Antioxidant and Anticholinesterase Properties of *Sideritis perfoliata* subsp*. athoa* (Papan. & Kokkini) Baden and *Sideritis trojana* Bornm. Teas from Mount Ida-Turkey and Their Phenolic Characterization by LC-MS/MS

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Abstract: The phenolic profile, antioxidant, and anticholinesterase activities of teas prepared from two *Sideritis* (L.) species: *Sideritis perfoliata* subsp. *athoa (Papan. & Kokkini*) Baden and *Sideritis trojana* Bornm., collected from Mount Ida (Kaz Dağı) Turkey, were determined. The teas were prepared by infusion and decoction methods. The quantitative amounts of the phenolic contents were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The significant phytochemicals were found to be fumaric acid for infusion and decoction samples of *S. trojana* and infusion of *S. perfoliata* subsp*. athoa*, chlorogenic acid for decoction of *S. perfoliata* subsp*. athoa.* The tea samples prepared by the decoction method were found to be rich in phenolics. Apart from the fact that the decoction sample of *S. trojana* exhibited the best antioxidant effect in 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene bleaching and cupric (Cu^{2+}) ion reducing power assay (CUPRAC) methods among the tested samples at all concentrations, and showed significant inhibition effect at 200 µg/mL against acetylcholinesterase and butyrylcholinesterase enzymes (59.74%, 64.99%, respectively).

Keywords: *Sideritis, Sideritis perfoliata* subsp*. athoa (Papan. &Kokkini*) Baden, *Sideritis trojana* Bornm., Antioxidant activity, Anticholinesterase activity, Phenolics.

Submitted: April 11, 2020. **Accepted:** June 15, 2020.

Cite this: CARIKCI S. Antioxidant and Anticholinesterase Properties of Sideritis perfoliata subsp. athoa (Papan. & Kokkini) Baden and Sideritis trojana Bornm. Teas from Mount Ida-Turkey and Their Phenolic Characterization by LC-MS/MS. JOTCSA. 2020;7(2):617–32.

DOI: https://doi.org/10.18596/jotcsa.718274.

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INTRODUCTION

For centuries, herbal teas have been used as herbal remedies to treat infections, ailments, and diseases. For the usage as a health-promoting beverage, the evaluation of the functional and bioactive composition of the tea, such as polyphenols, carotenoids, vitamin C, vitamin E, as well as essential major, minor, and trace elements are essential. Some studies have been reported their antioxidative, antimicrobial, anticarcinogenic, antihypertensive, antimutagenic, and antiangiogenic activities. These activities have shown that they are related to different bioactive components included in herbal teas (1–4).

The antioxidant activity is based on the availability of electrons to neutralize any free radicals. Phenolic compounds are known to be antioxidant compounds. The antioxidant activity of plants is attributed mainly to their phenolic compounds (1,3-4). Alzheimer's disease (AD) is the most common form of dementia. In addition to synthetic drugs, the treatment of this disease has gained significant importance with natural treatments. Therefore, finding natural sources to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which AD begins as a deficiency in the production of the neurotransmitter acetylcholine inhibitors, has been the subject of many studies recently.

The genus *Sideritis* (L.), a member of the Lamiaceae plant family, is distributed mainly in the Mediterranean area over 150 annual and perennial species (5,6). In Turkey, as well as in the world, *Sideritis* species is very widely consumed as herbal tea and has been used in folk medicine for their activities such as anti-inflammatory, antirheumatic, antispasmodic, and antimicrobial (7-11). In Turkey, the genus *Sideritis* is divided into three sections (Hesiodia, Burgsdorfia, and Empedoclia), and Turkey is the gene center of the Empedoclia section (5). They represented 46 species (52 taxa) with a very high endemism ratio (almost 80%) (9). Most of the studies carried on *Sideritis* species are focused on their bioactive constituents; essential oils, diterpenoids, glycosides- and activities of their extracts, and isolated pure compounds (1,3,6-13). Although they have been used as a tea for a long time, the studies of aqueous extracts of the genus *Sideritis* have recently become more popular. Traditionally, *Sideritis* teas are prepared via the infusion method using flowering branches of the plant. The studies showed that the main chemicals of the infusions are composed of water-soluble polyphenolic compounds, many of which have related to their potent antioxidant properties, also minerals and vitamins. The phytochemical composition and several activities of some *Sideritis* tea; *Sideritis scardica, S. raeseri, S. syriaca*, and S. *clandestina* subsp. *clandestina* have been reported previously (1,3,15–17).

Mount Ida, which is one of the wealthiest mountains concerning rareness and endemic species, is located between Balikesir and Canakkale boundaries in Turkey. *Sideritis perfoliata* subsp. *athoa* (Papan. & Kokkini) Baden., and *S. trojana* Bornm. are grown at Mount Ida. *S. trojana* is an endemic species, widely known as "Sarıkız çayı" and *S. perfoliata* subsp*. athoa* is known as "Kedi kuyruğu çayı". The teas prepared from both species are widely consumed with a view to its distinctive flavor and possible pharmacological effects such as common cold, including fever, flu, and bronchitis, and to relieve gastric disorders (18). Both species have been investigated for their essential oil, diterpenoids, and glycoside composition, and several activities in some studies (Table 1). There is no study on the detailed phenolic composition and anticholinesterase activity of teas prepared from *S. trojana* and *S. perfoliata* subsp*. athoa*.

