Pharmaceutical Sciences, 2023, 29(1), 100-110
doi:10.34172/PS.2022.27
https://ps.tbzmed.ac.ir/

Research Article

Phytochemical Analysis and Antioxidant Activity of Eight Cultivars of Tea (*Camellia sinensis*) and Rapid Discrimination with FTIR Spectroscopy and Pattern Recognition Techniques

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Article Info

Article History:
Received: 2 Feb 2022
Accepted: 26 Jun 2022
ePublished: 8 Jul 2022

Keywords:
-Antioxidant
-*Camellia sinensis*
-EGCG
-Pattern recognition
-Phenolic compound

Abstract

Background: Tea (*Camellia sinensis* L.O. Kuntze) is one of the most commonly consumed beverages globally, with several beneficial health effects. The composition of leaves is affected by different factors such as climate and tea cultivar.

Methods: In this study, the total phenolic, flavonoid, and tannin contents, and antioxidant activities of eight cultivars of tea growing in Iran were determined. The epigallocatechin gallate (EGCG) contents were measured by high-performance liquid chromatography (HPLC) analysis. The Fourier-transform infrared (FT-IR) spectra were used to construct a supervised pattern recognition model using a genetic algorithm-support vector machine (GA-SVM) for the classification of cultivars.

Results: The results indicated a wide variation of total phenolic content (178.04 to 316.09 mg gallic acid equivalents (GAE)/g extract). Also, the flavonoid contents ranged from 25.54 to 41.1 mg quercetin equivalents)QE(/g extract. All the samples had close tannin amounts (ranging from 40.32 to 45.90 mg as tannic acid equivalent (TAE)/g extract). There was a significant linear relationship among total phenolic and flavonoid contents and antioxidant properties. The cultivars DN, PK2, and C.Y.9 had the highest content of phenolic and flavonoid content as well as the best antioxidant activity. The EGCG contents were from 2.66 to 4.12%. The highest amount of EGCG was found in cultivars 282, PK2, and C.Y.9. The discriminative region of FT-IR spectra (1350-1650 cm^{-1}) was selected using a GA-SVM. This model showed 100% sensitivity and specificity for training and test sets.

Conclusion: The leaves' phytochemical compositions and antioxidant effect are deeply dependent on the type of tea cultivars. The cultivars PK2 and C.Y.9 can be considered richer sources of polyphenols, especially EGCG. The eight different cultivars can be classified based on chemical components using the recorded FT-IR spectra.

Introduction

The tea (*Camellia sinensis* L.O. Kuntze), family Theaceae, is one of the most prevalent beverages consumed globally.[1] It grows in tropical and subtropical regions, which provide sufficient rainfall, enough drainage, and somewhat acidic soil.[2] Statistics indicate that between 2005 and 2007, Iran consists of one of the major tea consuming countries in the world, with approximately 1.6 kg per capita tea consumption.[3]

About 34 thousand hectares of lands have been cultured for tea in the North of Iran (Guilan and Mazandaran provinces), which provides a humid temperate climate and adequate yearly precipitation.[3,4] Recently, tea has attracted considerable attention throughout the world because of different health benefits related to its consumption, including antioxidant, anti-arteriosclerotic, anti-obesity, antiallergic, anti-cancer, antimutagenic, anti-apoptotic, neuroprotective, antihyperglycemic, antimicrobial, and anti-inflammatory activities.[5,6] A large body of research demonstrated that overproduction of free radical species could lead to oxidative damage to vital biomolecules, such as DNA, lipids, and proteins. Consequently, they play a crucial role in many diseases such as cancer, heart disease, multiple sclerosis, neurodegenerative disorders (Alzheimer's disease and Parkinson's disease), diabetes mellitus, cardiovascular diseases, and autoimmune diseases.[5,7,8] In general, plant materials that are rich sources of natural antioxidants, especially polyphenols,

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can cause protective effects against such disorders.[2,7] The tea polyphenols can exert a significant antioxidant effect by scavenging reactive oxygen and nitrogen species as well as acting as metal chelators.[2]

Wide ranges of bioactive compounds like purine alkaloids (in particular, caffeine and theobromine) and polyphenols are responsible for the beneficial effects of tea.[6,9] It has been reported that green tea leaves contain 10–30% tannins or phenolic substances, such as catechins, flavonols, flavanones, phenolic acids, and glycosides.[10,11] The principal flavonols of tea leaves are quercetin, kaempferol, and myricetin, comprising 2-3% of the water-soluble extract materials of tea.[5] Furthermore, in fresh young leaves of *C. sinensis,* the main catechins (flavan-3-ols) are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), gallocatechin (GC), epicatechin (EC), and catechin.[2,12] Among these compounds, EGCG is considered the predominant one, which takes up around 3-13% of dry tea leaves, and 50-80% of the total catechins in green tea.[9,13] The composition of fresh tea leaves is affected by different factors such as climate, season, tea varieties, and cultivars, horticultural practices, and leaves age.[2,10,14] Therefore, the study of tea cultivars growing in the different regions is one of the global interests in order to determine samples that possess the highest health benefits.[2,12,15-17]

