, 5:95–30:70 at 3–20 minutes, and 30:70–90:10 at 20–36 minutes, v/v). The duration of re-equilibration was 5 minutes between individual runs and the flow rate was 0.4 mL/minute.

2.3.2. MS

Mass spectra were analyzed on a Finnigan LTQ-Orbitrap XL instrument with an ES source (Thermo Electron, Bremen, Germany). Nitrogen and helium were used as the sheath and auxiliary gas and the collision gas, respectively. Values of auxiliary gas flow rate and capillary voltage were set at 5 arbitrary units and 40 V in positive ion mode and 8 arbitrary units and −45 V in negative ion mode, respectively. The scan event cycle used a full scan mass spectrum at resolution of 15,000 (at *m/z* 400) and corresponding data-dependent MS/MS events acquired at a resolving power of 7500. The most intense ions detected in full scan MS were selected for data-dependent scanning. MS/MS activation parameters were set at an isolation width of 2 Da with normalized collision energy of 35% and an activation time of 30 milliseconds. An external calibration for mass accuracy was performed the day prior to the test. The mass spectrometric data were collected from *m/z* 100 to 1500 in positive and negative ion mode.

2.4. Antioxidant activity assay

The DPPH radical-scavenging activity of extracts of CCT was measured according to the literature [16]. Sample extracts were dissolved in 10 mL of deionized water to prepare a solution at a concentration equivalent to 1 g of raw herbs in 1 mL solvent. Then 200 μL of solution was placed into the tubes and 800 μL of 0.1 mM methanolic solution of DPPH was added and shaken vigorously. The tubes were allowed to stand for 20 minutes at 27°C. The absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radicals in the sample and was calculated using the formula:

$$\text{DPPH radical scavenging activity} = (\text{control OD} - \text{sample OD})/\text{control OD} \times 100\%$$

(OD-optical density).

2.5. Data analysis

Raw data from liquid chromatography (LC)-MS detection were imported to the MZmine 2 software (http://mzmine.sourceforge.net/) by means of peak detection, filtering, and normalization. The filter conditions were: chromatography peak intensity signal/noise >30, retention time tolerance ±0.1 minute, and *m/z* tolerance ±0.01. It allowed deconvolution, alignment and data reduction to give a list of mass and retention time pairs with corresponding intensities for all the detected peaks from each data file in the data set. The processed data were exported as CSV file. The resulting data set was analyzed by OPLS using SIMCA-P software (version 11.0, Umetrics AB, Umea, Sweden) after a Pareto-scaled procedure. To evaluate the correlation between the amounts of compounds identified and DPPH radical scavenging activity, canonical correlation analysis was performed by Stata software (version 10, StataCorp, Lakeway Drive, College Station, Texas, USA).

2.6. Validation of the quantitative analysis

The stock solutions of six reference compounds were diluted to six-point calibration levels for the construction of calibration curves. Each concentration of the standard solution was injected in triplicate. Calibration curves were established by plotting the peak area versus concentration of each analyte. The limitation of detection (LOD) and limitation of quantification (LOQ) for each standard were defined at signal-to-noise ratio of 3 and 10, respectively. To confirm the repeatability, five replicates of the same sample were extracted and analyzed. The stability was measured by analyzing one sample at five different time points over 24 hours. Variations were expressed as relative standard deviation (RSD). Intra and inter day variations were utilized to assess the precision of the method. The intra day variation was determined by analyzing five replicates within 1 day and the inter day variation was measured on 3 consecutive days. Recovery was used to evaluate the accuracy of the method. A certain amount of CCT sample was spiked with the standard solution. The mixture was processed and analyzed using the method mentioned above, and three samples analyzed. Variations were expressed as RSD in all three tests above.

3. Results and discussion

3.1. Optimization of LC and MS conditions

Mobile phase systems including acetonitrile-aqueous, methanol-aqueous, acetonitrile-aqueous with 0.1% formic acid,

Table 1 – DPPH radical scavenging activity of *Camellia chrysantha* (Hu) Tuyama in orthogonal experiment.

