Original Article

Study on the antioxidant activity of tea flowers (*Camellia sinensis*)

Ziying Yang AM, Yi Xu AB, Guoliang Jie AB, Puming He PhD and Youying Tu PhD

Department of Tea Science, Zhejiang University, Hangzhou, China

Major chemical compounds in different extracts from tea flowers (*Camellia sinensis*) were analyzed. Distilled water or 70% ethanol extracts were then fractionated with chloroform, ethyl acetate and n-butanol, respectively. Each extract fraction was tested its scavenging activities on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl free radicals. The results showed that ethyl acetate fraction of ethanol-extract of tea flower (EEA) exhibited the highest quenching activity to hydroxyl radicals (SC_{50} 11.6 μg/ml), followed by ethanol-extract (EE) of tea flower (SC_{50} 19.7μg/ml). Same tea flower extract showed big different scavenging activities on different free radicals. EEA quenched 80% of hydroxyl radicals generated by Fenton's reaction, however, only 40% of DPPH radical was scavenged in the Fe (II)-H_2O_2 -luminol system. The contents of flavones, polyphenols and catechins in EE and EEA fractions were higher than those in other fractions. We suggest that the stronger scavenging abilities to free radicals might be due to polyphenols, EGCG, ECG and flavones. However, the water extracts of tea flower and their fractions showed lower antioxidant activity for their inhibitory effect on hydroxyl radicals and DPPH radicals.

Key Words: tea flower, antioxidant activity, DPPH, hydroxyl radical, chemiluminescence

Introduction

Tea is one of the most widely consumed beverages in the world. Antioxidant activity of tea has been studied extensively. But so far, the study on antioxidant activity of tea flowers has been rarely reported. Tea flowers contained many nutrition compounds, such as protein, sugar, sucrose, vitamin, amino acid, tea polyphenols and caffeine.[1] Tea pollen was characterized as an excellent protein resource.[2] Compared with tea leaves, tea flowers have similar chemical compositions and contain less caffeine but comparable amounts of total catechins.[2-3]

In this study, we extracted tea flowers with distilled water and 70% ethanol respectively, the extracted solution were then successively extracted with chloroform, ethyl acetate and n-butanol, we got different fractions from the ethanol-extracted solution and the water-extracted solution. The contents of tea polyphenol and catechins in different fractions and extracts of tea flower were analyzed, and the antioxidant activities of different fractions and extracts of tea flower were tested by DPPH assay and Fenton-reaction system, which will provide useful information of antioxidant activity of tea flower.

Materials and methods

Preparation of tea flower extract

Freeze-dried tea flowers (from the Tea Resource Garden of Zhejiang Tea Science Research Academy, Hangzhou, China) were ground and extracted with 70% ethanol under reflux for 120 min (solvent: crude tea flower powders = 10:1, v/w) for three times at 50 ºC, then all the extracted solutions were combined. The solution was filtered with a 0.45 μm Millipore filter and concentrated under vacuum to remove ethanol, and the ethanol-extracted tea flower (EE) was obtained. EE was further fractionated by liquid-liquid partitions into four fractions using chloroform, ethyl acetate and n-butanol successively (water: organic solvent = 1:1,v/v). Each organic solvent fraction was concentrated under vacuum to remove the organic solvent, and the chloroform fraction (EEC), ethyl acetate (EEA) and n-butanol (EEB) were obtained. The residual aqueous fraction was lyophilized as the residue fraction of EE (EER). The liquid-liquid partitions were repeated three times. These fractions were used to test scavenging activities on DPPH and hydroxyl free radicals.

The freeze-dried and ground tea flower (30 g) was infused with 200 ml freshly boiled distilled water in an incubator for 30 min at 100 ºC. The water solution was concentrated by vacuum concentration, and a dark brown extract of water-extracted tea flower (WE) was then obtained. WE was dissolved in water and further fractionated by liquid-liquid partitions into four fractions using chloroform, ethyl acetate and 70% ethanol successively, thus four fractions such as chloroform fraction (WEC), ethyl acetate fraction (WEA), ethanol fraction (WEE), and the lyophilized residual fraction of WE (WER) were obtained. These fractions were also used to test the scavenging activities on DPPH and hydroxyl free radicals.