The objective of the present study is to determine phenolic composition as well as antioxidant and anticholinesterase activity of the *S. trojana* and *S. perfoliata* subsp*. athoa* herbal teas. Two methods were used to prepare tea samples: infusion and decoction. The determined phenolic contents, divided of mainly three groups: flavonoids and derivatives, phenolic acids, and dicarboxylic acid, were screened by LC-MS/MS. Antioxidant properties were determined based on 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene linoleic acid, and cupric (Cu^{2+}) ion reducing power assay (CUPRAC). The anticholinesterase activity was also evaluated. This study is the first one on the phenolic composition and activity of the *S. trojana* and *S. perfoliata* subsp*. athoa* herbal teas.

Table 1. Previous studies on *S. trojana* and *S. perfoliata* subsp. *athoa.*

	S. trojana	***S. perfoliata* subsp. *athoa***
Major comp. of Essential oil (EO)	β-pinene α-pinene (19) Valeranone α-bisabolol β-caryophyllene (20)	Myrcene β-pinene Ar-curcumene (25)
Diterpenoids	7-*epi*-Candicandiol Siderol Sideridiol Isocandol B Candol A acetate *Ent*-7α-acetoxy-kaur-15-ene *Ent*-7α-acetoxy-15β,16β-epoxy-kaurane (7-Acetyl-sideroxol) *Ent*-2α-hydroxy-8(14),15-pimaradiene (21)	Linearol Foliol Sidol *Ent*-3β,7α-dihydroxy-kaur-16-ene *Ent*-7α,18-dihydroxy-beyer-15-ene 7-epi-Candicandiol *Ent*-3β-hydroxy-kaur-16-ene Athonolone *Ent*-3α,18-dihydroxy-kaur-16-ene (26, 27)
Phenolics, Flavonoids and derivatives	Melittoside 10-*O*-(*E*)-feruloylmelittoside 10-*O*-(*E*)-p-coumaroylmelittoside Stachysoside E Stachysoside G Verbascoside	

	Isoacteoside Lamalboside Leonoside A Isolavandulifolioside Isoscutellarein 7-*O*-[6‴-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside 4′-*O*-Methylisoscutellarein 7-*O*-[6‴-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside 3′-Hydroxy-4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside Di-*O*-methylcrenatin (22)	
Activity	Antistress (23) Antimicrobial activity of EO (20) Antioxidant and antidiabetic activity (24)	*Candida albicans* Inhibitory (28) Insecticidal (29)

MATERIALS AND METHODS

General

LC-MS/MS experiments were performed by a Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometry, equipped with a Synergy Max C18 column (250 x 2 mm i.d., 5-μm particle size). The compounds used as standards in LC-MS/MS analyses and the experimental details are given in the supplementary data. For the antioxidant and anticholinesterase activities, absorbances (UV and visible range of 230 nm to 750 nm) were measured using a multiplate reader (Beckman Coulter DTX 880 Multimode Detector).

Plant Material

Sideritis perfoliata subsp.*athoa (Papan. &Kokkini*) Baden. were collected from natural habitats in B1, Balikesir-Edremit, Mount Ida (Kazdağı, Kapıdağ), around the fire tower, rocky areas, 1350 m altitude, July 2013, Turkey (herbarium number TD 3805).

Sideritis trojana Bornm., originated in Balikesir-Edremit, Mount Ida, near Sarıkız hill, limestone rocks, 1750 m altitude, July 2013, Turkey (herbarium number TD 3818). The species were identified by Prof. Dr. Tuncay Dirmenci (Balıkesir University), voucher specimens have been deposited at Balikesir University Necatibey Education Faculty Herbarium. The plant samples were allowed to dry in the shade.

Preparation of decoction and infusion samples

Herbal teas were prepared as following the traditional preparation method: infusion and decoction.

Infusion: Two grams of the dried aerial parts of the plant were added in a beaker which contained 100 mL of distilled boiled water and allowed to stay for 15 minutes. Then it was filtered through Whatman No.1 filters (Sigma-Aldrich). The filtrate (25 mL) was diluted with 25 mL of distilled water.

Decoction: Two grams of the dried aerial parts of the plant were added to 100 mL of distilled water in a beaker and brought to boiling. After the boiling was stopped, the mixture was left for 15 min and filtered. The filtrate (25 mL) was diluted with 25 mL of distilled water.

Infusion samples were named as **SPAI** (infusion sample of *S. perfoliata* subsp. *athoa* (Papan. & Kokkini) Baden.) and **STI** (infusion sample of *S. trojana* Bornm.), decoction was **SPAD** (decoction sample of *S. perfoliata* subsp. *athoa* (Papan. & Kokkini) Baden.) and **STD** (decoction sample of *S. trojana* Bornm).

Antioxidant Activities

The antioxidant activity was evaluated using in vitro the free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH·) (DPPH assay) (30), *β*-carotene-linoleic acid model system (31) and CUPRAC (32). BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) were used as standards in DDPH and *β*-carotene-linoleic acid assay. In CUPRAC assay ethanol was used as a negative control; whereas curcumin was used as a positive control. A detailed experimental procedure for the activity studies were given in the supplementary data.

Anticholinesterase activity

For the measurement of anticholinesterase activity, inhibition of AChE and BChE were measured by the slightly modified spectrophotometric method developed by Ellman, Courtney, Andres, and Featherston (33). A detailed procedure was given in supplementary material.

