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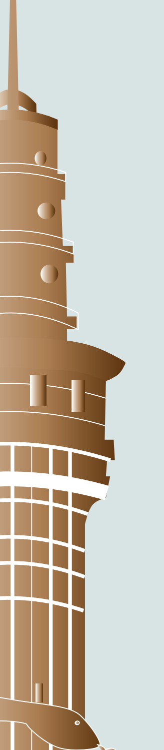

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Short Paper	1000	200	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	No tables	No media

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Journal Article: Orlacchio A, Campos-Cavieres M, Pashev I, Munn EA (1979) Some kinetic and other properties of the isoenzymes of aspartate aminotransferase isolated from sheep liver. *Biochem J* **177:** 583-593.

Book Section: Benn MH, Jacyno JM (1983) The toxicology and pharmacology of diterpenoid alkaloids. In: Pelletier SW (ed./eds.) Alkaloids: Chemical and Biological Perspectives, Vol. 1, John Wiley & Sons, New York, pp. 153-210.

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Biochemical constituents and antioxidant activities of some mushrooms from Turkey: *Agaricus* spp., *Pleurotus* spp., *Morchella esculenta* and *Terfezia boudieri*

Mehmet Akyüz[1]*, Ayşe Dilek Özşahin Kireçci[1], Zehra Gökçe[2], Sevda Kırbağ[3], Ökkeş Yılmaz[3]

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ABSTRACT

In this study,the vitamin, sugar, protein, fatty acid, total flavonoid, phenolic contents, and antioxidant activities (ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), and MDA (malondialdehyde) speciality of edible wild, cultured and commercial mushrooms (*Pleurotus* spp., *L. sajor-caju, Agaricus* spp., *Morchellaesculenta* and *Terfeziaboudieri*) from Turkey were researched. The levels of vitamins were 0.00-60.85 K1 (phylloquinone), 0.00-1.50 K2 (menaquinone), 1.35-7.20 D2 (ergocalciferol), 0.60-3.45 D3 (cholecalciferol), 8.35-68.20 α-tocopherol, 90.45-491.75 ergosterol, 0.00-110.45 stigmasterol, 0.00-1.30 β-sitosterol, 0.05-0.70 retinol and 0.05-0.15 retinolast (mg/kg) of dry weight. It was observed that *P. ostreatus* and *A. campestris* have a higher amount of glucose, sucrose and maltose, when compared with the other species. Furthermore, sugars such as maltose and arabinose were not detected in *Pleurotus* spp. It was determined that the amount of total flavonoid, total phenolic and protein were higher in *A. bisporus, A. campestris* and *L. sajor-caju* than other mushroom species according to literature.Unsaturated fatty acids, especially linoleic (C18:2) and oleic (C18:1) acids, were predominant (37.08-76.72% and 2.91-39.43%, between) whereas, other fatty acids were in smaller fractions. In addition, ABTS, DPPH and MDA were found to be 12.36-99.68% at 25-200 µL, 22.97-91.89% at 50-800 µL and 3.12-8.41 nmol/mL, respectively.

Keywords: Nutritive value, antioxidant activities *Pleurotus* spp., *Agaricus* spp., *M. esculenta, T. boudieri*

INTRODUCTION

Edible mushrooms are appreciated for their nutritional value and medical properties as well as their texture and flavor. They are considered healthy because they are low in fat and calories but rich in diatery fibre, vitamins, minerals and also protein. The nutritive components and taste properties of various mushrooms have been thoroughly studied (Barros et al. 2008; Grangeia et al. 2011; Reis et al. 2012; Kalogeropoulos et al. 2013; Heleno et al. 2015). In terms of their nutritional qualities, they have become increasingly important in the human diet; this may be explained by their antioxidant capacity to clear free radicals, which are responsible for the oxidative damage of lipids, proteins and nucleic acids of mushrooms (Wong and Chye 2009).

Oxidative stress caused by free radicals may be related to diseases such as aging, diabetes, cancer, cirrhosis and atherosclerosis etc. Organisms have developed antioxidant defenses and repair systems to protect against oxidative stress, but these systems are not sufficient to prevent damage completely (Wasser 2010; Wasser 2014; Sanchez 2017). However, antioxidant supplements or foods containing antioxidants can be used to help reduce the oxidative stress of the human body. Carotenoids, α-tocopherol,

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ascorbic acid and polyphenols are well protected against free radical damage by some oxidative enzymes (Tadhani et al. 2007). Epidemiological studies showed that the consumption of vegetable and fruits is associated with reduced risks of some diseases. Mushrooms accumulate a variety of tocopherols, steroids, phenols, polyketides, flavonoids, terpenesetc. (Wasser 2010; Vaz et al. 2011; Taofiq et al. 2016; Sanchez 2017). Nowadays, the use of edible mushrooms as nutrients have become more important due to the increase in diseases such as diabetes, fatty liver, obesity, heart disease, high blood pressure, cancer, immune systems etc. (Barros et al. 2008; Wasser 2014; Rathore et al. 2017).

Recently, it has been found that mushrooms are medically active in various treatments and used for diet therapy. Herein, we report the phenol, flavonoid, vitamin, fatty acid and sugar contents of mushrooms and their antioxidant capacity. For the identification of antioxidant properties, we also evaluated their DPPH, ABTS and MDA features.