Experimental run	Factors (levels)			Inhibition rate (%) of DPPH free radical ($n = 3$, mean ± SD)
	Soaking time (h)	Extraction time (h)	Amount of water (mL)	
1	0	1	100	35.6 ± 2.5
2	0	3	150	39.6 ± 3.0
3	0	5	200	76.8 ± 4.2
4	0.5	1	200	68.8 ± 3.6
5	0.5	3	100	55.8 ± 3.2
6	0.5	5	150	78.5 ± 3.9
7	1	1	150	70.1 ± 3.5
8	1	3	200	72.6 ± 3.6
9	1	5	100	69.8 ± 4.0

DPPH = 1,1-diphenyl-2-picrylhydrazyl.

and methanol-aqueous solutions with 0.1% formic acid were selected to optimize the chromatographic conditions. As a result, acetonitrile-aqueous solution with 0.1% formic acid on the optimized gradient mode gave a good separation and abundant signal response both in positive and in negative ion scan mode. For MS conditions, the flow rate of sheath gas, spray voltage, capillary temperature, and voltage of the tube lens were the main parameters affected by the ion intensity. These parameters were therefore optimized, with the flow rate of sheath gas at 30 arbitrary units, 20 arbitrary units, and 15 arbitrary units, spray voltage at 5, 4.5 and 4 in positive ion mode and −5, −4.5 and −4 in negative ion mode, voltage of tube lens at 80, 100 and 120 in positive ion mode and −80, −100 and −110 in negative ion mode, and the capillary temperature at 250°C, 275°C and 300°C. There was much less signal response in the positive ion scan mode than in the negative ion scan mode, despite the optimization; for negative ion mode, optimal conditions were sheath gas at 30 arbitrary units, spray voltage at −4.5 kV, capillary temperature at 275°C and tube lens at −100 V.

3.2. *LC-MS analysis and data conversion*

The total ion chromatograms of nine batches ($n = 3$) of CCT extracts in negative mode resulting from the optimal elution program were shown in Fig. S1 in the supplementary material online. The raw data of LC-MS detection were pretreated by the MZmine 2 software (http://mzmine.sourceforge.net/), as a result, a dataset with 27 observations and 1053 variables was built and imported to the SIMCA-P 11.0 software (Umetrics AP) for multivariate statistical analysis.

3.3. *Results of the antioxidant activity assay*

The radical scavenging activity of CCT following different extraction processes was investigated. As shown in Table 1,

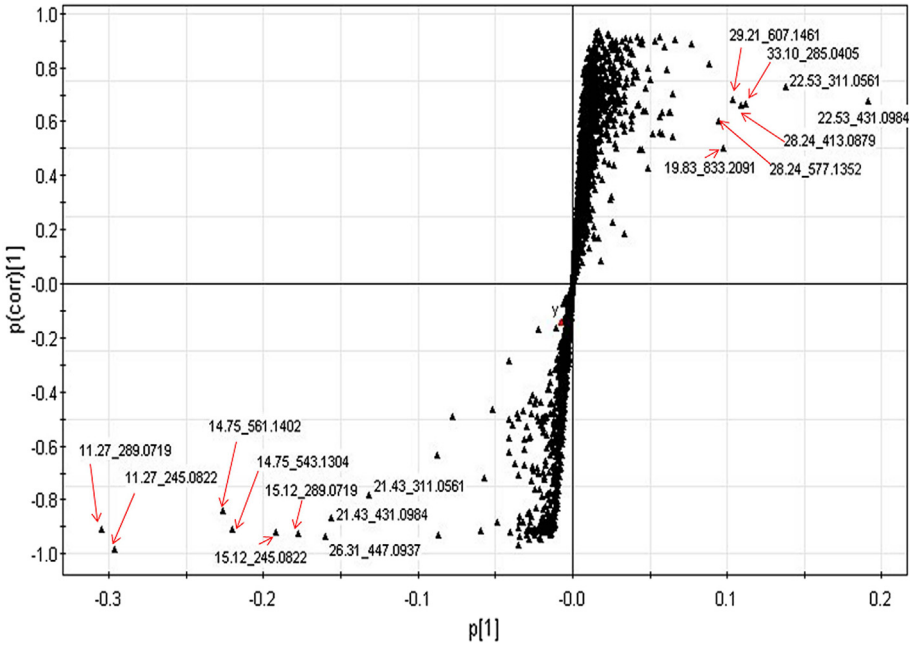

Fig. 1 – OPLS S-loading plot based on chemical profiling of the *Camellia chrysantha* (Hu) Tuyama extracts by SIMCA-P 11.0 ($n = 3$ in each batch, 27 samples totally). Sixteen variables far from the origin contributed significantly to differentiate the DPPH radical scavenging rate of 27 samples, which were considered as potential biomarkers.