LC-MS/MS experiments

In the LC-MS/MS experiments the mobile phase was composed of water (A, 0.1 % formic acid) in methanol (B, 0.1 % formic acid), the gradient program of which was 0-1.00 minute 55 % A and 45

% B, 1.01-20.00 minutes 100 % B and finally 20.01-23.00 55 % A and 45 % B. The flow rate of the mobile phase was 0.25 mL/min, and the column temperature was set to 30 ˚C. The injection volume was 10 µL.

The mobile phase was composed to be a gradient of acidified methanol and water system because of its fragmented ion stability. The optimum ESI (Electrospray Ionization) parameters were identical to those described in the recent study (13-14). Detailed information, validation of experiments and uncertainty evaluation is given in supplementary data.

Preparation of test solution for LC-MS/MS

A one mL of each sample was added 4 mL of the ethanol-water mixture (50:50 v/v). A portion of 1 mL of this stock solution was transferred into a 5 mL of another volumetric flask, and 50 mL of curcumin solution was added as internal standard and diluted to the volume with methanol and mixed. The solution was filtered through a 0.45 µm Millipore Millex-HV filter and the final solution (1 mL) was transferred into a capped auto sampler vial and 10 mL of sample was injected to LC for each run. The samples in the auto sampler were kept at 15 °C during the experiment.

Statistical Analysis

In both antioxidant and anticholinesterase activity tests, the experimental data were calculated as the mean ± standard deviation and analyzed. Variance ANOVA was studied, including one-way analysis. Significant differences between means were recorded by Duncan's multiple range tests. $p<0.05$ was regarded as significant, and $p<0.01$ was very significant.

RESULTS AND DISCUSSION

Table 2. Phenolic composition of *Sideritis trojana* and *S. perfoliata* subsp. *athoa*

	SPAI	SPAD	STI	STD
Flavonoids and derivatives				
Rutin (1)	1.64±0.11	-	1.70±0.11	-
Quercitrin (2)	-	15.17±0.97	-	-
Apigenin (3)	-	-	12.17±0.98	16.61±1.34
Penduletin (4)	16.73±1.7	58.53±5.93	2.92±0.3	59.30±6.01
Quercetagetin-3,6-dimethylether (5)	110.87±20.76	-	-	27.32±5.12
Luteolin-7-*O*-glucoside (6)	7.34±0.37	22.26±2.27	-	1.84±0.19
Luteolin-5-*O*-glucoside (7)	-	4.60±0.3	-	-
Pelargonin (8)	7.30±0.37		46.21±2.35	47.86±4.87
Total (mg/kg)	**143.88**	**100.56**	**63.00**	**152.93**
Phenolic acids				
p-Coumaric acid (9)	2.65±0.41	-	-	-
Caffeic acid (10)	34.65±6.86	28.36±5.61	22.89±4.53	15.53±3.07
t-Ferulic acid (11)	201.26±14.06	-	107.32±7.5	-
Chlorogenic acid (12)	205.28±28.43	**210.89±29.2**	18.20±2.52	184.86±25.6
Rosmarinic acid (13)	3.94±0.3	3.73±0.29	4.19±0.32	4.57±0.35
Gallic acid (14)	4.65±0.32	4.55±0.32	4.63±0.32	4.54±0.31
Syringic acid (15)	-	2.92±0.2	**-**	28.80±1.94
Total (mg/kg)	**452.43**	**250.945**	**157.23**	**218.30**
Dicarboxylic acid				
Fumaric acid (16)	**281.06±19.49**	206.49±14.32	**310.62±21.54**	**462.14±32.05**
Total (mg/kg)	**281.06**	206.49	**310.62**	**462.14**
Total (mg/kg)	**877.37**	**557.50**	**530.85**	**853.37**

Major compounds of the extracts showed as **bold**.

Phenolic Profile

The characterization of the phenolic components of decoction and infusion samples of *S. perfoliata* subsp. *athoa* and *S. trojana* was achieved by LC/MS analysis. The results were given in Table 2. In the tea samples, around 10 to 12 phenolic compounds were determined. *S. perfoliata* subsp. *athoa* was found to be rich in phenolic acids, whereas *S.*

trojana was rich in dicarboxylic acid. SPAI was found to be the richest in terms quantity (877.37 mg/kg) and numbers of determined components (12 phenolic compounds).

Fumaric acid was the main component for the infusion sample of *Sideritis perfoliata* subsp. *athoa*, infusion sample of *S. trojana* (STI) and decoction sample of *S. trojana* (STD) (281.06, 310.62, 462.14 mg/kg, respectively). For decoction sample of *S. perfoliata* subsp. *athoa* (SPAD), chlorogenic acid was determined as the main component (210.89 mg /kg). The three main components for tea samples were determined as follows: fumaric acid, chlorogenic acid, t-ferulic acid for SPAI; chlorogenic acid, fumaric acid, penduletin for SPAD; fumaric acid, t-ferulic acid, pelargonin for STI and fumaric acid, chlorogenic acid, penduletin for STD. It was previously shown that *Sideritis* extracts, especially methanolic, were rich in phenylpropanoids like verbascoside and martinoside; flavones like apigenin and penduletin; and flavone glucosides like isoscutellarein and hypolaetin (12,13,34–36). In the aqueous extract, in addition to these compounds, mainly chlorogenic acid, ferulic acid, cinnamic acid, and caffeic acid, were determined (3,15,37,35).The results are consistent with previous studies.

Antioxidant and Anticholinesterase Activities

Since a higher correlation between phenolics and antioxidant activity has been demonstrated by several studies in aromatic and medicinal plants (39–42), there is a growing interest in phenolic compounds and flavonoids, which are the most widely occurring chemicals in plants having strong antioxidant properties. Due to the toxic effect of synthetic derivatives humans prefer taking these compounds supplied by nutritional sources such as fruits, vegetables, and herbal tea, which have high phenolic content and good antioxidant capacity.