MATERIAL AND METHODS

Collection of Mushroom Samples

The mushrooms used in the present study were obtained from different cultural studies, collected from field work and purchased from a local grocery. Wet *Pleurotus eryngii* (DC. ex Fr.) Quel. var. *ferulae* Lanzi, *Pleurotus eryngii* (DC. ex Fr.) Quel. var. *eryngii*, *Pleurotus ostreatus* (Jacq.) P. Kumm., *Lentinus sajor-caju* (Fr.) Fries. (syn. *Pleurotus sajor-caju* (Fr.) Sing.), and *Pleurotus floridanus* Singersamples were obtained from the Cultured Mushroom Laboratory of Bitlis Eren University, Bitlis - Turkey. In addition, commercial samples of *Agaricus bisporus* (J.E. Lange) Imbach were purchased from Bitlis - Turkey, wild samples of *Morchella esculenta* (L.) Pers.from Antalya, *P. ostreatus* and *Agaricus campestris* L.from Elazığ,and *Terfezia boudieri* Chatinfrom Şanlıurfa were collected and purchased from Turkey, respectively.The samples were dried at 25 °C for 15-20 days, and then used in the study.

Methods

One gram of dry mushroom samples was homogenised with 10 ml of 80% methanol using a blender, and then the residues were filtered. After centrifugation (5000 rpm, 5 min.), the supernatant was separated from the residue, and the solvent removed with a rotary vacuum evaporator. The evaporated residue was dissolved in DMSO and stored until analysis.

Selected biochemical components and antioxidant activites were determined with appropriate methods, as described below: Flavonoid (DAD detector following RP-HPLC) by the chromatographic analysi sand total phenolic contents (The absorbance of the mixture was measured spectrophotometrically at 765 nm) (Singleton and Rossi 1965; Zu et al. 2006; Barros et al. 2007; Song et al. 2010), fatty acids (Methyl esters were analyzed with the SHIMADZU GC 17 Ver. 3 gas chromatography (Kyoto, Japan))by modified by Hara and Radin (1978), Christie (1992), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (The absorbance of the mixture was measured spectrophotometrically at 517 nm), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (The absorbance of the mixture was measured spectrophotometrically at 734 nm), and malondialdehyde (MDA) (The equipment consisted of a pump (LC-10 ADVP), a UV-visible detector (SPD-10AVP), a column oven (CTO-10ASVP), an autosampler (SIL-10ADVP) a degasser unit (DGU-14A) and a computer system with class VP software (Shimadzu, Kyoto Japan) according to the method of Shimoi et al. (1994), Brand-Williams et al. (1995) andRe et al. (1999) , free sugars (high performance liquid chromatography (HPLC) with a refractive index detector (RID) and vitamins (DGU-14A and Class VP software (Shimadzu, Kyoto Japan) were determined by HPLC based on the method used by Sánchez-Machado et al. (2004) and Lopez-Cervantes et al. (2005), and the content of proteins was analysed according to Lowry procedure (The absorbance of the mixture was measured spectrophotometrically at 750 nm) (Lowry et al. 1951).

RESULTS AND DISCUSSION

Vitamin A, D, E and K are essential micronutrients that have a wide variety of functions throughout the human body; the first helps the eyes adjust to light changes (blindness), in gene expression, cell division, reproduction, bone growth, tooth development, antioxidant, and regulation of the immune system; the second plays a critical role in the body's use of Ca^{2+} and P in the maintenance of healthy bones and teeth; the third one benefits the body by acting as an antioxidant, protecting red blood cells, and essential fatty acids from destruction; and the fourth plays an essential role in promoting bone health, normal blood clotting, and other functions (Combs 2008). While there is a wealth of literature data regarding studies on the vitamin A, E, and C contents of mushroom species, those studies devoted to vitamin K, D and sterol contents are limited. Mushrooms rich in vitamins A, D, E and K along with ergosterol content are thought to be the only vegetarian source for vitamin D (Rathore et al. 2017). It has been well established as critical for bone health, in immune function and the prevention of certain types of cancer (Bischoff-Ferrari et al. 2006; Holick 2007; Urashima et al. 2010).

Vitamin (K1, K2, D2, D3, α-tocopherol, retinol and retinolast) and sitosterol (ergosterol, stigmasterol and β-sitosterol) contents of Pleurotus spp., L. sajor-caju, Agaricus spp., M. esculenta and T. boudieri samples are shown in Table 1. The highest vitamin K1, K2, D2, D3, α-tocopherol, ergosterol and stigmasterol contents were 60.85 µg/g in T. boudieri, 1.50 µg/g in P. floridanus, 7.20 µg/g in L. sajor-caju, 3.45 µg/g in M. esculenta, 68.20 µg/g in T. boudieri, 491.75 µg/g in M. esculenta and 110.45 µg/g in M. esculenta as shown in Table 1. The presence of sterols in mushrooms has previously been reported. The predominance of ergosterol and the presence of the minor related sterols in *Russula delica* (0.07-12.51 µg/100 g fw), *Suillus bellinii* (0.05-12.31 µg/100 g fw) and *Lactarius* species (0.02-18.0 µg/100 g fw) was reported by Kalogeropoulos et al. (2013). Mushrooms contain several primary vitamins such as vitamin D, riboflavin, niacin, thiamine and tocopherol (Cheung, 2010). For various species, the niacin, ascorbic acid, thiamine and riboflavin content can vary (Zhu et al. 2007; Yin and Zhou 2008; Zhou and Yin 2008; Xu et al. 2012). The reported vitamin contents were 19.16-400.36 µg/100 g