Plant genetic diversity is essential for developing novel and improved cultivars with appropriate characteristics, such as excellent yield potential, and large seeds.[18] Approximately, 2650 accessions of genetic resources from the tea have been recognized in China, Japan, India, Kenya, and other parts of the world.[14,19] Tea germplasms have typically been characterized by biochemical and morphological descriptors. While these descriptors are valuable for tea varietal group identification, they indicate limited levels of inter and intra-varietal polymorphism and they may not account for all the diversity in the species.[20] Numerous previous studies have been developed for the classification of tea cultivars using pattern recognition techniques based on chemical composition data, like gas chromatographic (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis.[21] However, these methods are costly and complicated to be applied in the routine work.[22] Infrared (IR) spectroscopy methods have been extensively used because it is a rapid and non-destructive method for qualitative and quantitative analyses of many materials.[23] It is extremely fast, chemical-free, and produces no resultant waste. Fourier-transform infrared spectroscopy (FT-IR) combined with attenuated total reflection (ATR) accessory, can be applied for analysing a vast number of samples in a short time without any sample preparation.[24] The advance in chemometric technologies linked with ATR-FT-IR spectroscopy provides an efficient method for determining the physical and chemical properties of products.

Multivariate data analysis and machine learning techniques are excellent strategies for foodstuff discrimination.[25] Cebi *et al.*[26] used ATR-FTIR to detect sibutramine adulteration in tea and coffee, based on hierarchical clustering and principal component analysis (PCA). Classification of unifloral honey was attempted by ATR-FTIR and random forest.[25] Gori *et al.*[27] applied a combination of linear discriminant analysis (LDA) and PCA with a moving window algorithm for the classification of grated cheese, based on their ATR-FTIR data.

In the present study, the total phenolic, flavonoid, and tannin contents as well as antioxidant activities of eight different cultivars of tea (*Camellia sinensis*), including PK2, C.Y.9, D.N, D.T.1, 100, 261, 270, and 282, growing in the North of Iran, were determined for the first time. Furthermore, the quantitative analysis of EGCG, as the main catechin in tea leaves, was performed by developing an HPLC method. Finally, the classification of the eight different tea cultivars was performed based on the ATR-FTIR recorded spectra of the extracted samples using chemometric techniques, including partial least squares-discriminant analysis (PLS-DA) and a genetic algorithm-support vector machine (GA-SVM).

Materials and Methods

Reagents and chemicals

Quercetin, tannic acid, EGCG, gallic acid, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH•), Folin–Ciocalteu's phenol reagent, Butylated hydroxy anisole (BHA), and vitamin E were purchased from Sigma-Aldrich chemical company (USA). Sodium bicarbonate, aluminium trichloride, and polyvinylpolypyrrolidone (PVPP) were purchased from Merck, Germany. HPLC-grade acetonitrile and acetic acid were obtained from DUKSAN, Korea. Other used reagents and solvents were of analytical grade. HPLC grade water was obtained from a Millipore Direct-Q purification system.

Plant material and extraction

According to the reports of Tea Research Centre of Iran (TRCI), Fashalem station, Rasht, Iran, the most common and popular imported tea cultivars, including P.K.2, C.Y.9, D.N, and D.T.1 were chosen. Also, four Iranian genotypes, growing in this center (100, 261, 270, and 282) were chosen to have a comparison among foreign and Iranian cultivars. The youngest two leaves, plus a terminal bud, were hand-plucked from selected eight different tea cultivars, from TRCI, in June, 2019. Samples were all propagated at the TRCI under the same environmental conditions. The tea cultivars differed in their shoot colour and size of the leaves. The voucher specimens were deposited in the herbarium of the school of pharmacy, Guilan University of medical sciences, Rasht, Iran.

The fresh green tea leaves (100 g, each cultivar) were shade dried at room temperature before powdering. The extraction was performed by the method described by Hu *et al.*[28] The leaves of each cultivar were brewed with ethanol (75%) in a water bath (30°C for 10 min). Then, the extract was filtered (with Whatman No. 1 filter paper) and the remained leaves were extracted by ethanol (35%) at 90°C for 60 min. The extracts obtained from two steps

were mixed and dried entirely under the reduced pressure by rotary evaporator.

Determination of total phenolic compounds

Total phenolic contents (TPC) of extracts were measured by the Folin-Ciocalteu method.[29,30] In the beginning, each extract (1 ml, in the concentration of 1 $mg.ml^{-1}$) was separately mixed with freshly prepared Folin-Ciocalteu reagent (5 ml, diluted ten-fold with distilled water). After 10 min, sodium bicarbonate solution (4 ml, 75 $g.l^{-1}$) was added. The samples were placed in a dark cabinet for 30 min, at room temperature. The absorbance of each sample was measured using a PerkinElmer ultraviolet-visible (UV-Vis) spectrophotometer (LAMBDA 25) at 765 nm. The absorbance of five known concentrations of gallic acid standard solutions (25, 50, 70, 100, and 200 $\mu g.ml^{-1}$) was measured at the same wavelength, and the calibration curve was plotted. The TPC were expressed as mg gallic acid equivalents (GAE)/ g extract.[8,31]

Determination of total flavonoid content

Total flavonoid contents (TFC) of extracts were determined, using the technique described by Saeidnia and Gohari.[32] At first, tea extracts (5 ml, 0.5 $mg.ml^{-1}$ each) were mixed with 5 ml of aluminium trichloride ($AlCl_3$) (2% in methanol) and stand for 10 min. After that, the absorbance of each mixture was measured at 415 nm using a UV/ Vis spectrophotometer. Quercetin was used in different concentrations (10, 25, 50, 75, and 100 $\mu g.ml^{-1}$) to plot the standard curve. Finally, the TFC was expressed as mg quercetin equivalents (QE)/ g extract.[33]