Antioxidant activities of teas were determined according to three methods: DPPH, β-carotene, and CUPRAC. The results are given in Table 3. For DPPH and β-carotene, the activity tests were carried on four different concentrations: at 10 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL. In the DPPH method, for all concentrations, SPAI, SPAD, and STD showed very high radical scavenging activity (up to 60%), while STI had relatively lower activity. STI has showed the best inhibition activity at a concentration of 100 μg/mL with 42.34%, while BHA was 62.39% and BHT was 80.82%.

Considering that these three tea samples are rich in fumaric acid and chlorogenic acid, it can be said that these compounds are responsible for the significant antioxidant activity of the teas. Most studies on phenolic contents of herbal teas showed that the antioxidant activity of the teas could be attributed to the presence of phenolic compounds. Notably, the antioxidant capacities of the teas having chlorogenic acid were found to be reasonably high (3,43,44). Although it is not a phenolic compound, fumaric acid is a compound commonly detected in plant extracts, and plants carrying this compound at a high amount have been found to exhibit high antimicrobial and antioxidant activity (14,45). Furthermore, in the β-carotene-linoleic acid method, the SPAI has had the best activity value, like the standard BHA. In the CUPRAC, $TEAC_{CUPRAC}$ values of the teas were calculated by using curcumin as a reference. $TEAC_{CUPRAC}$ of curcumin was found as 0.9 mmol TR g^{-1}(in Trolox mM equivalents of 1 mM antioxidant solution which studied). The tea samples prepared by the decoction method were showed higher activity (for SPAD 2.21 mmol TR g^{-1} and for STD 2.23 mmol TR g^{-1}), while the lowest activity was showed by STI (0.43 mmol TR g^{-1}), which was the lowest tea sample in terms of total phenolic content (Table 3).

The anticholinesterase activities of the tea samples were determined at a concentration of 200 μg/mL, and inhibition % values were calculated against AChE and BChE activities. The results are given in Table 4. AChE and BChE were inhibited by galantamine, which was used as a standard, at a rate of 86% and 77%, respectively. Among the tea samples, the best inhibition values against the AChE enzyme were shown by STD of 59.74% and SPAI of 58.30%. For the BChE enzyme, the results were similar. STD and SPAI had the best inhibition values; 64.99% and 53.60%, respectively. STI has the lowest inhibition against both enzymes. Although the antioxidant activities of the tea samples resulted from their high phenolic acid contents, Orhan et al., (2007) (46) reported that there was no correlation between AChE and BChE enzyme inhibition with phenolic contents. They had reported that some of these compounds are not inhibitor for AChE and BChE. Rather than phenolic acids, flavonoid derivatives such as quercetin, genistein, luteolin-7-*O*-rutinoside were found to be more effective inhibitors. If the flavonoid content of the tea samples were compared, it was found that STD and SPAI samples with higher flavonoid content (in total 152.93 and 143.88 mg/kg, respectively) were showed higher enzyme inhibition. STI, with the lowest amount of flavonoid content, has the lowest inhibition. These results are consistent with the literature.

Table 3. Activity results of SPA and ST.

		10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
DPPH (Inhibition %)	**SPAD**	68.92±0.70	68.98±0.68	62.78±0.65	65.83±0.30
	STD	69.38±0.93	68.47±0.40	65.92±0.90	62.98±1.20
	SPAI	66.25±0.72	67.31±2.70	64.73±2.54	62.33±1.24
	STI	4.88±0.84	4.99±1.18	8.85±2.63	42.34±3.26
	BHA	22.75±2.15	30.97±4.14	48.17±3.94	62.39±2.99
	BHT	73.09±2.62	77.68±0.74	78.79±0.76	80.82±1.56
β-carotene-linoleic acid (Inhibition %)	**SPAD**	53.44±1.55	61.27±1.12	59.25±0.67	47.82±0.23
	STD	70.47±1.55	69.88±2.13	66.34±2.15	54.83±2.55
	SPAI	39.96±2.6	85.87±0.46	70.49±3.77	76.95±0.54
	STI	5.77±1.45	68.38±6.47	54.72±1.73	62.24±2.58
	BHA	81.90±1.95	85.54±1.73	85.98±2.42	79.54±4.13
	BHT	82.56±5.03	72.38±11.8	77.12±2.93	71.02±1.01
CUPRAC		**mmol TR/g**			
	SPAD	2.21			
	STD	2.53			
	SPAI	1.43			
	STI	0.43			
	Curcumin	0.90			

Table 4 Anticholinesterase Activity of *S. trojana* and *S. perfoliata* subsp. *athoa*

Tea Samples	Anticholinesterase Activity	
	AChE	**BChE**
SPAI	58.30 ± 0.48	53.60 ± 0.33
SPAD	46.82 ± 0.74	32.40 ± 0.22
STI	30.06 ± 0.67	22.78 ± 0.04
STD	59.74 ± 0.80	64.99 ± 0.36
Galantamine	86.73 ±1.25	77.13 ± 1.48

% inhibition values at 200 µg/mL concentration of tea samples.
Galantamine was used as a standard.