dw ascorbic acid and tocopherol in wild edible mushroom (Grangeia et al. 2011; Vaz et al. 2011), 0.18-10.65 µg/g dw tocopherol in commercial and wild mushrooms (Barros et al. 2008), 1.81-11.16 µg/100 g fw in *Flammulina velutipes*, *A. bisporus*, *Pleurotus* spp. and*Lentinula edodes* (Reis et al. 2012), 0.70-5.1 mg/100 g ascorbic acid in *Terfezia* and *Tirmania* species (Sawaya et al. 1985; Hussain and Al-Ruqaie 1999), 4.7-194 mg/100 g dm tocopherol and vitamin D2 in *Boletus* species and *Thelephora ganbajun* (Wu et al. 2005; Zhou and Yin 2008). The wide variation in vitamin contents in edible wild, culture and commercial mushrooms might arise from the variety of the growing areas, stage of ripening, sample preparation, methods of analysis, as well as other factors (climate condition, sample collection, transportation, host plant) as stated in the aforementioned studies.

The sugar, total flavonoid, phenol and protein contents of the studied mushrooms, expressed on a dry weight basis, are presented in Table 2. It was observed that *P. ostreatus* and *A. campestris* have a higher amount of glucose, sucrose and maltose, when compared with the other species. Furthermore, sugars such as maltose and arabinose were not detected in *Pleurotus*-species (see Table 2). The accumulation of arabinose, mannitol, fructose, sucrose, trehalose, mannose, glucose in the fruit bodies of other species were already reported. The observed sugar values can vary within those considered as typical for culture, commercial and edible wild mushrooms (Barros et al. 2008; Grangeia et al. 2011; Vaz et al. 2011; Reis et al. 2012; Heleno et al. 2015). These differences might have been dependent on growing habitats, mushroom types and geographical areas as stated ın the aforementioned reports.

It was determined that the amount of total flavonoid and phenol that *Agaricus* spp. (*A. bisporus* and *A. campestris*) contain are higher than the amount that other mushroom species (*Pleurotus* spp., *L. sajor-caju*, *T. boudieri* and *M. esculenta*) see Table 2. It has also been suggested that the antioxidant properties contribute to their vitamin, flavonoid and phenolic compounds

Table 1. Vitamin contents of some edible mushrooms from Turkey (dry weight)

	Vitamin (µg/g)							Sitosterol (µg/g)		
Mushrooms	K1	K2	D2	D3	α-tocopherol	Retinol	Retinolast	Ergosterol	Stigmasterol	β-sitosterol
P. eryngii var. ferulae *	-	1.00	4.50	0.85	25.15	0.30	0.10	259.10	5.10	-
P. eryngii var. eryngii *	2.65	0.15	2.90	1.15	8.35	0.10	0.10	90.45	1.35	1.30
L. sajor-caju *	0.95	-	7.20	1.45	19.70	0.05	0.10	431.10	2.60	-
P. floridanus *	1.70	1.50	3.55	0.70	28.45	0.15	0.15	372.25	-	0.20
P. ostreatus *	0.90	-	3.95	0.60	34.60	0.10	0.05	448.65	5.55	-
P. ostreatus [b,] **	2.60	-	5.05	0.75	31.20	0.05	0.05	374.80	6.40	0.10
A. bisporus [d,] *	2.00	-	4.85	1.10	56.35	0.70	0.05	403.65	16.95	0.45
A. campestris [b,] **	20.75	1.15	1.35	1.05	24.90	0.20	0.10	169.75	3.10	0.15
T. boudieri [a,] * *	60.85	0.60	3.95	0.60	68.20	0.55	0.05	103.70	36.30	1.10
M. esculenta [c,] **	21.05	0.95	6.75	3.45	43.65	0.10	0.05	491.75	110.45	-

*: culture, **: wild,
[a]: Şanlıurfa, [b]: Elazığ, [c]: Antalya, [d]: Bitlis
K1 : phylloquinone, K2 : menaquinone, D2 : ergocalciferol, D3 : cholecalciferol

Table 2. Sugar, flavonoid,phenol and protein contents of some edible mushroom from Turkey (dry weight)

	Sugar (µg/g)							
Mushrooms	Glucose	Sucrose	Fructose	Maltose	Arabinose	Total Flavonoid (µg/g)	Total Phenol (µg/mL)	Protein (mg/g)
P. eryngii var. ferulae *	5.58	0.92	-	-	-	199.00	1.87±0.10	82.82
P. eryngii var. eryngii *	1.77	5.17	0.42	-	-	88.00	1.88±0.13	96.05
L. sajor-caju *	-	-	-	-	-	2.00	1.39±0.18	138.60
P. floridanus *	27.57	-	-	-	-	4.00	1.11±0.04	137.37
P. ostreatus *	266.03	11.00	1.61	-	-	-	1.24±0.13	137.36
P. ostreatus [b,] **	207.72	17.19	0.64	-	-	43.00	2.09±0.12	114.21
A. bisporus [d,] *	-	1.42	0.48	31.16	-	611.00	2.11±0.07	95.78
A. campestris [b,] **	-	28.63	-	230.97	-	18.00	3.78±0.46	101.15
T. boudieri [a,] **	39.30	4.66	-	-	3.99	39.00	1.88±0.20	81.05
M. esculenta [c,] **	74.27	1.01	-	52.98	1.79	6.00	2.02±0.26	107.10

*: culture, **: wild,
[a]: Şanlıurfa, [b]: Elazığ, [c]: Antalya, [d]: Bitlis

Table 3. Fatty acid composition of some edible mushroom from Turkey (dry weight)