Determination of total tannin content

The determination of total tannin content (TTC) was performed using PVPP as the insoluble matrix, which binds to tannin phenolics, to measure tannins.[34-36] The test comprised two steps. In the first step, the stock solution of each sample (1 $mg.ml^{-1}$) was prepared in distilled water. Next, 1 ml of each sample and 0.5 ml of Folin-Ciocalteu reagent (1 N) were mixed. Then, 2.5 ml of sodium carbonate (20%) was added, and the mixture was shaken. The absorbance of samples was measured at 725 nm after 40 min. The calibration curve was plotted using different concentrations of tannic acid (0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.2 $mg.ml^{-1}$). The amount of total phenols (X), as tannic acid equivalent (TAE) was calculated based on the calibration curve.

In the second step, the tannins were removed from tannin-containing samples by adding PVPP (100 mg PVPP is sufficient to bind 2 mg of total phenols). The samples were vortexed and kept at 4° C for 15 min before being centrifuged (3000 g for 10 min). In this stage, the supernatants were collected. In this supernatant, only simple phenols other than tannins remained. The measurement of the phenolic contents of the supernatants was performed as mentioned in step 1 to determine the contents of non-tannin phenols (Y). Finally, X-Y indicated mg of tannin as TAE/g extracts.[37]

Antioxidant activity

The DPPH radical scavenging activities of tea extracts were measured using the method reported by Golfakhrabadi *et al.*[29] Tea leaves extracts were prepared in a series of test tubes. After that, 1 ml of each sample (in different concentrations) was mixed with 2 ml of DPPH solution. After 30 min, the absorbance was read at 517. The control contained sample (1 ml) and methanol (up to 3 ml), without DPPH. The blank consisted of 1 ml of methanol added to 2 ml of DPPH solution. Furthermore, the antioxidant activities of BHA, vitamin E, and EGCG were tested as the standard antioxidants. The experiments were repeated three times. The inhibition activity was calculated using the following formula.[38]

The IC_{50} values (designate the concentration of the sample ($\mu g.ml^{-1}$) causing 50% inhibition) were calculated from the graph plotted for inhibition against sample concentration.[31,39,40]

Quantification of EGCG in the tea extracts

The quantitative analysis of EGCG was carried out by a Waters Alliance e2695 HPLC apparatus. The detector was a Waters 2998 photodiode array (PDA). A C_{18} reversed-phase column (Waters Spherisorb: S5 ODS2, 4×250 mm, particle size 5 µm) equipped with a guard column (Waters Spherisorb, S5 ODS2 4.6×10 mm) was used. The Empower 3 software (Waters, USA) was used for data acquisition, peak integration, and calibrations. All the tea samples were dissolved in methanol: distilled water (1:1) (5 $mg.ml^{-1}$). The mobile phases consisted of (A): acetic acid/water (1:99 v/v) and (B): acetonitrile. Elution was performed using the following gradient program: The solvents composition started at 97% A and changed to 0% A during 50 min. A constant flow rate of 1 $ml.min^{-1}$ and a temperature of 25 °C were used. The equilibration time was 10 min. The injection volume for all samples was 100 µl. UV absorption spectra were recorded during the HPLC analysis over the wavelength range of 210–400 nm.

The identification was performed by comparing the retention times (Rt) of each tea sample and their obtained PDA absorbance spectra with the EGCG standard. Different concentrations of EGCG (10, 20, 30, 40, 50, and 60 $\mu g.ml^{-1}$) were prepared. The EGCG contents were quantified with a 6-point calibration curve of standard. All data are reported as means ± standard deviations of triplicate independent analyses.

Linearity, limit of detection, and limit of quantitation

Linearity was assessed through the relationship between the concentrations of EGCG and the absorbance found from the PDA detector using the determination coefficient (R^2).[41] The calibration lines were plotted by three replicates of each concentration of EGCG (10, 20, 30, 40, 50, and 60 $\mu g.ml^{-1}$) to recognize the extent of the total variability of the response that could be described by the linear

regression model. Limits of detection (LODs) and limits of quantitation (LOQs) were determined using the expressions 3.3σ/S and 10σ/S, respectively, where σ is the standard deviation of the residues and S is the slope of the calibration curve.[42]

Classification of tea extracts

Sample spectra were recorded using ATR-FTIR (Spectrum Two FT-IR spectrometer with ATR accessory (PerkinElmer)) in 4000–780 cm^{-1}. Each cultivar was extracted five times, and five FT-IR spectra were recorded for each extract. Spectral baselines were corrected using asymmetric least squares (P.H.C. Eilers, Analytical Chemistry, 75 (2003) 3631). Noise reduction was performed by Saviztky-Golay filter with second-order polynomial and window size of 21. Each cultivar data was randomly divided into training (70%) and test (30%) sets. The training set (144 samples for all cultivars) was used for obtaining the classification model, and a test set (56 samples for all cultivar classes) was used for external validation. For building the classification models, PLS-DA, and SVM with linear kernel function were applied to the training set for the pattern recognition. Monte Carlo cross-validation (100 times, calibration (70%): validation (30%)) was performed for internal assessment of the models. The ratio of misclassified validation set was used for evaluation of the models. The best model was further combined with a GA to reduce the number of variables and to find the informative spectral regions. Finally, the constructed model was assessed by the test set for its validity. Furthermore, a chance correlation test was also applied to evaluate robustness of the model. All data processing was performed in MATLAB (version 9.8 R2020a, the MathWorks, Natick, MA).