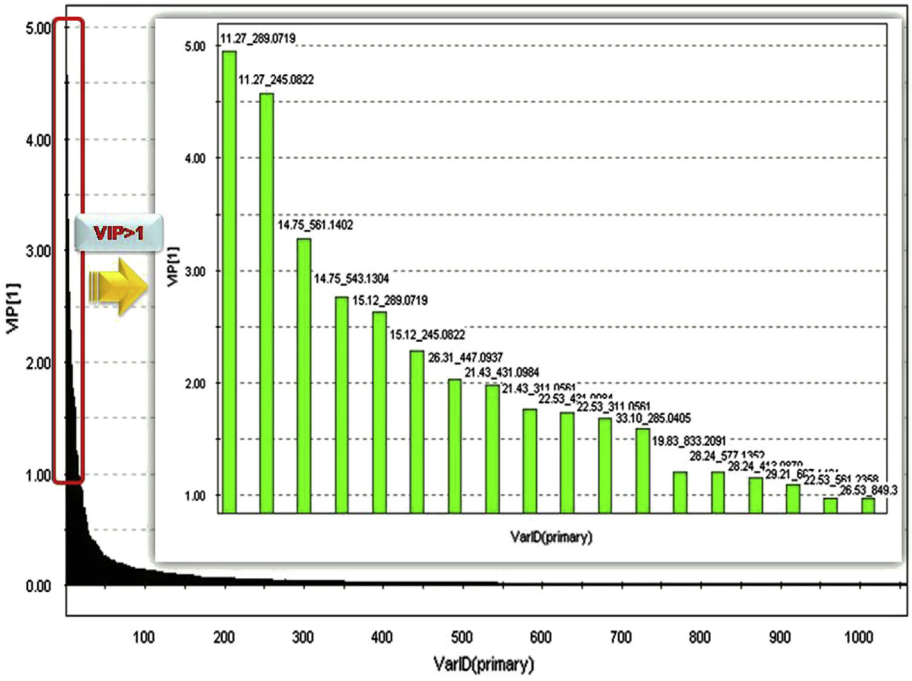

Fig. 2 – Variable importance in projection (VIP) from OPLS model of 16 characteristic variables.

the extraction process with 0.5-hour soaking and 5-hour extraction times and 150 mL of extraction solvent had the highest scavenging activity. The DPPH radical scavenging activity of the CCT samples was in the range 35.6%–78.5%, which indicated that radical scavenging activity was related to the extraction process and there were different compositions of antioxidant constituents that contributed to their antioxidant activities.

3.4. *Identification of active constituents*

The 1053 variables from 27 observations produced by data conversion (X) and the data from the radical scavenging activity of CCT (Y) were imported into the SIMCA-P 11.0 software (Umetrics AP) to build a matrix. Three principal components were extracted using the OPLS method, and the parameters for the model were 0.842 (R^2Y) and 0.731 (Q^2Y). From the S-loading