Mushrooms	Fatty acids (%, dry weight)																	
	C14:0	C15:0	C15:1	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:1	C20:2	C20:3	C20:5	C21:0	C22:0	C22:6	C23:0	C24:0
*P. eryngii var. ferulae**	-	1.31	0.13	13.55	1.78	-	2.77	39.43	37.08	0.32	0.18	1.60	-	-	0.23	0.55	-	0.40	0.67
*P. eryngii var. eryngii**	-	1.43	0.15	12.83	2.11	-	3.01	35.07	43.58	0.18	-	0.68	-	-	0.22	0.36	-	0.28	0.11
L. sajor-caju *	-	-	-	10.49	1.19	-	1.96	13.75	70.04	-	-	-	-	1.62	-	0.95	-	-	-
P. floridanus *	-	1.67	-	13.07	1.97	-	1.62	14.70	65.20	-	-	-	-	0.77	-	-	-	-	0.98
P. ostreatus *	-	-	-	8.94	0.99	-	1.32	11.33	76.72	-	-	-	-	0.70	-	-	-	-	-
P. ostreatus [b], **	-	1.68	-	11.01	1.97	-	1.34	12.69	69.85	-	-	-	-	1.05	-	-	-	-	0.42
A. bisporus [d], *	-	1.16	-	10.64	1.75	0.53	5.53	2.91	71.98	2.12	-	-	0.35	-	1.14	1.21	-	-	0.67
A. campestris [b], **	-	-	-	18.14	3.59	-	4.43	4.09	59.02	1.61	-	-	-	0.63	1.10	2.95	-	2.98	1.47
T. boudieri [a], **	-	-	-	16.35	3.40	-	2.89	21.42	53.88	0.52	0.15	0.28	0.13	-	0.15	0.61	0.10	0.12	-
M. esculenta [c], **	-	-	-	12.54	3.49	-	2.51	8.31	71.41	0.45	-	0.28	-	-	0.99	-	-	-	-

*: culture, **: wild,
[a]: Şanlıurfa, [b]: Elazığ, [c]: Antalya, [d]: Bitlis
C14:0 myristic acid, C15:0 pentadecanoic acid, C15:1 pentadesenoic acid, C16:0 palmitic acid, C16:1 palmitoleic acid, C17:0 margaric acid, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid, C18:3 linolenic acid, C20:1 eicosenoic acid, C20:2 eicosadienoic acid, C20:3 eicosatrienoic acid, C20:5 eicosapentaenoic acid, C21:0 heneicosanoic acid, C22:0 behenic acid, C22:6 docosahexaenoic acid, C23:0 tricosanoic acid, C24:0 lignoseric acid

Previous studies suggested a strong correlation between the vitamin, phenolic compounds and flavonoid in mushrooms and their antioxidant activity ((2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), ferric reducing antioxidant power (FRAP) assay, oxygen radical absorbance capacity (ORAC) assay, antiradical, reducing power, chelating ability etc. (Grangeia et al. 2011; Vaz et al. 2011; Kalogeropoulos et al. 2013; Sanchez 2017). The total flavonoid and phenolic contents of the mushrooms was determined by growing conditions, time and manner of harvesting, maturity, variety and species. Even after harvest, many factors may affect composition. These include,variation in analytical methods, sample preparation, storage time and conditions and processing procedures as shown by others.

L. sajor-caju, P. floridanus and *P. ostreatus* (culture) were the mushrooms with the highest amounts of protein (137.3-138.6 mg/g), while *T. boudieri*, *P. eryngii* var. *ferulae*, *A. bisporus* and *P. eryngii* var. *eryngii*were had relavitely lower protein contents (81.0-96.0 mg/g) than the other samples in our study as seen in Table 2. Mushroom protein was reported to vary according to especially analytical methods, the genetic structure of the species and the chemical and physical differences in growing habitat. It seems that the quantity of crude protein values are different to those reported by other researchers (Barros et al. 2008; Vaz et al. 2011; Reis et al. 2012; Heleno et al. 2015; Rathore et al. 2017).

The results for fatty acid composition of edible wild, culture and commercial mushrooms are shown in Table 3. Up to 18 fatty acids were found in mushroom lipids. The analysis of the obtained profiles showed that linoleic (37.08-76.72%), oleic (2.91-39.43%), palmitic (8.94-18.14%), stearic (1.32-5.53%) and palmitoleic acid (0.99-3.59%) were the main fatty acids in the species studied (Table 3). Other fatty acids were present in only low levels. The studied species revealed that linoleic acid was an important fatty acid. It was the preponderant fatty acid in *P. ostreatus*, *A. bisporus*, *M. esculenta*, *L. sajor-caju*, *P. floridanus*, *A. campestris*, *T. boudieri*, while oleic acid was the main component in *P. eryngii* var. *ferulae*, *P. eryngii*-var. *eryngii*and *T. boudieri* species. *Agaricus* species were the mushroom with the highest amounts of stearic acid, while *A. campestris* and *T. boudieri* were the mushrooms with the highest amounts of palmitic acid. The fatty acid profiles of the different mushroom species appeared to be distinct. An abundance of these essential fatty acids in other edible wild, culture and commercial mushrooms has been described (Barros et al. 2008; Vaz et al. 2011; Reis et al. 2012; Kalogeropoulos et al.2013). Regarding the species described above, their qualitative and quantitative fatty acids profiles have, to some extent, been found to be different from those described in literature. These are consistent with the observation that, in mushrooms, the unsaturated fatty acids predominate over the saturated, in the total fatty acids contents.