Statistical analyses

All the experiments were done in triplicates. The results were expressed as mean ± standard deviation (SD). Comparison between groups was carried out using One-way ANOVA followed by Tukey *post hoc* test (SPSS software). Differences were considered significant at $p<0.05$. The correlation between antioxidant properties and phytochemical contents were determined by the Pearson procedure, using the SPSS program (Version 22.0. SPSS Inc., Chicago, USA). p-Values<0.05 were considered significant.

Results and Discussion

Total phenol, total flavonoid, and total tannin contents

The TPC of tea extracts from eight cultivars were determined by the Folin- Ciocalteu method. The results are shown in Table 1. The analysis of cultivars showed wide variation from 178.04±0.06 mg GAE/g extract (for 100 cultivar) to 316.09±0.01 mg GAE/g extract (for DN cultivar), based on the standard curve of gallic acid ($y = 0.00096x - 0.0365$, $R^2 = 0.995$). The cultivars DN, PK2, and C.Y.9 contained the highest concentrations of phenolic compounds (316.09±0.01, 313.94±0.03, and 301.04±0.04 mg GAE/g extract, respectively). Statistical analysis showed that the difference in TPC was significant ($p< 0.05$) when compared among tea cultivars. A 1.77-fold difference in TPC was observed between the highest and lowest-ranked cultivars.

Additionally, the total flavonoid content was determined using the standard curve of quercetin ($y =0.0187x- 0.0254$, $R^2 = 0.975$). The flavonoid contents ranged from 25.54±0.17 to 41.1±0.12 mg QE/g extract. Statistical analysis depicted that the TFC in three cultivars, including DN, PK2, and C.Y.9, has no significant difference ($p>0.05$). There was a 1.6-fold difference between cultivars with the highest (C.Y.9) and lowest (282) total flavonoid contents.

The TTC were calculated using calibration curve ($y = 0.0005x- 0.0281$, $R^2 = 0.995$). The results indicated that all the samples had very close amounts of tannins (ranged from 40.32±0.04 to 45.90±0.03 mg TAE/ g of extract). The difference in TTC was statistically significant ($p< 0.05$) when compared among tea cultivars, with the exception of 270 and P.K.2 cultivars ($p>0.05$).

So far, the total phenolic compounds of tea cultivars have been investigated in different studies. Chan *et al.*[2] reported that the amount of total phenols in young tea leaves, growing in Malaysia, was 72.80 mg GAE/g. Also, the DPPH radical scavenging activity was 30 $\mu g.ml^{-1}$. Kerio *et al.*[15] studied the TPC of 10 green and 18 purple leaf-coloured cultivars, growing in Kenya. Their results

Table 1. Total phenolic, total flavonoid, and total tannin contents of eight different cultivars of *Camellia sinensis*.

Sample	Total phenol[1]	Total Flavonoid[2]	Total tannin[3]
100	178.04±0.06[a]	25.54±0.17[a]	40.32±0.04[a]
261	244.7±0.02[b]	32.32±0.03[b]	42.70±0.01[b]
270	228.72±0.01[c]	23.51±0.11[c]	45.60±0.04[c]
282	288.64±0.02[d]	22.77±0.11[d]	45.90±0.03[d]
P.K.2	313.94±0.03[e]	40.59±0.03[e]	45.49±0.07[c]
C.Y.9	301.04±0.04[f]	41.1±0.12[e]	43.94±0.01[e]
D.N	316.09±0.01[g]	40.69±0.4[e]	43.38±0.04[f]
D.T.1	275.46±0.11[h]	37.92±0.08[f]	42.38±0.01[g]

[1]mg of GAE/g extracts; [2]mg of QE/ g of extracts; [3]mg of TAE /g of extracts; the results are expressed as mean ± SD of three independent experiments. Values with no letters in common within each column were significantly different ($p < 0.05$).

indicated that the average amount of total phenols ranged between 16.8% and 32%. Their data is inconsistent with the finding of the present study (17.8% -31.6%). Moreover, the determination of TPC in 27 tea cultivars, growing in China revealed a wide range of variation (121.6–223.7 mg of GAE/g extract).[16]

In another study, several cultivars from Sri Lanka were investigated for their TPC. A range from 178 to 251 $mg.g^{-1}$ was reported among different samples. The cultivars DN, PK2, and C.Y.9 had 218.62, 238.03, and 205.65 $mg.g^{-1}$ total phenols, respectively.[43] Compared to our findings, these three cultivars have a higher amount of phenolic compounds in Iran.