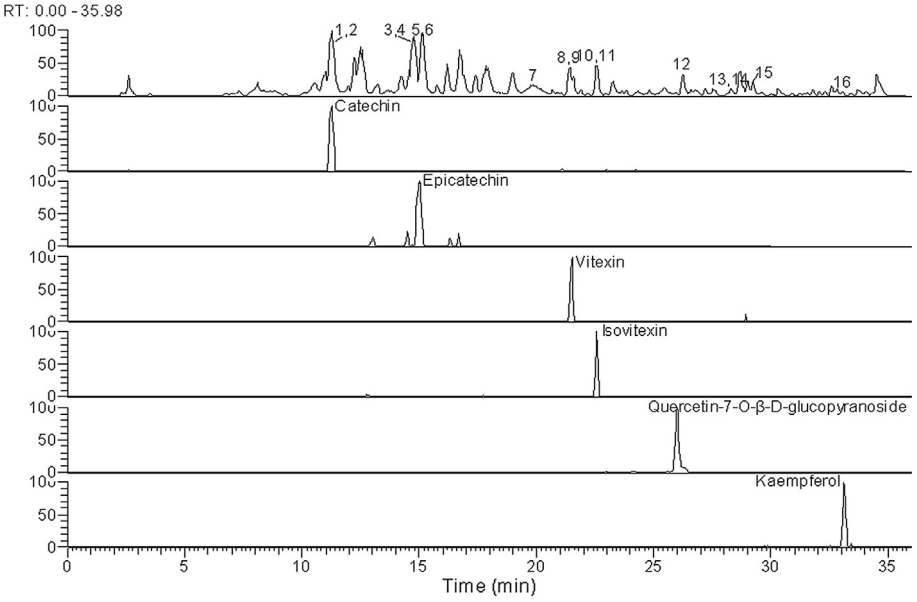

Fig. 3 – Base peak spectra of *Camellia chrysantha* (Hu) Tuyama extract and authentic compounds including catechin, epicatechin, vitexin, isovitexin, quercetin-7-O-β-D-glucopyranoside and kaempferol in negative ion mode.

Table 2 – Liquid chromatography mass spectrometry analysis of retention time, precursor ion and main fragment ions of the reference compounds in negative ion mode.

Authentic compounds	Retention time (min)	Quasi molecular ion (measured)	Error (ppm)	Formula	MS^2 data (measured)
Catechin	11.31	289.0719 $[M-H]^-$	4.376	$C_{15}H_{14}O_6$	245.0822, 205.0509
Epicatechin	15.06	289.0720 $[M-H]^-$	4.480	$C_{15}H_{14}O_6$	245.0822, 205.0509
Vitexin	21.43	431.0984 $[M-H]^-$	2.659	$C_{21}H_{20}O_{10}$	341.0667, 311.0561
Isovitexin	22.56	431.0984 $[M-H]^-$	2.729	$C_{21}H_{20}O_{10}$	341.0670, 311.0563
Quercetin-7-O-β--D-glucopyranoside	26.17	447.0936 $[M-H]^-$	3.225	$C_{21}H_{20}O_{11}$	301.0353, 285.0404
Kaempferol	33.03	285.0406 $[M-H]^-$	4.298	$C_{15}H_{10}O_6$	257.0456, 229.0507

plot (Fig. 1) and variable importance in projection (Fig. 2; the data of variable importance in projection >1 were chosen [17]), we found that 16 variables produced with retention time and *m/z* pairs [t_R_*m/z* pair, i.e., 11.27_289.0719 (**1**), 11.27_245.0822 (**2**), 14.75_561.1402 (**3**), 14.75_543.1304 (**4**), 15.12_289.0719 (**5**), 15.12_245.0822 (**6**), 19.83_833.2091 (**7**), 21.43_431.0984 (**8**), 21.43_311.0561 (**9**), 22.53_431.0984 (**10**), 22.53_311.0561 (**11**), 26.31_447.0937 (**12**), 28.24_577.1352 (**13**), 28.24_413.0879 (**14**), 29.21_607.1461 (**15**), and 33.10_285.0405 (**16**)] made significant contributions to the radical scavenging activity. By comparing individual peak retention times (Fig. 3) and the MS, MS/MS data (Table 2) with those of authentic compounds, six components were identified: catechin (**1**), epicatechin (**5**), vitexin (**8**), isovitexin (**10**), quercetin-7-O-β-D-glucopyranoside (**12**) and kaempferol (**16**). Corresponding quasi-molecular ions and their fragment ions in the MS/MS spectra are summarized in Table 3. In terms of the six constituents identified, their antioxidant activities have been reported as follows: catechin (**1**) could have a role as a physiological antioxidant in human plasma [18]; catechin and epicatechin (**5**) in grape seeds contributed to the peroxyl radical scavenging activities measured as Oxygen radical absorbance capacity (ORAC) [18,19]; vitexin (**8**) could be effectively used for the prevention of UV-induced adverse skin reactions, such as free radical production and skin cell damage [20]; isovitexin (**10**) inhibited xanthine oxidase with an IC_{50} value of 15.2 μM and protected cells from oxidative stress [21]; quercetin-7-O-β-D-glucopyranoside (**12**) exhibited antioxidant activities and possessed anti-inflammatory activity, inhibiting the expression of inducible nitric oxide synthase and release of nitric oxide by lipopolysaccharide-stimulated RAW 264.7 macrophages in a dose-dependent manner [22]; and kaempferol (**16**) was reported to have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, anxiolytic, analgesic, and antiallergic activities [23]. Even if some constituents that made significant contributions to the radical scavenging activity could not be identified exactly by comparison with authentic compounds in this survey, some structural information such as accurate molecular weights and fragmentation patterns could still be obtained for the further characterization. For instance, variable **3** with quasi-molecular ion at *m/z* 561.1402 ($[C_{30}H_{25}O_{11}]^-$, calculated 561.1391) in 14.75 minutes gave an ion at *m/z* 289.0717 ($[C_{15}H_{13}O_6]^-$, calculated. 289.0707) in MS^2 spectrum indicating that catechin or epicatechin moieties were involved in the structure, which may be tentatively identified as the conjugates of trihydroxyflavanone and catechin or epicatechin. Variable **7** showed a quasi-molecular ion at *m/z* 833.2091 in 19.83 minutes ($[C_{45}H_{37}O_{16}]^-$, calculated 833.2076), which generated ions at *m/z* 561.1402 and 289.0717 ($[C_{30}H_{25}O_{11}]^-$, calculated 561.1391 and $[C_{15}H_{13}O_6]^-$, calculated