Corresponding Author: Professor Youying Tu, Department of Tea Science, Zhejiang University, 268 Kaixuan Road, Hangzhou, Zhejiang, China 310029
Tel: 86 571 8697 1743; Fax: 86 571 8697 1260
Email: youytu@zju.edu.cn

Assay for the scavenging effect on Hydroxyl radical generated from a Fenton-reaction system

The Hydroxyl radical scavenging activity was assessed according to the methods reported by Cheng and Zhao.[4, 5] The Hydroxyl radical was generated by the Fenton-type reaction at room temperature. The reaction mixture (1.0 ml) contained 500 µl Na_2CO_3-$NaHCO_3$ (pH 10.2, 0.05 M)- buffered solution, 100 µl luminol (2 mM), 100 µl sample solution (with different concentration, replaced with water in the control), 200 µl Fe^{2+}-EDTA (3 mM), and 100 µl H_2O_2 (4.4 mM). Initiation of reaction was achieved by adding Fe^{2+}-EDTA and then H_2O_2 into the mixture, in this trail ascorbic acid was used as a positive control. The dynamic curves were recorded immediately after the initiation of chemiluminescene (CL) reaction.

The hydroxyl radicals scavenging abilities of the extracts of tea flower and their fractions were assessed on an Luminescence Analyzer (Lumat LB9587, German). The CL peak values were recorded in the absence (CLck) or presence (CLsample) of tea flower fractions. The Scavenging rate (S_R) was obtained according to the formula: Scavenging rate (%) = 100 × (CL_{ck} - CL_{sample}) / CL_{ck}.

Assay for the scavenging effect on DPPH radicals

The DPPH radical scavenging activity was determined according to the method of Zhu.[5] Briefly, 1 mM solution of DPPH in ethanol was prepared, and then 1 ml of this solution was mixed with 3 ml of samples dissolved in ethanol containing 5-1000 g of freeze-dried extract; the mixture was then mixed vigorously and maintained at room temperature in the dark. The absorbance was measured at 517 nm (Lengguang model-752, Lengguang Optical Instrument Ltd. Co., Shanghai, China) at regular intervals. A control sample containing the same amount of water and DPPH radical was prepared and measured at the same wavelength as $O.D_0$. This activity is given as change of absorbance and is calculated according to the following equation: Change of absorbance %= ($O.D_0$-$O.D_1$) × 100 / $O.D_0$

Analysis of caffeine and catechins by HPLC [6-8]

One gram of tea flower fractions was dissolved in 100 ml freshly distilled water and filtered through a 0.45 µm Millipore filter for analysis of caffeine and catechin by HPLC. *Analyses of tea polyphenols and flavones:* Tea polyphenols and flavones were analyzed by the spectrophotometric method described by Zhong.[9] Each fraction (0.5 ml) was transferred into a 25 ml test tube to react with 10 ml 1% $AlCl_3 \cdot 6H_2O$ and reacted solution was measured at 420 nm (OD_1). Tea flower sample (0.5 ml) was transferred into a 25 ml test tube, react with 10 ml 1% $AlCl_3 \bullet 6H_2O$, the absorbance (OD_1) was measured at 420 nm.

Statistical analysis: Data analysis was performed using a SAS System (Windows software 8.01, SAS Institute Inc., Cary, NC) as described by Tu.[7] Descriptive statistics were initially performed. Data were presented as mean ± SD. The values are reported as mean ± SD. *p* values were two tailed and $p < 0.05$ was considered as significant.

Results

Scavenging effect on hydroxyl radical

The effect of hydroxyl radical scavenging activities of tea flower is shown in Figure 1and Figure 2. High scavenging activity was obtained from the fractions EE and EEA, and relatively low scavenging activity was found from WE. It was 20% difference between the lowest (EER) and the highest scavenging activities (EE) in all ethanol extracts, and 15% difference between the highest scavenging activities (EE) in ethanol extracts and the highest scavenging activities (WER) in water extracts, as well as 15% difference between the highest scavenging activities (WER) and the lowest scavenging activities (WE) in all water extracts as shown in Fig 1and Fig 2.