DPPH radical scavenging activity

Polyphenols in green tea have been described to be potent antioxidant agents. In the recent years, there has been rising interest in using green tea leaves in food industries as a natural antioxidant, to take the place of the synthetic ones, including BHA, and butylated hydroxytoluene (BHT), which are doubted to cause cancer and their usage has been limited in food products.[2,8,44] These phenolic compounds have strong free-radical scavenging activities due to their one-electron reduction potential. They have the ability to act as hydrogen or electron donors.[2]

DPPH radical scavenging assay has been extensively used to measure the radical scavenging power of plant extracts because of its simplicity, affordability, stability, and reproducibility. The DPPH molecule is a stable free radical with an odd electron, which causes an absorption band at about 517 nm and a visible deep violate colour.[45-47] In this method, the DPPH captures an electron from a free radical scavenging antioxidant (hydrogen/electron donor) that leads to a reduction in the intensity of absorption and the solution is decolorized based on the number of electrons accepted.[46,48]

As is presented in Figure 1, the best radical scavenging activity reported from C.Y.9 cultivar (IC_{50}= 22.95 $\mu g.ml^{-1}$) followed by P.K.2 and D.N cultivars (IC_{50}= 25.99 and 29.63 $\mu g.ml^{-1}$). The C.Y.9 with the highest antioxidant activity showed 2-fold activity of the lowest-ranked one, 270. Furthermore, the IC_{50} values of EGCG, BHA, and vitamin E were 7.1, 7.77, and 14.03 $\mu g.ml^{-1}$, respectively. The antioxidant activity of all cultivars had significant difference ($p<0.05$) compared to vitamin E and BHA. The comparison among other groups is represented in Figure 1.

Furthermore, correlations and regression analysis were performed between total phenolic, flavonoid, and tannin contents and antioxidant activities of tea extracts. Results indicated that DPPH radical scavenging activity had negative correlations and regression with the phenolic, flavonoids, and tannin contents because that the results are expressed as IC_{50} values.[49]

Interestingly, the concentration of flavonoids exhibited significant correlation with radical scavenging effect (r= -0.817, R^2=0.668, p=0.007). Moreover, the phenolic contents were significantly correlated and influenced the radical scavenging activity in tea extracts (with r= -0.646, R^2=0.417, p= 0.042). However, the TTC did not reveal a significant correlation with antioxidant effect (r=- 0.079, R^2= 0.0062, p=0.426).

In a study, the IC_{50} value of green tea extract collected from Japan was reported as 194.6 ± 20.5 $\mu g.ml^{-1}$.[50] Bastos *et al.*[46] investigated the antioxidant activity of green tea, growing in Brazil, and they reported an IC_{50} of 88.36 ±0.7 $\mu g.ml^{-1}$. Jain *et al.*[51] reported that the green tea, grown in India, had the IC_{50}= 90.86 $\mu g.ml^{-1}$, in this essay. Chan *et al.*[2] revealed that the tea leaves growing in Selangor, Malaysia, had an antioxidant activity with an IC_{50} of 35 $\mu g.ml^{-1}$.

Quantitative measurement of EGCG in different tea cultivars

EGCG, as the most common catechin in green tea leaves, consists of about 50% of the total catechin content and shows the highest antioxidant effect among other catechins. In this study, the quantitative analysis of EGCG content in different tea samples was performed by developing an HPLC method. Figure 2 depicts a typical HPLC chromatogram of tea extracts. The EGCG was detected at R_t= 26 min. The amount of EGCG ranged from 2.66 to 4.12 %, with an average of 3.06%. As is presented in Table 2, the highest concentration of this compound was detected in

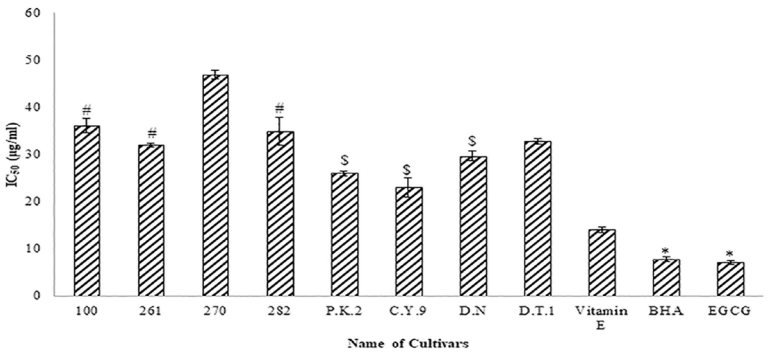

Figure 1. The IC_{50} values of eight tea cultivars, vitamin E, BHA and EGCG in DPPH radical scavenging test. The IC_{50} values are expressed as mean ± SD of three independent experiments. Different letters above each bar show that the difference between cultivars was significant ($p < 0.05$).