Table 3 – Identification of the chemical constituents of *Camellia chrysantha* (Hu) Tuyama by liquid chromatography mass spectrometry analysis.

No.	Retention time (min)	Quasi molecular ion (measured)	Error (ppm)	Variable importance in projection	Formula	MS^2 data (measured)	Identification
1	11.27	289.0719	4.376	4.96	$C_{15}H_{14}O_6$	245.0822, 205.0509	Catechin
2	11.27	245.0822	5.608	4.55	$C_{14}H_{14}O_4$	—	Catechin fragment
3	14.75	561.1402	1.892	3.28	$C_{30}H_{26}O_{11}$	543.1299, 289.0718	Unidentified
4	14.75	543.1304	3.418	2.78	$C_{30}H_{24}O_{10}$	—	Unidentified
5	15.12	289.0719	4.376	2.65	$C_{15}H_{14}O_6$	245.0822, 205.0510	Epicatechin
6	15.12	245.0822	5.608	2.28	$C_{14}H_{14}O_4$	—	Epicatechin fragment
7	19.83	833.2091	1.798	1.23	$C_{45}H_{38}O_{16}$	707.1765, 543.1295	Unidentified
8	21.43	431.0984	2.659	1.99	$C_{21}H_{20}O_{10}$	341.0667, 311.0561	Vitexin
9	21.43	311.0561	3.553	1.76	$C_{17}H_{12}O_6$	—	Vitexin fragment
10	22.53	431.0984	2.659	1.75	$C_{21}H_{20}O_{10}$	341.0668, 311.0561	Isovitexin
11	22.53	311.0561	3.553	1.72	$C_{17}H_{12}O_6$	—	Isovitexin fragment
12	26.31	447.0937	3.292	2.02	$C_{21}H_{20}O_{11}$	301.0353, 285.0405	Quercetin-7-O-β--D-glucopyranoside
13	28.24	577.1352	1.970	1.23	$C_{30}H_{26}O_{12}$	431.0982, 413.0877	Unidentified
14	28.24	413.0879	2.932	1.19	$C_{21}H_{18}O_9$	—	Unidentified
15	29.21	607.1461	2.375	1.14	$C_{31}H_{28}O_{13}$	431.0985, 413.0880	Unidentified
16	33.10	285.0405	4.088	1.63	$C_{15}H_{10}O_6$	257.0459, 229.0504	Kaempferol