Table 4. Antioxidant activies of some edible mushroom from Turkey (dry weight)

	ABTS				DPPH					MDA (nmol/mL)		
Mushrooms	25µL	50 µL	100 µL	200 µL	50µL	100 µL	200 µL	400 µL	800 µL	MDA	FeCl	Control
P. eryngii var. ferulae *	55.71	72.92	92.95	99.53	36.48	30.88	40.13	79.92	91.89	3.12±0.11	7.09±0.37	1.104±0.06
P. eryngii var. eryngii *	44.33	70.26	95.77	97.96	54.05	70.65	64.67	80.50	91.69	4.23±0.22		
L. sajor-caju *	45.38	77.93	92.48	97.65	57.14	76.44	77.60	80.30	91.69	5.95±0.46		
P. floridanus *	64.47	90.29	97.33	99.21	68.53	87.06	83.39	79.15	90.34	7.21±0.34		
P. ostreatus *	44.60	74.33	93.11	98.59	48.84	63.89	70.84	80.30	91.89	4.94±0.67		
P. ostreatus [b], **	42.09	67.91	78.56	96.40	25.86	22.97	70.84	65.63	91.50	7.30±0.26		
A. bisporus [d], *	12.36	88.41	96.87	99.53	47.10	71.81	45.94	77.99	85.32	5.93±1.47		
A. campestris [b], **	73.86	94.83	98.12	99.16	77.41	90.92	76.44	78.37	86.87	8.41±0.94		
T. boudieri [a], **	74.80	69.32	84.50	97.18	42.47	42.85	44.98	72.00	86.29	7.04±0.54		
M. esculenta [c], **	76.21	96.71	91.70	92.33	40.92	42.08	37.83	73.93	83.39	6.79±0.37		

*: culture, **: wild,
[a]: Şanlıurfa, [b]: Elazığ , [c]: Antalya, [d]: Bitlis
ABTS: 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH: 2,2-diphenyl-1-picrylhydrazyl, MDA: malondialdehyde

It was determined that their effect (A. bisporus, P. eryngii var. ferulae, P. ostreatus and P. eryngii var. eryngii) to remove the ABTS and DPPH radical was more efficient in groups to which samples of 25-200 µL (12.36-99.53%) ABTS and 50-800 µL (36.48-91.89%) DPPH were dose dependent (see Table 4). The present findings reveal that the extract of A. bisporus, L. sajor-caju, P. eryngii var. eryngii, P. ostreatus and P. eryngii var. ferulae possesses a profound antioxidant effect as seen in Table 4. Several studies suggested a strong correlation between the vitamin, flavonoid and phenolic compounds in mushrooms and their antioxidant activity (DPPH, ABTS, FRAP, ORAC, antiradical, reducing power, chelating ability etc.(Grangeia et al. 2011; Vaz et al. 2011; Kalogeropoulos et al. 2013; Sanchez 2017). Our data (see Table 4) is different to that reported by other researchers. Large quantitative differences (probably due to the analytical methods used), and the heterogeneity of the samples analysed were found to be so in the cited studies. The geographic effect, climatic conditions, growing habitats and species are thought to be responsible for these differences. Our findings were supported by previous findings in the aforementioned studies.

In conclusion, the study results generally show that mushrooms can be a good antioxidant source to help an organism increase its overall antioxidant capacity and protect it against lipid peroxidation.

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Original Article

Qualitative and quantitative phytochemical analysis and *in-vitro* biological activity of *Rheum ribes* L. different parts

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ABSTRACT

The methanol extracts from different parts of *Rheum ribes* were subjected to qualitative and quantitative phytochemical and *in vitro* biological activity (antioxidant, anti-urease, anticholinesterase). Qualitative phytochemical tests were performed using standard analysis methods and these studies revealed the presence of phenolics and tannins. Following this, a quantitative determination of total phenolics and tannins contents was carried out. The antioxidant activity of the extracts were assayed using DPPH, FRAP, TEAC/ABTS and CUPRAC techniques. In addition, the anti-urease and anticholinesterase activity of the extracts were examined using indophenol and Ellman methods, respectively. In this study, it was determined that the macerated flowers extract contained higher total phenolic and tannins contents than the other extracts. According to the results obtained from the antioxidant experiment, the macerated extract of flowers showed the strongest ABTS.+ scavenging and ferric reducing antioxidant power activity. The macerated leaves and Soxhlet radix extracts exhibited the strongest DPPH. scavenging and cupric reducing antioxidant activity, respectively. The young shoots extracts obtained using the Soxhlet methods showed the highest anticholinesterase activity. All extracts obtained from different parts of the plant were found to have very low anti-urease activity when compared to the anti-urease activity of standard compound. Therefore, methanol extracts from plant's flowers, leaves and young shoots can be used as a natural antioxidant and anticholinesterase agent respectively, for the pharmaceutical and food industry in the future.

Keywords: *Rheum ribes*, antioxidant, anti-uresae, anticholinesterase, phytochemical analysis

INTRODUCTION

Free radicals are produced continuously in our bodies (naturally or due to environmental impacts), and play a role in many diseases (cancer, Parkinson's disease, Alzheimer's disease, aging etc). Antioxidants are agents that clear free radicals and prevent them from doing damage. Because of the side effects of synthetic antioxidants, it has been more meaningful to use natural antioxidant sources such as fruits, vegetables, and grain foods (Baskar et al. 2011). It is widely known today that gastric and duodenal ulcers a usually caused by *Helicobacter pylori*. This organism releases urease that converts urea into ammonia and the released ammonia protects it from the acidic environment of the stomach. For this reason, the natural source compounds that inhibit urease activity are very important (Amin et al. 2013).