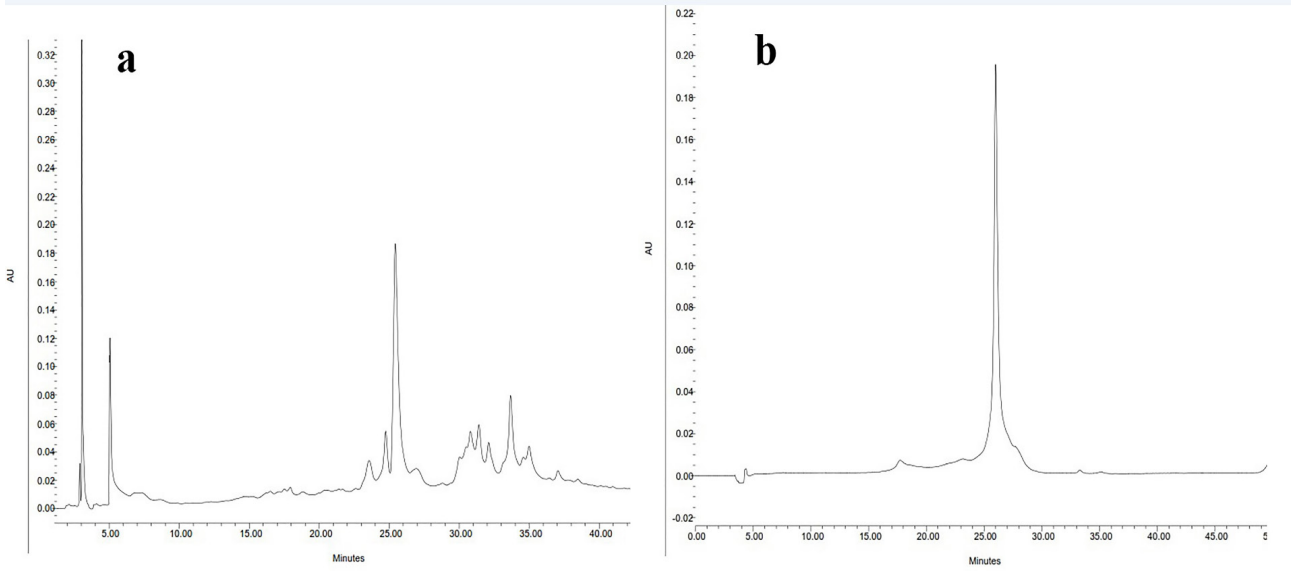

Figure 2. a) HPLC chromatogram of a typical tea extract (10 mg.ml^{-1}; injection volume: 100 µl); b) HPLC chromatogram of pure EGCG (60 µg.ml^{-1}). UV detection was performed from 210 to 400 nm; the other analytical conditions are designated in the methodology section. Peak identification: EGCG (Rt=26 min).

Table 2. The amount of EGCG in eight different cultivars of tea leaves, determining by analytical HPLC.

Sample	EGCG[1]
100	30.29±0.12^{a}
261	27.91±0.07^{b}
270	26.68±0.02^{c}
282	41.27±0.02^{d}
P.K.2	32±0.01^{e}
C.Y.9	31.28±0.21^{f}
D.N	30.33±0.15^{g}
D.T.1	27.08±0.12^{c}

[1] mg/g dried extract ± SD, Values with no letters in common within each column were significantly different (p<0.05).

cultivar 282 (41.24 mg/g extract), followed by cultivar PK2 and C.Y.9 (32 and 30 mg/g extract, respectively).

The comparison among different groups is represented in Table 2. No significantly correlative relationships were found between EGCG content and antioxidant activity (r=- 0.197, R^2= 0.3067, p=0.32).

In another study, Kerio *et al.*[15] reported the amount of EGCG in eleven different cultivars of green leaves tea ranged from 0.46% to 2.92%, with an average of 1.28%. Bazinet *et al.*[52] showed that the tea samples from Quebec, Canada, had 4% EGCG. In another study, Areba *et al.*[53] demonstrated that green tea samples in Kenya had 7% EGCG. The results of our work are in line with the previous studies.

The results of method validation for analysis of EGCG according to linearity, LOD and LOQ indicated that the developed method was valid (Table 3). Outstanding linearity was between 10 and 60 µg.ml^{-1}, respectively, with R^2= 0.994 for EGCG.

FT-IR spectroscopy

FT-IR spectra of cultivars were used for the determination of functional groups of biomolecules, existing in tea extracts. The FT-IR spectrum of all tea extracts (Figure 3) showed a broad peak from 3050 to 3600 cm^{-1}, which can be assigned to O-H and N-H stretching modes. It can prove the presence of some polyphenol, polysaccharides, and amide groups of proteins. The peaks at 2924 and 2858

Table 3. Linear range, LOD, and LOQ parameters of EGCG.

Compound	Regression equation	R^2	LOD (µg.ml^{-1})	LOQ (µg.ml^{-1})	Linear range (µg.ml^{-1})
EGCG	614789x-998265	0.994	0.88	2.66	10-60