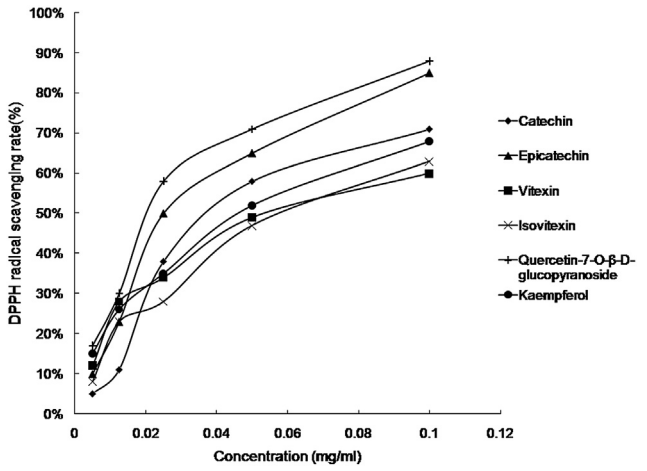

Fig. 4 – The assay of DPPH radical scavenging activity of six identified potential antioxidant constituents (catechin, epicatechin, vitexin, isovitexin, quercetin-7-O-β-D-glucopyranoside, and kaempferol) that screened by the OPLS analysis.

289.0707) in MS^2 spectrum, suggesting the structure consisted of catechin or epicatechin and the dimer of trihydroxyflavanone. Similarly, variable **13** was presumed to be the dimer of catechin or epicatechin. An ion at *m/z* 431.0985 ($[C_{21}H_{19}O_{10}]^-$, calculated 431.0973) produced in MS^2 spectrum of quasi-molecular ion at *m/z* 607.1461 ($[C_{31}H_{27}O_{13}]^-$, calculated. 607.1446) in 29.21 minutes (variable **15**), which may be presumed to be the conjugate of vitexin or isovitexin and a moiety with a formula of $[C_{10}H_8O_3]$.

The OPLS analysis was performed again with the relative peak intensities of the 16 variables screened with potential active constituents considered to be *X* to verify their contributions to the bioactivity, and the model parameters were 0.841 (R^2Y) and 0.707 (Q^2Y) after the extraction of the three principal components. The revised model still has a strong ability to explain *Y* (the percentage of DPPH free radicals inhibited) compared with the above model, which used the 1053 peaks to determine Y. These 16 variables can therefore be treated as the main active constituents that contribute to the DPPH radical scavenging activity.

3.5. Validation of the active constituents

The DPPH radical scavenging activity assay was performed to validate the six potential antioxidant constituents identified by the OPLS analysis. As shown in Fig. 4, the relationship between activity and concentration was described. According to the IC_{50}, catechin (**1**), epicatechin (**5**), vitexin (**8**), isovitexin (**10**), quercetin-7-O-β-D-glucopyranoside (**12**), and kaempferol (**16**) all demonstrated DPPH radical scavenging activity. Their strength of scavenging activity was as follows: quercetin-7-O-β-D-glucopyranoside (**12**) > catechin (**1**) > epicatechin (**5**) > kaempferol (**16**) > vitexin (**8**) > isovitexin (**10**). As reported by Sowndhararajan and Kang [16], the phenolic hydroxyl groups on the B-ring of flavonoids contributed mostly to its antioxidant activity. Two phenolic hydroxyl groups in the ortho-position on the B-ring had stronger radical scavenging activity than other forms. In our study, the DPPH radical scavenging activities of compounds **1**, **5**, and **12** (2 phenolic hydroxyl groups in the 4′, 5′ position on the B-ring) were stronger than compounds **8**, **10**, and **16** (one phenolic hydroxyl group in the 4′ position on the B-ring), which was in accordance with previous studies. As to the C-ring of flavonoids, the existence of hydroxyl group in position 3 (3-OH) had a stronger radical scavenging activity than those flavonoids without 3-OH [24]. This was the reason why the results of validation demonstrated that compound **12** had stronger radical scavenging activity than compounds **1** and **5**, and compound **16** presented a lower IC_{50} than compounds **8** and **10**. Further study is required to establish a sensitive and reliable quantitative method for quantitative analysis of these compounds in CCT samples from different extraction procedures.