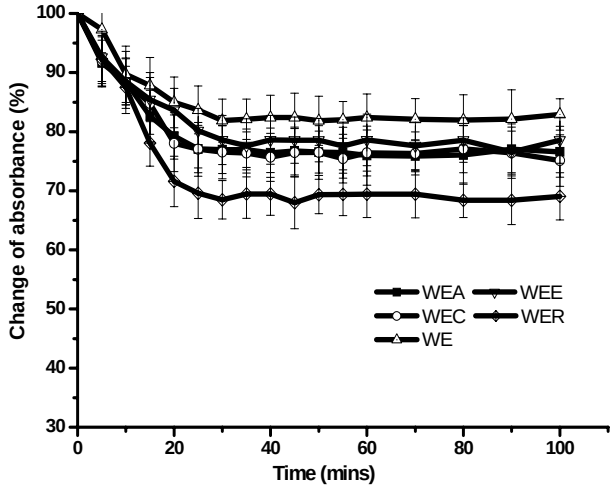

Figure 1. The scavenging effects of tea flower fractions using ethanol to extract on hydroxyl radicals generated by Fenton's reaction.

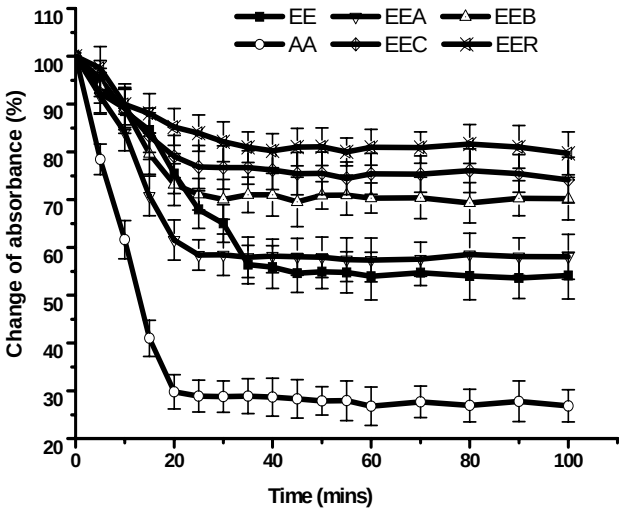

Figure 2. The scavenging effects of tea flower fractions using water to extract on hydroxyl radicals generated by Fenton's reaction. Data are expressed as mean±SD (n = 4). The symbols on solid lines denote as follow: open circle, ascorbic acid (AA) was used as a positive control; solid circle, ethanol extract (EE); open triangle, chloroform fraction of EE (EEC); solid triangle, ethyl acetate fraction of EE (EEA); open square, n-butanol fraction of EE (EEB); solid square, residue fraction of EE (EER). The symbols on dotted lines denote as follow: cross mark, water-extracted tea flower (WE); open circle, chloroform fraction of WC (WEC); solid circle, ethyl acetate fraction of WE (WEA); open triangle, filtrate after added with 70% ethanol of WE (WEE); solid triangle, residue after added with 70% ethanol of WE (WER).

Table 1. Scavenging ability of tea flower extracts and fractions on the chemiluminescence signal caused by hydroxyl radicals

Fraction	Regression equation[a]	R^2	SC_{50} (μg/ml)
AA	*y = 17.3x + 18.7*	0.97**[b]	6.09A[c]
EE	*y = 12.4x + 13.1*	0.93**	19.7C
EEC	*y = 13.4x + 1.63*	0.84**	37.1E
EEA	*y = 14.5x + 14.4*	0.92**	11.6B
EEB	*y = 12.7x + 3.84*	0.87**	38.1F
EER	*y = 8.17x + 8.09*	0.95**	169K
WE	*y = 12.4x - 3.42*	0.90**	74.4G
WEC	*y = 8.60x+ 6.50*	0.90**	157I
WEA	*y = 7.58x + 11.3*	0.93**	165J
WEE	*y = 11.6x - 2.39*	0.91**	92.2H
WER	*y = 11.5x + 9.91*	0.94**	32.5D

[a] y, scavenging rate (%); x, natural logarithm values of corresponding concentrations of tea flower extracts and fractions. [b]** $p < 0.01$. [c] Different letters in the same column indicate a significant variance among these fractions with statistically analyzed by Fisher's Least Significant Difference ($p < 0.01$).