LOD: Limit of detection; LOQ: limits of quantitation

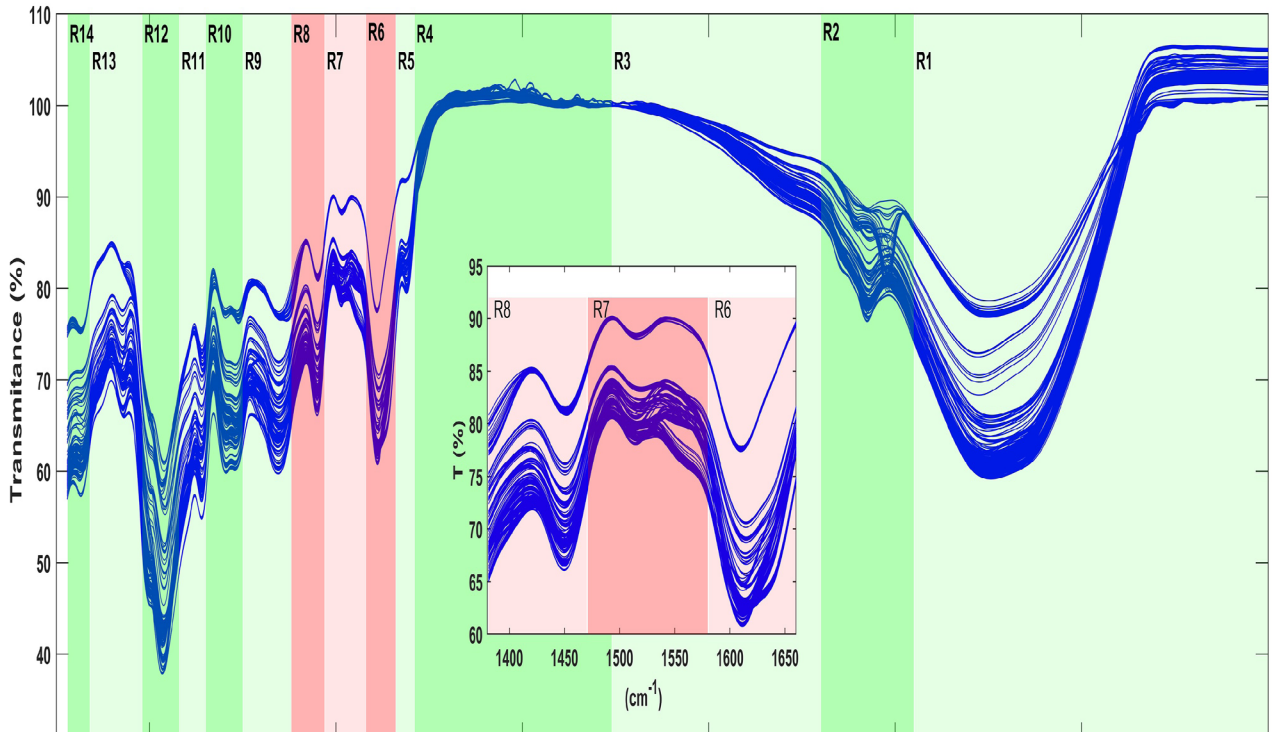

Figure 3. ATR-FT-IR spectra of all extracted cultivars. 14 regions are highlighted and labelled accordingly. Inset: magnified the GA selected regions.

cm^{-1} result from asymmetric and symmetric stretching vibrations of aliphatic C-H. Another band at 1688 cm^{-1} is related to the C=O stretching vibration. The characteristic peak at 1613 cm^{-1} corresponds to the bending vibration of amide I (N-H) of proteins, stretching vibration of C=C groups, and C-H bending vibration of alkenes. The out-of-plane bending vibration of N-H amide II and III, as well as C-N stretching vibration of proteins appear at 1537 and 1345 cm^{-1}, respectively. The absorption band at 1448 cm^{-1} can be ascribed to the bending vibration of CH_2 alkanes and COO^- of carboxylate moiety from proteins and lipids, and rocking vibrations of C-H bonds of cis-disubstituted alkenes. Two peaks at 1233 and 1245 cm^{-1} are related to the stretching vibration of C-O group of esters. The bands in 1140 and 1205 cm^{-1} can be attributed to C-H bending and C-H deformation vibrations of fatty acids. The characteristic peak at 1060 shows the stretching vibrations of C-O. Also, all peaks below 1000 cm^{-1} correspond to the bending modes of sp^2 C-H of alkenes and aromatic rings.

Pattern recognition

PLS-DA as a linear classification technique is a combination of the characteristics of PLS regression with the discrimination power of a classification method. This method is based on searching by PLS regression algorithm for latent variables (LVs) with the highest covariance for the dependent variables. Using PLS-DA, the original variables combine linearly as LVs which reflect the relevant sources of data variability. Subsequently, the graphical visualization, a better understanding of the various data patterns as well as their relations with LV scores and loadings are possible. The coefficients of variables are defined as the loadings in the linear combinations. Consequently, they can be explained as the impact of each variable on each LV, while the coordinates of each sample can be interpreted using the score in the LV projection hyperspace.[54] Zhu *et al.*[55] used PLS-DA identification of pesticide residues in tea.

SVM, as a statistical learning method, was introduced by Vapnik.[56] It is an efficient method for prediction and classification of various data sets. GA simulates the process of evolution and the natural genetics based on randomized searches and optimization methods. It is an acceptable method for classification, prediction, regression estimation, variable selection, and data reduction. Also, a

Table 4. Parameters of genetic algorithm-support vector machine (GA-SVM).

SVM	**C parameter**		**Kernel Scale**	**Box Constraint**		**Kernel Function**
	1		0.006	302.3		Linear
GA	**Crossover Fraction**	**Constraint Tolerance**	**Population**	**Selection Function**	**Elite Count**	**Mutation Function**
	0.8	10^{-3}	50	Stochastic Uniform	3	Gaussian