Rheum ribes L. (Rhubarb) belonging to Polygonaceae family is an annual species that is distributed across the temperate and subtropical regions of the world. This species is grown between 2300 and 2700 altitude in the rocky countryside of Turkey and known as "Işgın, Işkın, Uşgun and Uçgun". The edible parts of the plant are the young shoots and petiols, which were eaten raw or cooked (Davis and Cullen, 1967; Bulut et al. 2016). *Rheum* species are valuable to the pharmaceutical industry due to the presence of phytochemical contents (anthracene derivatives, tannins and phenolic compounds). *R.*

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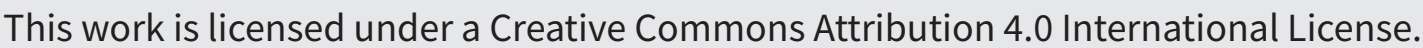

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ribes's young shoots and petioles are used against diarrhea and vomiting. The roots of the plant have been used in the treatment of diabetes, hypertension, ulcer and diarrhea. *Rheum ribes* contains vitamins (A, B1, B2 and C), some elements (potassium, magnesium and calcium) organic acids (citric acid and malic acid), anthraquinones (chrysophanol, physcion and emodin), flavonoid compounds (quercetin, 5- desoxyquercetin, quercetin 3-*0*-rhamnoside, quercetin 3-*0*- galactoside and auercetin 3-0-rutinoside), and tannins (Tosun and Kızılay, 2003; Andiç et al. 2009; Sindhu et al. 2010; Shafaghat et al. 2014; Polat et al. 2015; Shahi et al. 2016).

In recent years, the number of studies on plant extracts showing antioxidant, anticholinesterase and anti-urease activity has increased. In addition, it is known that extraction methods are very important in biological activities. Therefore, the aim of this study was to analyse qualitative and quantitative phytochemicals and to determine the antioxidant, anticholinesterase and anti-urease activities of *R.ribes* extracts obtained using different extraction methods.

MATERIAL AND METHODS

Plant material and extract preparation: The *R. ribes* was collected on 20th May 2016 from Van-Gürpınar, Turkey and identified by Dr. Gizem Bulut from Marmara University. The voucher specimen was deposited in the Pharmacy Faculty Herbarium (MARE) and the voucher specimen number was MARE 18817. The young shoots, leaves, radix and flowers of the plant were cut into small pieces. The small pieces (10 g) were extracted using the maceration, Soxhlet and ultrasonic bath methods with a methanol solvent. After extraction was complete, the samples were filtered through filter paper, the solvents were evaporated with a rotary evaporator and the crude extracts were stored in a refrigerator at 4 °C.

Preliminary qualitative phytochemical analysis: Phytochemical analysis of *R. ribes* was carried out using standard procedure to identify the possible bioactive compound(s) (Trease and Evans, 2002; Sharma and Agarwal, 2015). The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals (Table 1).

Table 1. Preliminary qualitative phytochemical analysis of different parts of *R. ribes*

Phytochemicals	Radix	Flower	Leaves	Young shoots
Alkaloids	-	-	-	-
Glycosides	-	-	-	-
Saponins	-	-	-	-
Tannins	+	+	+	-
Cardiac glycosides	-	-	-	-
Phenols	+	+	+	+

+: Presence of the phytochemical, –: absence of the phytochemical from the extract

Quantitative determination of chemical constituents

Extract yield percentage and total phenolic contents: The extraction yield was calculated to determine the effectiveness of the solvents in extracting the active compounds from the plant material. The total phenolic contents of the 12 different plant extracts were determined using the FCR method (Ozsoy et al. 2008). The total phenolic contents in the extracts were given as µg gallic acid equivalents/mg extract.

Determination of tannins content: The amount of tannin contained in the different extracts was determined using the Folin-Ciocalteu method (Vijay and Rajendra, 2014). The tannin contents in the extracts were expressed as µg tannic acid equivalents in microgram per milligram of extract (µgTAE/ mg extract).

In vitro evaluation of antioxidant assays

DPPH radical scavenging activity: The free radical scavenging ability of 12 different extracts was examined with the DPPH· method. The results obtained in the DPPH radical experiment were given as IC_{50} = µg/mL (Wei et al. 2010).

Trolox equivalent antioxidant activity: The $ABTS^{\cdot+}$ scavenging activity of the different extracts from the plant was evaluated using the TEAC/ABTS method. The standard curve was prepared using trolox and the data obtained in the experiment was expressed as mM trolox/mg extract (Re et al. 1999).

Ferric reducing/antioxidant power (FRAP) assay: The ferric reducing/antioxidant power of the different extracts was evaluated using the FRAP method. The standard curve was prepared using $FeSO_4.7H_2O$ and the data obtained in the experiment was expressed as mM Fe^{2+}/mg extract (Benzie and Strain, 1996).

Cupric reducing antioxidant capacity (CUPRAC): The cupric reducing antioxidant capacity of the different extracts was evaluated using the CUPRAC method. The CUPRAC values of the plant extracts were reported as trolox equivalents (mM trolox/mg extract) (Taskin et al. 2017).

In vitro anti-urease activity: In this study, the anti-urease activities of 12 different extracts obtained from the plant were evaluated according to the method of Ghous et al., 2010 and the results were given as a percentage of enzyme inhibition (Ghous et al. 2010).