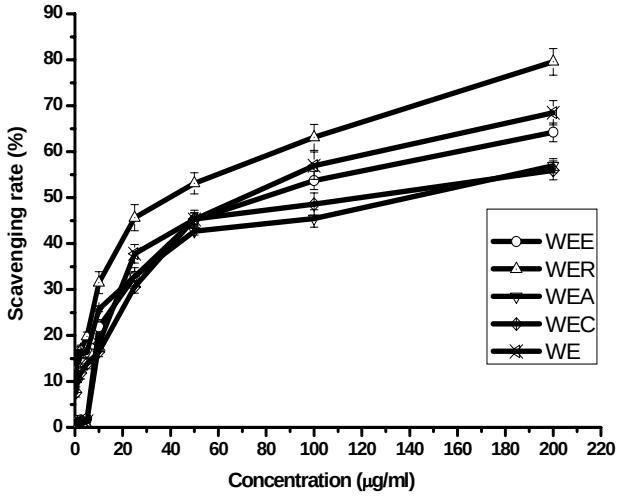

Figure 3. The scavenging effects of tea flower fractions using ethanol to extract on DPPH radical.

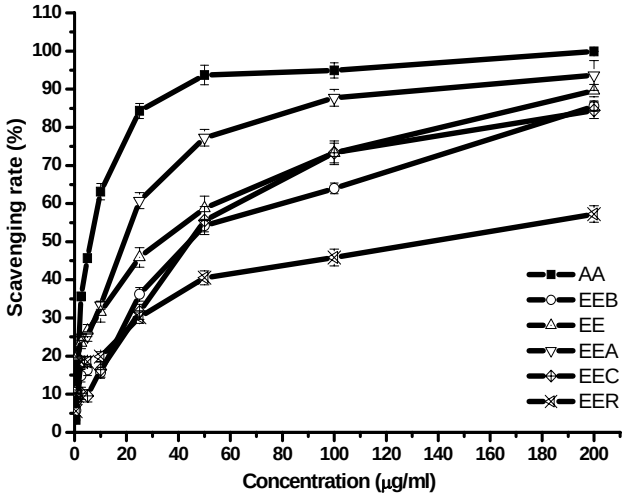

Figure 4. The scavenging effects of tea flower fractions using water to extract on DPPH radical. Data are expressed as mean±SD of (n=4). The symbol denotes is the same as above.

Quenching ability of tea flower extracts and fractions on the CL signal, indicated their potentials to scavenge hydroxyl radicals produced in the Fe (II)-H_2O_2 -luminol system, was represented by 50%-scavenging concentrations (SC_{50}). The lower the SC_{50} value, the higher activity of tea flower extracts and fractions. A good linear relationship was observed between hydroxyl radical scavenging rate (%) and the natural logarithm of the concentrations of tea flower extracts, indicating that the hydroxyl radical scavenging activities of tea flower fractions were dose-dependent. The regression equations and correlation coefficients are listed in Table 1. The SC_{50} values of each extract and fraction were calculated using the regression equations. Based on the comparison on the SC_{50} values of each fraction, EE and EEA exhibited the highest quenching activity to hydroxyl radicals, followed by WER, EEC, EEB, WE, WEE, WEC, WEA and EER. However, and ascorbic acid showed much higher quenching activity than those of tea flower fractions. According to the statistic analyses by Fisher's Least Significant Difference we found that all fractions appeared significant different quenching activities to hydroxyl radicals at $p < 0.01$ level.

Scavenging activity on DPPH radicals

DPPH is a stable free radical that can accept an electron of hydrogen radical to become diamagnetic molecule. The reduction in DPPH radical was determined by the decrease of its absorbance at 517 nm (in ethanol) induced by antioxidants.[10] DPPH reaction has been widely used to test the ability of compounds to act as free-radical scavengers or hydrogen donors and to evaluate the antioxidant activity of foods and plant extracts.[11-13] There was a good linear relationship between the absorbance of DPPH and its concentration. The regression equation was y = 0.0083x ($R^2 = 0.988$, $p < 0.01$, linear range 0 - 420 μM) in this study, where y is the absorbance of DPPH, x is the concentration of DPPH (μM). Each fraction could react completely with DPPH within 30 min and be stable for more than 30 min in Fig 3 and Fig 4. There was 40% difference between the lowest scavenging activities (EER) and the highest scavenging activities (EEA) in all ethanol extracts, and 20% difference between the highest scavenging activities (EEA) in ethanol extracts and the highest scavenging activities (WER) in water extracts, as well as 10% different between the highest scavenging activities (WER) and the lowest scavenging activities (WE) in all water extracts as shown in Fig 3 and Fig 4.