CONCLUSION

In conclusion, *Sideritis perfoliata* subsp. *athoa* and *S. trojana* were found as a crucial antioxidant source in different *in vitro* tests like DPPH, β-carotene linoleic acid, and CUPRAC assays. Also, they have the best inhibition results on two enzymes, which have essential roles in Alzheimer's Disease. The quantities of some phenolics of the infusion and decoction of *S. perfoliata* subsp. *athoa* and *S. trojana* were successfully characterized by LC-MS/MS, and chlorogenic acid, t-ferulic acid, penduletin, and fumaric acid were determined as the most abundant chemicals for tea samples. The antioxidant and anticholinesterase activities of the *S. perfoliata* subsp. *athoa* and *S. trojana* were probably their phenolic and flavonoid composition. Furthermore, the activity could be attributed to the possible synergistic interaction between phenolic and non-phenolic components in its chemical composition. According to the results, it can be said that *S. trojana* and *S. perfoliata* subsp*. athoa* can be used as a supportive food for an antioxidant source for daily take.

Supplementary data
Method validation parameters of LC-MS/MS measurements, standards chromatogram of secondary metabolites, structures of the determined phenolics and procedures of biological activity assays can be found in the Supplementary Information section of this document.

Conflict of interest
The author declares no competing financial interest.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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SUPPLEMENTARY DATA

Antioxidant and Anticholinesterase Properties of *Sideritis perfoliata* subsp. *athoa* (Papan. & Kokkini) Baden and *Sideritis trojana* Bornm. Teas from Mount Ida-Turkey and Their Phenolic Characterization by LC-MS/MS

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Table S1. Method Validation Parameters of LC-MS/MS Measurements

	Compounds	Linear regression equation	R^2	LOD (mg/L)	LOQ (mg/L)	RSD (%)
	Kaempferol	y=0.2040x+0.0573	0.9925	0.002	0.008	5.47
	Kaempferon-3-O-Rutinoside	y=0.1080x+0.0135	0.9977	0.014	0.045	8.15
	Salvigenin	y=0.0355x+0.8620	0.9912	0.036	0.119	5.21
	Penduletin	y=0.1630x+0.0262	0.9965	0.089	0.297	9.47
	Isorhamnetin	y=0.0739x+0.5100	0.9608	0.088	0.294	3.67
	Quercetin	y=0.1150x+0.0078	0.9938	0.001	0.002	0.11
	Quercetagetin-3,6-dimethylether	y=0.0181x+0.0202	0.9924	0.022	0.074	0.1
	Isoquercetin	y=0.0115x+0.0215	0.9959	0.199	0.665	9.42
	Quercitrin	y=0.0290x+0.0058	0.9918	0.001	0.002	4.28
	Luteolin	y=0.2120x+0.0699	0.9937	0.062	0.207	0.16
	Luteolin-7-glucoside	y=0.1350x+0.0246	0.9957	0.022	0.072	8.56
	Luteolin-5-glucoside	y=0.2300x+0.0413	0.9926	0.01	0.034	1.12
	Apigenin	y=0.1780x+0.0850	0.9961	0.15	0.501	4.01
	Rutin	y=0.0232x+0.0008	0.9969	0.01	0.034	7.9
	p-Coumaric acid	y=0.2670x+0.1810	0.9774	0.006	0.021	6.39
	Caffeic Acid	y=0.3300x+0.0036	0.9924	0.028	0.093	8.04
	t-ferulic acid	y=0.0655x+0.0266	0.9925	0.047	0.158	5.21
	Chlorogenic Acid	y=0.2620x+0.0674	0.998	0.445	1.483	5.45
	Rosmarinic acid	y=0.1960x+0.0043	0.9982	0.022	0.072	3.73
	Fumaric Acid	y=0.0569x+0.0177	0.9912	0.003	0.01	5.44
	Pyrogallol	y=0.0438x+0.0073	0.9803	0.001	0.002	5.47
	Ellagic acid	y=0.0244x+0.0048	0.9951	0.02	0.068	0.11
	Vanillin	y=0.0982x+0.0158	0.9982	0.019	0.064	6.57
	Syringic acid	y=0.0305x+0.0079	0.9973	0.022	0.073	8.39
	Salicylic acid	y=0.0255x+0.1780	0.9701	0.211	0.704	0.21
	p-OH benzoic acid	y=0.1230x+0.0280	0.9939	0.002	0.007	4.78
IS*	**Curcumin***			369.3	176.9	20