3.6. Method validation of the quantitative analysis

The calibration curves, linear ranges, LOD, LOQ, and repeatability of six analytes were investigated using the HP-MS method developed (Table 4). Correlation coefficient values ($r^2 > 0.999$) indicated good correlations between the standard concentrations investigated and their peak areas within the ranges tested. The ranges of LOD and LOQ for all the analytes were from 0.003206 μg/mL to 0.01458 μg/mL and 0.01122 μg/mL to 0.05105 μg/mL, respectively. The repeatability of the six compounds present as RSD ($n = 5$) was between 0.69% and 2.86%. The variations (RSD) of stability for the six analytes

Table 4 – Summary of quantitation ion, calibration curves, linear range, limitation of detection (LOD), limitation of quantification (LOQ), and repeatability for six analytes analyzed with the liquid chromatography mass spectrometry system.

Analyte	Quantitation ion (*m/z*)	Linear range (μg/mL)	Calibration curve ($n = 6$)[a]	r^2 ($n = 6$)	LOD (μg/mL)	LOQ (μg/mL)	Repeatability, RSD (%)
Catechin (**1**)	289.0719	0.51–51.05	$Y = 10^7X + 3 \times 10^6$	0.999	0.014580	0.05105	1.68
Epicatechin (**5**)	289.0719	1.25–62.60	$Y = 9 \times 10^6X - 4 \times 10^6$	0.999	0.007150	0.02504	1.25
Vitexin (**8**)	431.0984	0.24–23.60	$Y = 10^7X - 80686$	0.999	0.006740	0.02360	0.69
Isovitexin (**10**)	431.0984	0.15–15.40	$Y = 2 \times 10^7X + 2 \times 10^6$	0.999	0.004400	0.01540	1.72
Quercetin-7-O-β-D-glucopyranoside (**12**)	447.0937	0.06–5.61	$Y = 6 \times 10^7X - 10^6$	0.999	0.003206	0.01122	2.86
Kaempferol (**16**)	285.0405	0.018–1.82	$Y = 10^8X + 6 \times 10^{-9}$	1.000	0.005200	0.01820	2.31

RSD = relative standard deviation.
[a] *Y* and *X* stand for the peak area and the concentration (μg/mL) of each analyte, respectively.

Table 5 – Precisions and recoveries of six analytes.

Analyte	Intra day ($n = 5$) (μg/mL)		Inter day ($n = 3$) (μg/mL)		Recoveries ($n = 3$)				
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Initial (μg)	Added (μg)	Detected (μg)	Recovery (%)	RSD (%)
Catechin (**1**)	1.110 ± 0.036	3.49	1.05 ± 0.04	3.55	22.44	10.21	31.85	97.54	1.05
	20.420 ± 0.210	1.02	20.55 ± 0.26	1.27		20.42	42.58	99.34	1.02
	51.080 ± 0.057	0.11	51.08 ± 0.05	0.10		51.05	71.69	97.55	0.98
Epicatechin (**5**)	6.246 ± 0.093	1.49	6.27 ± 0.09	1.50	28.75	12.52	40.26	97.55	1.88
	25.290 ± 0.260	1.04	25.41 ± 0.28	1.11		25.04	51.96	96.59	1.85
	62.670 ± 0.250	0.41	62.61 ± 0.27	0.44		50.08	78.96	100.20	2.69
Vitexin (**8**)	2.320 ± 0.045	1.95	2.33 ± 0.06	2.37	9.62	2.36	12.32	102.80	1.54
	11.740 ± 0.210	1.76	11.67 ± 0.24	2.09		5.90	15.69	101.10	2.36
	23.590 ± 0.082	0.35	23.61 ± 0.05	0.19		11.80	21.36	99.71	1.89
Isovitexin (**10**)	1.550 ± 0.052	3.36	1.53 ± 0.04	2.62	4.40	1.54	5.70	95.95	1.16
	7.540 ± 0.080	1.06	7.54 ± 0.03	0.41		3.75	8.00	98.15	2.85
	15.430 ± 0.051	0.33	15.41 ± 0.05	0.35		7.50	11.50	96.63	3.21
Quercetin-7-O-β-D-glucopyranoside (**12**)	0.560 ± 0.007	1.25	0.57 ± 0.01	2.21	1.09	0.56	1.58	95.69	2.36
	1.410 ± 0.010	0.70	1.41 ± 0.01	0.87		1.40	2.50	100.30	3.55
	2.800 ± 0.004	0.14	2.81 ± 0.0049	0.18		2.80	3.80	97.56	3.45
Kaempferol (**16**)	0.093 ± 0.003	3.29	0.09 ± 0.003	3.36	0.07700	0.018	0.09	94.53	2.89
	0.180 ± 0.002	1.15	0.180 ± 0.001	0.85		0.091	0.16	95.23	3.21
	0.450 ± 0.004	0.97	0.45 ± 0.005	1.13		0.18	0.24	94.59	3.88