At a concentration of 1 mg/ml, EE and EEA had higher DPPH-scavenging activities than the other fractions, similar results on hydroxyl radical-scavenging ability. Higher DPPH-scavenging activities of EE and EEA

Table 2. The constituents and their content existed in the extract of tea flower fractions and their fractions (mg/g)

	Flavone	Polyphenols	Caffeine	GC	EGC	C	EC	EGCG	GCG	ECG	CG	Total catechins
EE	28.8±0.50	145±1.06	1.02±0.12	1.12±0.12	5.12±0.23	0.45±0.08	2.34±0.16	12.6±0.54	2.36±0.06	12.3±0.60	1.09±0.12	37.4±1.03
EEC	25.1±0.34	129±0.80	25.6±0.25	ND	ND	ND	2.97±0.30	8.75±0.23	ND	6.51±0.41	ND	18.2±0.94
EEA	36.1±0.66	298±1.54	2.29±0.12	5.36±0.35	11.1±0.56	1.79±0.31	9.31±0.67	51.6±0.99	8.14±0.31	42.4±0.21	4.07±0.17	134±1.21
EEB	24.9±0.69	122±2.15	ND	ND	ND	ND	1.30±0.10	5.58±0.27	0.86±0.09	6.32±0.65	0.44±0.01	14.5±0.76
EER	3.31±0.06	20.3±0.27	0.20±0.00	ND	ND	ND	ND	0.26±0.01	ND	0.76±0.02	ND	1.02±0.06
WE	22.2±0.31	37.4±1.06	1.31±0.15	1.61±0.10	1.26±0.09	0.30±0.06	1.57±0.21	ND	ND	0.55±0.00	ND	5.29±0.33
WEC	23.9±0.55	33.8±0.99	34.4±0.64	ND	0.81±0.05	ND	0.54±0.04	ND	ND	ND	ND	1.35±0.06
WEA	25.7±0.19	41.3±1.25	ND	2.99±0.21	1.78±0.12	0.87±0.05	1.85±0.19	ND	ND	0.84±0.04	ND	8.33±0.62
WEE	16.5±1.06	28.3±0.78	ND	0.92±0.01	0.15±0.00	0.11±0.03	ND	ND	ND	ND	ND	1.18±0.05
WER	34.5±1.69	126±1.36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Each value represents the mean±SD (n = 4); ND, not detectable.

were related to the polyphenol and flavone contents (Table 2).

The constituents and their content detected in tea flower

EEA had the highest contents of polyphenols (measured by the spectrophotometric method of colour reaction) or total catechins (analysed by the HPLC method), as shown in Table 2. Especially, the total concentration of epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) was 70% of the total concentration of catechins in EEA. Caffeine and eight kinds of catechins: catechin (C), catechin gallate (CG), epicatechin (EC), ECG, epigallocatechin (EGC), EGCG, gallocatechin (GC), and gallocatechin gallate (GCG) were found in EE, EEA, EEB and EEC. However, gallic acid conjugated catechins, such as EGCG, GCG, ECG and CG, was scarcely in EER, WE, WEA, WEC, WER and WEE.

Discussion

According to the property of chemical compound, caffeine and pigments has a good solubility in chloroform, flavones and polyphenols are a good solubility in ethyl acetate, and n-butanol can precipitate the polysaccharide. In this study we used distilled water or 70% ethanol to extract tea flower then isolated caffeine, pigments, flavones, polyphenols and polysaccharide with chloroform, ethyl acetate and n-butanol, respectively. We measured the contents of caffeine, flavones and polyphenols including catechins in all extracts.

Flavonoids, including flavonols, flavanols, flavones, and isoflavones have capacity to act as antioxidants.[14] Flavonoids can scavenge free radicals directly and stabilize the reactive oxygen species by reacting with the reactive compounds. It is well known, flavones have a good solubility in ethyl acetate, ethanol and *n*-butanol, therefore, the concentration of flavones in EE was 30% higher than in WE. We found another result in table 2, the concentrations of flavones in EEA was the highest in ethanol extracts, as well as WEA in water exracts. It is the same tendency to scavenging activities, the higher concentration of flavones in fraction, the stronger of scavenging activities, which implies that flavones was one of the important reasons for scavenging free radicals in this study. On the other hand, the concentrations of flavones in EER were much lower than in WER, which means the extraction efficiency of using ethanol to extract flavones from tea flower was better than that of using water.