*** Used as internal standard**

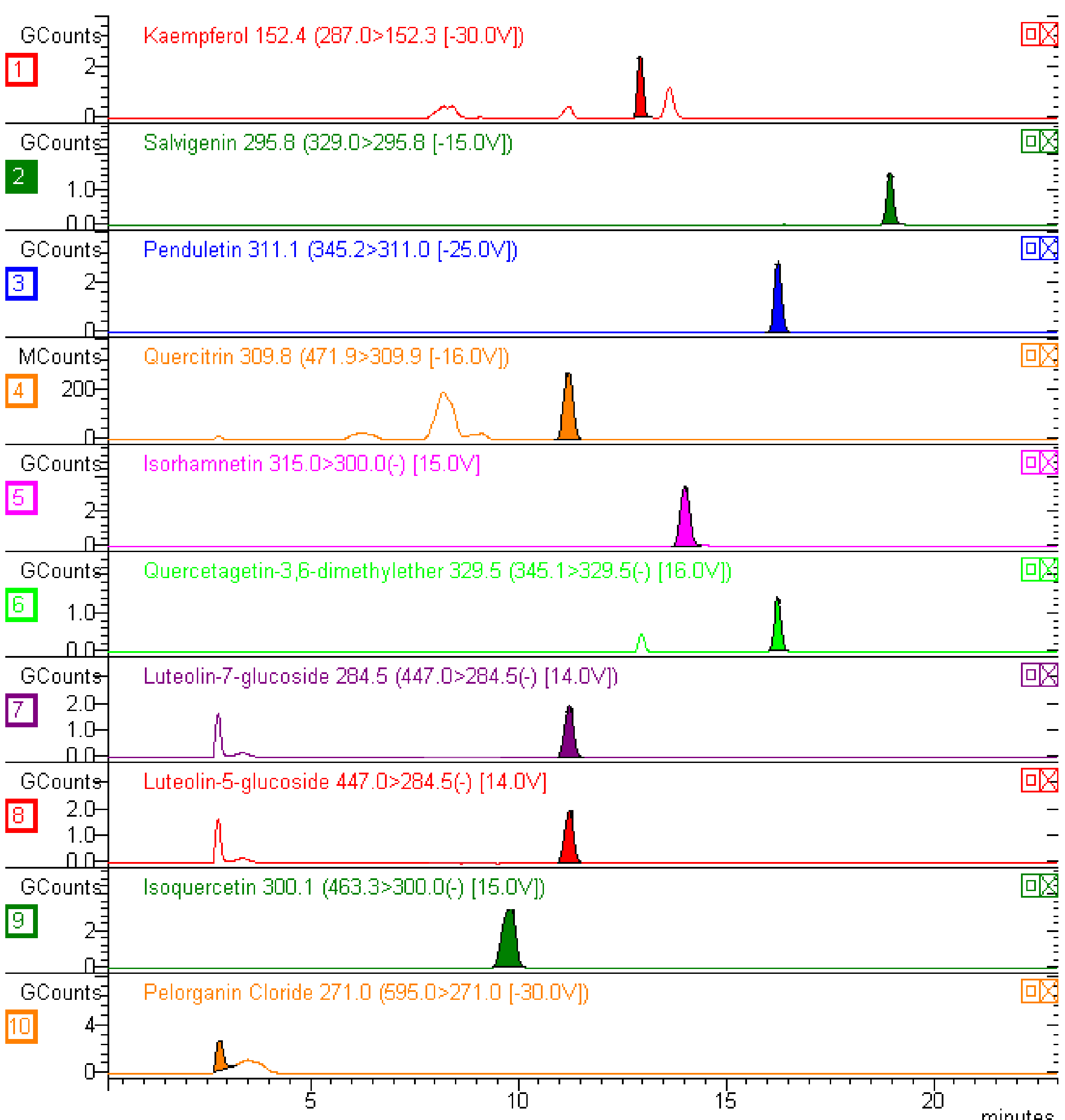

Figure S1 : Standards Chromatogram of Secondary Metabolites (Flavonoids) by LC-MS/MS (5 mg/L)