RSD = relative standard deviation; SD = standard deviation.

were from 1.36% to 3.87% in 24 hours. The overall intra and inter day variations (RSD) of the six analytes were ranged from 0.11% to 3.49% and from 0.096% to 3.55% (Table 5), respectively. The method developed showed good accuracy, with recoveries of between 94.53% and 102.83% (Table 5). The results therefore demonstrated that the LC-electrospray ionization (ESI)-MS method was sensitive, precise, and accurate enough for quantitative evaluation of multi-compound in CCT.

3.7. *Quantitative determination of CCT samples*

Typical chromatograms for the quantitative determination of the six compounds developed in CCT samples are shown in the supplementary materials (see Fig. S2 in the supplementary material online). A total of nine different batches of CCT samples from different extraction procedures were tested using the LC-ESI-MS method. The contents ($n = 3$) of the compounds are summarized in Table 6. The compounds were found in all of the samples examined, and the amounts of each compound varied between CCT samples. To evaluate the correlation between the quantities of the six compounds and DPPH radical scavenging activity, canonical correlation analysis was used to analyze the data. As shown in Table 6, compounds **1**, **5**, **8**, **10**, **12**, and **16** had a strong correlation with the inhibition rate, which was in accordance with the results obtained from the OPLS analysis.

4. Conclusion

In this study, a composition–activity relationship approach used to investigate the antioxidant components in CCT found that 16 variables significantly contributed to the DPPH radical

Table 6 – The contents (μg/g) of six analytes in nine *Camellia chrysantha* (Hu) Tuyama samples and canonical correlation coefficient between six characteristic compounds and DPPH radical scavenging activity ($n = 3$, the data are presented as an average of triplicates).

Samples	Compound **1**	Compound **5**	Compound **8**	Compound **9**	Compound **12**	Compound **16**	DPPH radical scavenging rate (%)
1	30.14	37.62	12.56	5.950	1.350	0.06500	35.6
2	32.23	36.93	13.67	6.110	0.9500	0.07400	39.6
3	43.56	52.56	19.12	8.340	2.010	0.1360	76.8
4	36.67	43.67	14.56	6.550	1.450	0.08300	68.8
5	35.32	41.46	13.89	6.560	1.220	0.07600	55.8
6	44.89	55.56	19.24	8.800	2.180	0.1560	78.5
7	38.35	48.35	16.28	7.740	1.760	0.1120	70.1
8	40.45	45.45	17.32	7.880	1.850	0.1200	72.6
9	37.32	45.42	15.88	6.780	1.550	0.08600	69.8
Correlation coefficient	0.9242	0.8927	0.8695	0.8389	0.8834	0.7998	

DPPH = 1,1-diphenyl-2-picrylhydrazyl.

scavenging activity. Six of the variables were identified as catechin (**1**), epicatechin (**5**), vitexin (**8**), isovitexin (**10**), quercetin-7-O-β-D-glucopyranoside (**12**), and kaempferol (**16**). According to the IC_{50} values in the validation assay, the strength of DPPH radical scavenging activity was **12** > **1** > **5** > **16** > **8** > **10**. A LC-ESI-MS method was then established for an assay of the six marker compounds found in CCT samples from different preparations. The method was found to have good accuracy, sensitivity, and repeatability. This study not only helps to reveal the antioxidant constituents in CCT but can also assist in the discovery of active constituents and facilitate the quality control of medicinal herbs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfda.2014.02.003.

Conflicts of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled: “Characterization and determination of antioxidant components in the leaves of Camellia chrysantha (Hu) Tuyama based on composition–activity relationship approach”.

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