Anticholinesterase activity of extracts: Inhibition of cholinesterases was evaluated using a 96-well microplate reader based on the method of Ellman et al. with some modifications (Ellman et al. 1961). The experiments were performed in triplicate in each case and the results were given as a percentage of enzyme inhibition. Galantamine was used as a reference.

Statistical analysis

The antioxidant, anticholinesterase and anti-urease experiments were done in triplicate and all the data was shown as mean±SD. The data was analyzed using the Graphpad Prism 5 program. Statistical differences between the experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Mean values were considered statistically significant when $p<0.05$.

Table 2. Extract yield percentage, total phenolic and total tannins contents of different parts of *R.ribes*

Samples	Total phenolic (µgGAE/mg extract)			Extract yield (%)			Tannins (µgTAE/mg extract)		
	Ultrasonic bath	Soxhlet	Maceration	Ultrasonic bath	Soxhlet	Maceration	Ultrasonic bath	Soxhlet	Maceration
Radix	135.00 ± 0.007^{a}	122.00 ± 0.003^{a}	83.00 ± 0.008^{a}	5.21^{a}	20.46^{a}	18.44^{a}	123.00 ± 0.003^{a}	109.00 ± 0.005^{a}	61.00 ± 0.010^{a}
Flowers	105.00 ± 0.003^{b}	140.00 ± 0.001^{b}	167.00 ± 0.002^{b}	10.72^{b}	17.44^{b}	11.82^{b}	184.00 ± 0.005^{b}	140.00 ± 0.018^{b}	229.00 ± 0.005^{b}
Leaves	163.00 ± 0.007^{c}	158.00 ± 0.004^{c}	139.00 ± 0.005^{c}	12.62^{c}	65.03^{c}	55.80^{c}	210.00 ± 0.007^{c}	$141.00\pm0.006^{c,b}$	178.00 ± 0.014^{c}
Young shoots	58.00 ± 0.002^{d}	67.00 ± 0.001^{d}	53.00 ± 0.004^{d}	8.74^{d}	30.79^{d}	19.62^{d}	ND	ND	ND

Values are mean of triplicate determination (n=3) ± standard deviation
Means with different superscripts (a-d) are significantly different, $p<0.05$
GAE-Gallic acid equivalents.
TAE-Tannic acid equivalents
ND: Not determined

RESULTS AND DISCUSSION

Qualitative phytochemical analysis of *R. ribes*

The phytochemical screening of different parts of a plant exhibited negative test for alkaloids, glycosides, saponins, cardiac glycosides (Table 1). Although all the plant's different parts showed positive test for phenols, and tannins, only young shoots showed negative test for tannins. It was known that the phytochemical compounds (phenols, tannins) that were qualitatively analyzed in *R. ribes* were medically important. Tannin-containing drugs have traditionally been used to protect inflamed surfaces of the mouth and throat. In addition, recent studies have shown that tannins are effective as antitumor and anti-HIV agents. Phenols are important compounds of some medicinal plants, and are used as coloring agents, flavored aromatizators and antioxidants (Trease and Evans, 2002; Buzzini et al. 2008). The phenols and tannins compounds identified in the methanol extract from *R.ribes'* different parts may be responsible for the biological activities. It is known that *R. ribes* contain tannins and phenol compounds (Amiri et al. 2015). The results of our study are consistent with the literature.

Quantitative phytochemical analysis of *R. ribes*

Extract yield percentage, total tannins and total phenolic contents: The total phenolic, tannins contents and yield percentage of methanol extracts from different parts of plant were analysed and are presented in Table 2. Leaf extracts obtained using ultrasonic bath and Soxhlet (163.00 µgGAE/mg extract, 158.00 µgGAE/mg extract) showed higher total phenolic contents than the macerated leaves extract (139.00 µgGAE/mg extract), respectively. In addition, it was found that the flowers extract (167 µgGAE/mg extract) obtained using the maceration method exhibited the highest total phenolic contents. When compared to all the data obtained in this study, the young shoots extracts obtained from the three methods were found to exhibit the lowest total phenolic contents. When the yields percentage of the different extracts were compared, the leaf extract obtained using the Soxhlet method was found to have a higher recovery over the other extracts. The total phenolic contents of the chloroform and methanol extracts from the roots and stems of *R. ribes* have been reported before (Öztürk et al. 2007). In this study, it was found that the roots's chloroform extract (48.66 ± 1.23 µg pyrocatechol equivalent/mg extract) had higher phenolic contents than the others, while the one containing least phenolics was the stems's chloroform extract (22.68 ± 1.10 µg pyrocatechol equivalent/mg extract). When we compared our study with data in literature, we found that methanol extract of radix (83.00 µgGAE/mg extract) showed higher total phenolic contents than radix's chloroform extract (48.66 µg pyrocatechol equivalent/ mg extract).