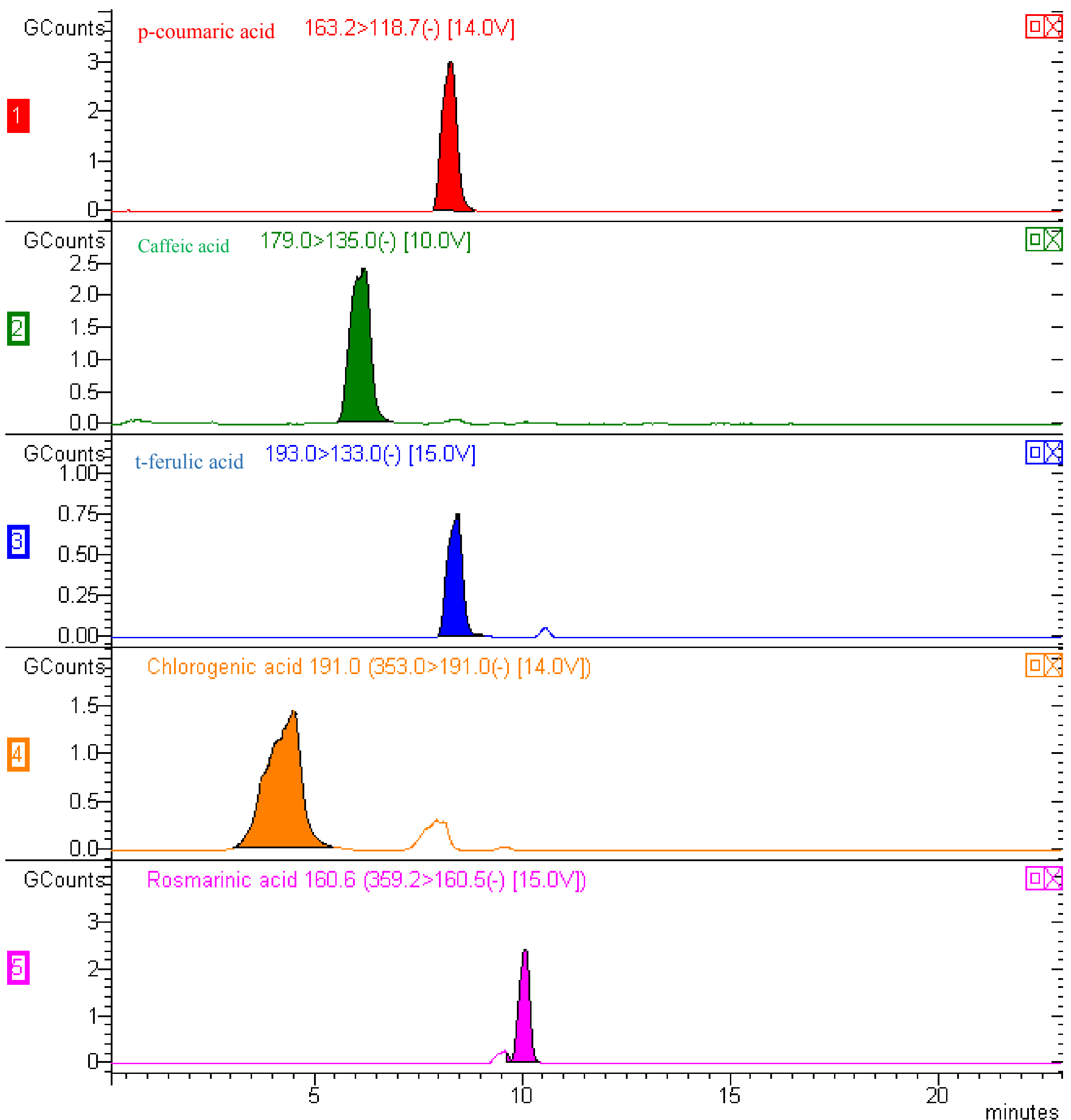

Figure S2: Standards Chromatogram of Secondary Metabolites (Phenolics and Others) by LC-MS/MS (5 mg/L)

Figure S2 (Continued)

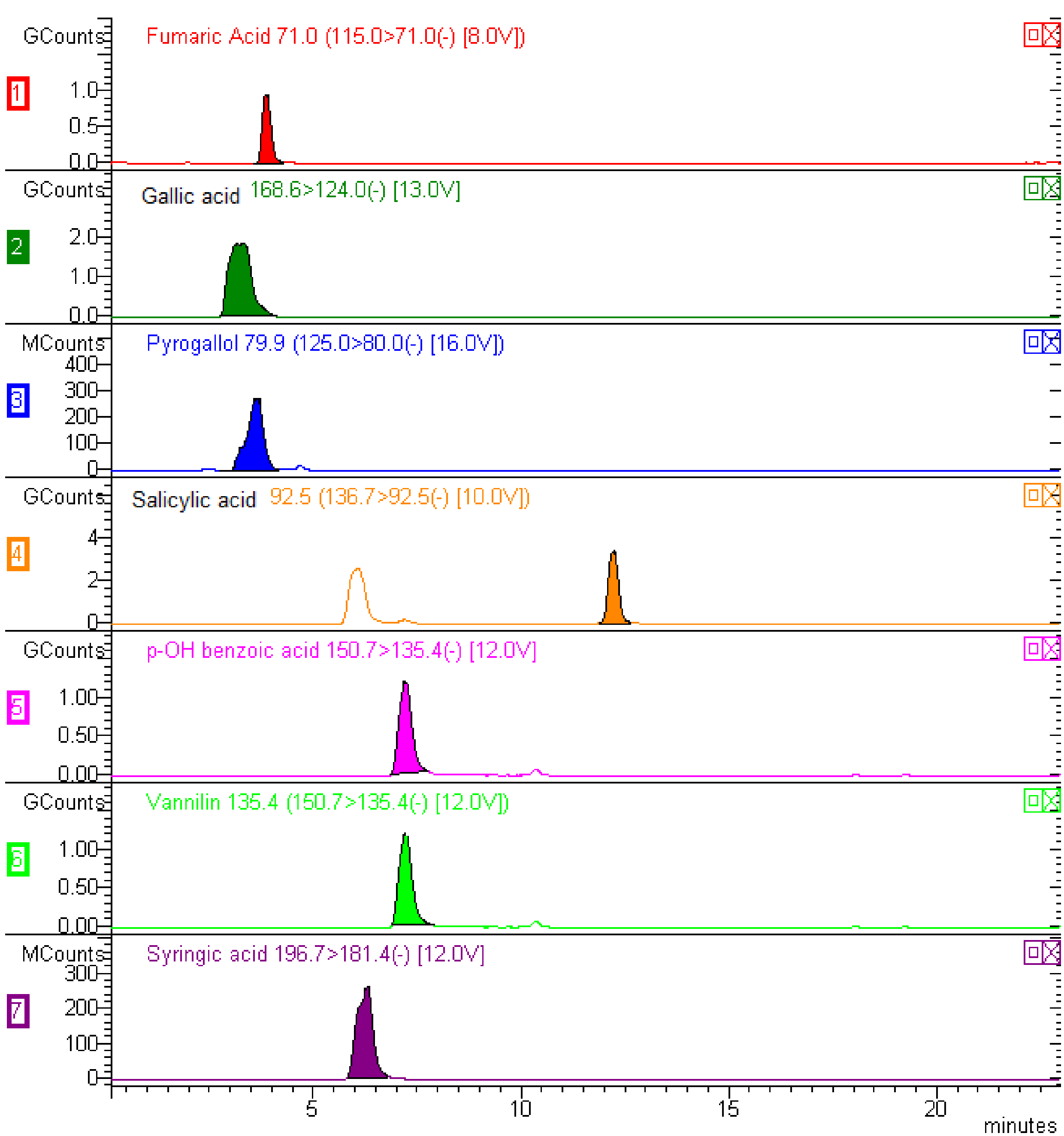

Table S2. Structures of the determined phenolics

1	Rutin	
2	Quercitrin	
3	Apigenin	
4	Penduletin	
5	Quercetagetin-3,6-dimethylether	
6	Luteolin-7-*O*-Glucoside	

Table S2 (continued)		
7	Luteolin-5-O-Glucoside	
8	Pelargonin	
9	p-coumaric acid	
10	Caffeic acid	
11	t-ferulic acid	
12	Chlorogenic acid	