The results showed that polyphenols, EGCG, and ECG were the main compounds to affect the free radical scavenging activities in this study, for example, polyphenols, EGCG, and ECG showed the highest concentrations in all fractions (Table 2), and EEA appeared the highest scavenging hydroxyl radicals according to the SC_{50} values 11.6μg/ml (Table 1). The SC_{50} values of EE for scavenging hydroxyl radicals was19.7μg/ml flowed by EEA, EE has the good ability for scavenging free radical, the concentrations of polyphenols, EGCG and ECG in all fractions were ranked on the second. Because the ortho- trihydroxyl group in the B ring and the galloyl moiety at 3 position of flavan-3-ol skeleton are the most important structural features for displaying an excellent scavenging ability on the DPPH radical.[15]

The same tea flower extract showed big different scavenging activity for different free radicals. For example, EEA quenched 80% of hydroxyl radicals generated by Fenton's reaction, however, only 40% of DPPH radical was scavenged in the Fe (II)-H_2O_2-luminol system. Whatever, EEA has the highest scavenging activities in all extracts.

In the present study, we have demonstrated that the tea flower extract and their fraction had inhibitory effects on the hydroxyl radicals and DPPH radicals, similar with the results reported by Yung SL.[5] Compared with the ethanol extract and its fractions, the water extract of tea flower and its fractions showed lower antioxidant activity for their inhibitory effect on hydroxyl radicals and DPPH radicals, It might be the abundant of polysaccharide and protein were came out when using water as solvent to extract the tea flower, and polysaccharide and protein were combined with catechins in water extracts by hy-

droxyl group, which caused HPLC could not isolate and measure catechins.

References

1. Wu XY, Xiong B Z, He ML, Miao AQ, Li Q. Studies on the utilization of tea (*Camellia sinensis*) flowers and fruits. Guangdong Tea (in Chinese) 2003; 1: 11-23.
2. Su SK, Chen SL, Lin XZ, Hu FL, Shao M. The determination of ingredient of tea (*Camellia sinensis*) pollen. Apiculture of China (in Chinese) 2000; 51: 3-5.
3. Yung SL, Sang SW, Jen KL. Determination of tea polyphenols and caffeine in tea flowers (Camellia sinensis) and their hydroxyl radical scavenging and nitric oxide suppressing effects. J Agric Food Chem 2003; 51: 975-978.
4. Cheng ZY, Yan GT, Li YZ, Chang WB. Determination of antioxidant activity of phenolic antioxidants in a Fenton-type reaction system by chemiluminescene assy. Anal Bioanal Chem 2003; 375: 376-380.
5. Zhu QY, Robert MH, Jodi LE, Roberta RH, Carl LK. Antioxidant activities of oolong tea. Agric Food Chem 2002; 50: 6929-6934.
6. Liang YR, Lu JL, Zhang LY. Comparative study cream in infusions of black tea and green tea [*Camellia sinensis* (L.) O. Kuntze]. Int J Food Sci Technol 2002; 34: 627-634.
7. Tu YY, Xu XQ, Xia HL, Watanabe N. Optimization of theaflavin biosynthesis of tea polyphenols using an immobilized enzyme system and response surface methodology. Biotechnol Lett 2005; 27: 269-274.
8. Tu YY, Xia HL, Watanabe N. Changes in catechins during the fermentation of green tea. Appl Biochem Microbiol 2005; 41: 574-577.
9. Zhong L. Analysis methods of tea polyphenols. In "Methods of chemical and physical evaluation of tea quality" (in Chinese). Shanghai Science and Technology Press, Shanghai1, 1989.
10. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958; 26: 1199-1200.
11. Porto CD, Calligaris S, Cellotti E, Nicoli MC. Antiradical properties of commercial cognacs assessed by the DPPH test. J Agric Food Chem 2000; 48: 4241-4245.
12. Cotelle N, Bernier JL, Catteau JP, Pommery J, Wallet JC, Gaydou EM. Antioxidant properties of hydroxyl-flavones. Free Radical Biol Med 1996; 20: 35-43.
13. Soares JR, Dinis TC P, Cunha AP, Almeida LM. Antioxidant activities of some extracts of thymus zygis. Free Radical Res 1997; 26: 469-478.
14. Robert JN, Els N, Danny EH, Petra GB, Klaske N, Paul AL. Flavonoids: A review of probable mechanisms of action and potential applications. Am J Clin Nutri 2001; 74: 418-425.
15. Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1- diphenyl-2-picrylhydrazyl radical. Free Radic Biol Med 1996; 21:895-902.