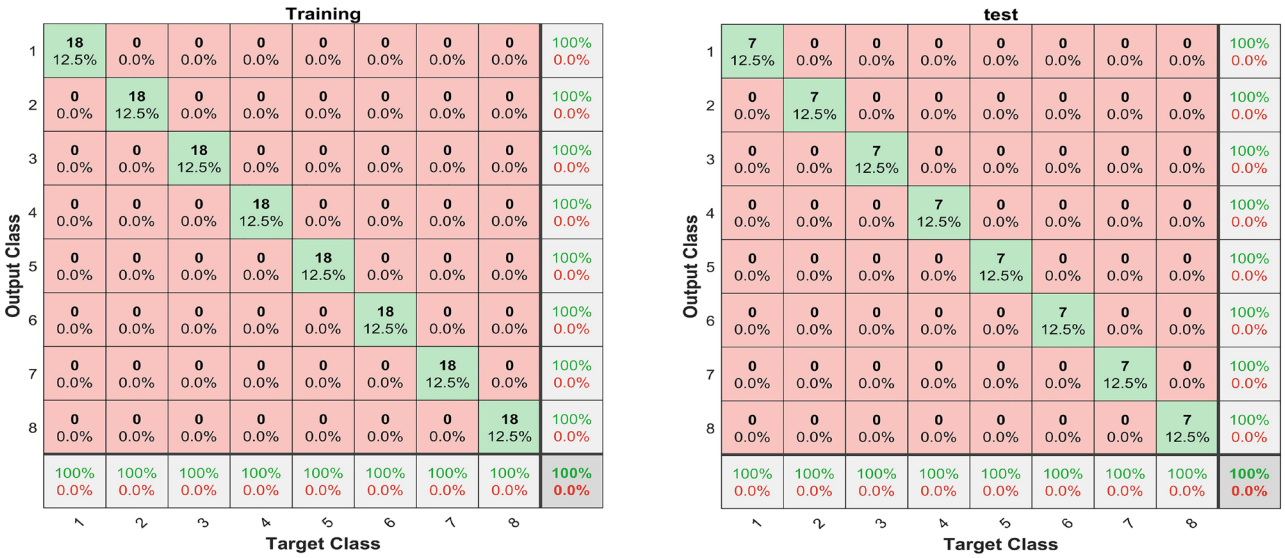

Figure 4. Confusion matrix of GA-SVM for training set and test set. (Classes: 1:D.N, 2: 100, 3: 282, 4:PK2, 5: 261, 6: D.T.1, 7: 270, 8: C.Y.9.

combination of SVM and GA defined as GA-SVM is an efficient tool for pattern recognition and classification.[57-59]

Average error values of 100 times Monte Carlo cross-validation (MCCV) applied on the training set were 0.01 and 0.14 for SVM and PLS-DA, respectively. It should be mentioned that the PLS-DA latent variables and SVM hyperparameters, including box-constraint and kernel scale, were optimized on each iteration of MCCV. As SVM showed a lower error value, it was chosen for further analysis. Table 4 shows the optimized parameters of GA-SVM.

Then, GA-SVM was applied to the training set to select informative regions. Due to the continuity of spectral data, the procedure of selecting well-defined regions is superior to single variables scattered throughout the spectrum. The spectral data were divided into 14 regions. The IR spectra, together with the corresponding 14 regions are presented in Figure 4. The spectral regions between 1660 and 1380 were selected by GA.

Table 5 indicates the accuracy of the established model for train and test sets. The model sensitivity, specificity and accuracy are surprisingly 100%. The model successfully predicts all samples in their own classes. The confusion plot for the training and test sets is presented in Figure 4. The

Table 5. Classification results of GA-SVM.

	100	261	270	282	P.K.2	C.Y.9	D.N	D.T	Correct	Correct Identification Rate (%)
					Train set					
100	18								18	100%
261		18							18	100%
270			18						18	100%
282				18					18	100%
P.K.2					18				18	100%
C.Y.9						18			18	100%
D.N							18		18	100%
D.T								18	18	100%
					Test set					
100	7								7	100%
261		7							7	100%
270			7						7	100%
282				7					7	100%
P.K.2					7				7	100%
C.Y.9						7			7	100%
D.N							7		7	100%
D.T								7	7	100%

most important area in the spectrum for distinguishing the origin of the samples was the region 1380–1660 cm^{-1}, which contains mainly the bands of proteins, and unsaturated lipids.

Conclusion

Tea polyphenols are responsible for multiple and varied health benefits of tea, including antioxidant and chemopreventive effects. This study showed a considerable variation in total phenolic and flavonoid contents, and antioxidant activities among the eight tested cultivars. This data approved that chemical composition of fresh tea leaves is affected by the types of tea cultivars. A significant correlation was detected between antioxidant activity and total phenolic and flavonoid contents. The cultivars DN, PK2, and C.Y.9 had the highest content of phenolic and flavonoid content as well as the best antioxidant activity. Consequently, these three cultivars can be suggested as better dietary sources of natural antioxidants, mainly polyphenols, to be used as a functional drink or dietary supplements. All evaluated cultivars had a considerable amount of EGCG (especially 282**,** DN, PK2, and C.Y.9), which make them better candidates for isolation and purification of EGCG, as the main tea catechin, in further investigations. Although the morphological characteristics of eight cultivars are very similar, their classification can be performed based on chemical components, using chemometric methods linked with ATR-FTIR recorded spectra which is a simple, time-saving, and precise method.

Ethical Issues

This study was approved by the Ethical Committee of Guilan University of Medical Sciences (ID: IR.GUMS.REC.1398.289).

Acknowledgements

This work was supported by Guilan University of Medical Sciences.

Author Contributions

FY: Design of the work, data analysis, project administration, drafting the manuscript; HEN: Data analysis; SD: Data analysis; SS: The acquisition of data; FB: The acquisition of data; FAM: The acquisition of data; SG: Project administration, supervisor, design of the work, data analysis, interpretation of data.

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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