Table S2 (continued)		
14	Gallic acid	
15	Syringic acid	
16	Fumaric acid	
IS	Curcumin-IS	

MATERIAL AND METHODS

Chemicals

The following compounds were used as standards in LC-MS/MS analyses: fumaric acid (99%, Sigma-Aldrich), pyrogallol (98%, Sigma-Aldrich), rutin (94%, Sigma-Aldrich), chlorogenic acid (95%, Sigma-Aldrich), gallic acid (99%, Merck), syringic acid (95%, Sigma-Aldrich), t-ferulic acid (99%, Sigma-Aldrich), caffeic acid (98%, Sigma-Aldrich), pelargonin chloride (98%, Sigma-Aldrich), quercitrin (97%, Sigma-Aldrich), salicylic acid (99%, Sigma-Aldrich), p-coumaric acid (98%, Sigma-Aldrich), luteolin-7-*O*-glu (99%, AppliChem), rosmarinic acid (96%, Sigma-Aldrich), pyrogallol (98%, Sigma-Aldrich), apigenin (95%, Sigma-Aldrich), kaempferol (96%, Sigma-Aldrich), and isorhamnetin (98%, ExtraSynthese, Genay-France).

LC-MS/MS experiments

Due to its fragmented ion stability, the best mobile phase solution was determined to be a gradient of acidified methanol and water system. The optimum ESI parameters were determined as 2.40 mTorr CID gas pressure, 5000.00 V ESI needle voltage, 600.00 V ESI shield voltage, 300.00 °C drying gas temperature, 50.00 °C API housing temperature, 55 psi Nebulizer gas pressure, and 40.00 psi drying gas pressure.

Procedures of Biological Activity Assays

Determination of the Anticholinesterase Activity

Inhibitory activities of acetyl- and butyryl-cholinesterase were measured by slightly modified spectrophotometric method, developed by Ellman, Courtney, Andres and Featherston (1). Acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates of the reaction and DTNB method was used for the measurement of the anticholinesterase activity. Hundred and fifty microlitres of 100 mM sodium phosphate buffer (pH 8.0), test tea samples at different concentrations. Solution of AChE or BChE were mixed and incubated for 15 min at 25 °C, and 0.5 mM DTNB was added. The reaction was then initiated by the addition of acetylthiocholine iodide (0.71 mM) or butyrylthiocholinechloride (0.2 mM). The hydrolysis of these substrates were monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, at a wavelength of 412 nm. Methanol was used as a solvent to dissolve the controls. Percentage of inhibition of AChE or BChE was determined by a comparison of the rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH 8.0) using the formula:

$$\text{Inhibition } \% = [(E-S)/E] \times 100$$

where E is the activity of enzyme without test sample. and S is the activity of enzyme with test sample. Galanthamine was used as a reference compound.

Determination of the Antioxidant Activity with the β-Carotene Bleaching Method (2)
β -Carotene (0.5 mg) in 1 mL of chloroform was added to 25 µL of linoleic acid, and 200 mg of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, was added through vigorous shaking. A mixture of four thousand microliters was transferred into different test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero-time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50 °C. A blank, devoid of β-carotene, was prepared for background subtraction. BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) were used as standards. The bleaching rate (R) of β-carotene was calculated using the following equation: R = ln (a/b) / t, where: ln=natural log, a=absorbance at time (0 min), and b=absorbance at time t (120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using the equation below:

AA = [(Rcontrol - Rsample) / Rcontrol] x 100

Determination of Antioxidant Activity with the DPPH Free Radical Scavenging Method (3)
The free radical scavenging activity of the methanol extract was determined spectrophotometrically by the DPPH (1.1-diphenyl-2-picrylhydrazyl) assay. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 160 µL of this solution was added to 40 µL of sample solutions in methanol at different concentrations. After 30 min, the absorbance was measured at 517 nm. The lower absorbance of the reaction mixture indicates the higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)}=[(A_{control}-A_{sample})/A_{control}]\times 100$$

The CUPRAC Experiment (4)
The CUPRAC method was performed as described. with some minor modification. since experiments were performed 96-well plates.

Briefly, 1 mM dimethylformamide (DMF), 10 mM $CuCl_2$, 7.5 mM neocuproine, 1 M NH_4CH_3COO (pH 7.0) solution, and distilled water were mixed in a volume ratio of 1:1:1:0.6. Then, 180 µL of the mixture was dispersed into the wells. 25 µL diluted compounds (dilution ratio 1:20) in EtOH. After waiting for 30 minutes, the absorbance at 450 nm was measured against a reagent blank by Beckman Coulter DTX 880 Multimode Detection System. Ethanol was used as a negative control; whereas curcumin was used as a positive control.

$TEAC_{CUPRAC}$ of curcumin was found as 0.9 mmol TR g^{-1}. by using calculation formula.

$TEAC_{CUPRAC}$ values of compounds were calculated by using references. TEAC of plant extracts (mmol TR g^{-1}) = (Absorbance/ ε_{TR}) (205/25) (20/1) (2/0.02). Here, absorbance comes from the instrument; ε_{TR} = 16700 (1); 205 is total reaction volume; 25 is compound volume added to the reaction; 20/1 is the dilution factor; 2 is the solvent volume (mL) in which plant extracts; 0.02 is weight of plant extract as grams.

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