The amounts of tannins contained in different parts of the plant were ascertained in the following order: macerated flower extract (229.00 ± 0.005 µgTAE/mg extract)>ultrasonic bath leaves extract (210.00 ± 0.007 µgTAE/mg extract)>Soxhlet leaves extract (141.00 ± 0.006 µgTAE/mg extract). The results from the total tannins assay showed that the macerated flowers extract had the highest amount of tannins. According to

Table 3. Effects of extracting solvents/methods on the antioxidant activity of *R. ribes* extracts

Samples	DPPH (IC_{50}; µg/mL)			ABTS (mM trolox/mg extract)			FRAP assay (mM Fe^{2+}/mg extract)			CUPRAC assay (mM trolox/mg extract)		
	Ultrasonic bath	Maceration	Soxhlet	Ultrasonic bath	Maceration	Soxhlet	Ultrasonic bath	Maceration	Soxhlet	Ultrasonic bath	Maceration	Soxhlet
Radix	28.00±0.01^{a}	75.00±0.02^{a}	36.00±0.02^{a}	51.70±0.01^{a}	48.80±0.2^{a}	51.60±0.02 ^{a}	0.13±0.08^{a}	0.18±0.1^{a}	0.15±0.03^{a}	0.78±0.11a	0.62±0.06^{a}	0.91±0.02^{a}
Flowers	5.00±0.02^{b}	4.80±0.05^{b}	23.00±0.02^{b}	51.10±0.02^{b,a}	51.90±0.1^{b}	51.40±0.01 ^{b,a}	0.15±0.04^{b,a}	0.29±0.6^{b}	0.17±0.02^{b,a}	0.62±0.05b,a	0.61±0.02^{b,a}	0.62±0.07^{b}
Leaves	7.00±0.02^{c,b}	3.00±0.01^{c,b}	20.00±0.02^{c,b}	51.40±0.03^{c,a,b}	49.10±0.1^{c,a}	50.80±0.03^{c,a,b}	0.15±0.02^{c,a,b}	0.21±0.2^{c,a}	0.22±0.04^{c,a,b}	0.67±0.02c,a,b	0.71±0.02^{c,a,b}	0.72±0.05^{c,b}
Young shoots	144.00±0.09^{d}	98.00±0.03^{d}	85.00±0.05^{d}	13.10±0.01^{d}	12.80±0.4^{d}	12.70±0.03^{d}	0.28±0.08^{d}	0.14±0.3^{d,a,c}	0.16±0.01d,a,b,c	0.58±0.17^{d,a,b,c}	0.27±0.07^{d}	0.74±0.01^{d,c}
Ascorbic acid	6.00±0.01^{e,b,c}	6.00±0.01^{e,b,c}	6.00±0.01^{e}									
BHT					1.10±0.12^{e}							
BHA		52.63±0.01^{e,a}	52.63±0.01^{e,a,b}					1.62±0.12^{e}				
		52.63±0.01^{e,b}										

Values are mean of triplicate determination (n=3) ± standard deviation
Means with different superscripts (a-e) are significantly different, $p<0.05$

the obtained results, it was determined that young shoots obtained using all extraction methods did not contain tannin compounds and also the radix extracts obtained using all extraction methods contained the lowest tannin compounds. To the best of our knowledge, there have been no reports in literature on the total tannins contents of methanol extract from a plant's different parts. Therefore, for the first time in this study, the amount of tannins contained in the different parts of plant was determined and the effects of this compound on biological activity were examined.

In vitro antioxidant activity assays: The DPPH· scavenging activity of different extracts from *R. ribes* different parts are shown in Table 3. Ascorbic acid was used as a positive control. According to the results obtained from the DPPH experiment, the flowers and leaves extracts obtained using the three extraction methods were very close to each other and had a stronger DPPH scavenging activity than the other extracts. Ultrasonic bath (IC_{50} 5.00 µg/mL), maceration (IC_{50} 4.80 µg/mL) flowers and maceration leaves (IC_{50} 3.00 µg/mL) extracts exhibited a stronger DPPH radical scavenging activity than ascorbic acid (IC_{50} 6.00 µg/mL). When comparing extractions methods, it was found that the maceration and ultrasonic bath methods were more suitable methods for the DPPH activity of *R.ribes*. In addition, the young shoots extracts obtained using the three extraction methods exhibited the lowest free radical scavenging activity. The DPPH method was usually applied to measure the activity of polar compounds. The obtained results showed that flowers and leaves extracts were rich in polar compounds. Since these extracts exhibited the highest phenolic contents and DPPH radical scavenging activity, it was found that there was a linear relationship between phenolic compounds and free radical scavenging activity.

The TEAC/ABTS was a widely used method for measuring the activity of polar and nonpolar compounds in plants. The maceration extract of flowers (51.90 mM trolox/mg extract) exhibited the strongest $ABTS^{\cdot+}$ scavenging activity. In addition, the radix, flowers and leaves extracts obtained using all the extraction methods showed antioxidant activity close to each other and BHA, but the young plant shoots exhibited lower antioxidant activity than BHA and other extracts. When comparing extractions methods, it was found that the all extraction methods were a suitable method for the TEAC/ABTS activity of this species.

In the ultrasonic bath method, the young shoots extract (0.28 mM Fe^{2+}/mg extract) showed a stronger ferric reducing/antioxidant power activity than the other extracts. In the maceration method, the flowers extract (0.29 mM Fe^{2+}/mg) had the highest FRAP values. In addittion, the leaves extract (0.22 mM Fe^{2+}/mg extract) obtained using the Soxhlet method showed a stronger ferric reducing activity than the other extracts. According to the obtained results, the macerated flowers and ultrasonic bath young shoots extracts were found to have stronger ferric reducing activity than the other extracts. The radix extract (0.13 mM Fe^{2+}/mg extract) obtained using the ultrasonic bath method exhibited the lowest ferric reducing/antioxidant power activity. All the extracts from the plant's dif-

Table 4. The anti-urease inhibitory activity of different parts of *R. ribes*

	Urease inhibition (%) (12.5 μg/mL)		
Samples	Ultrasonic bath	Maceration	Soxhlet
Radix	2.33±0.1[a]	12.46±1.06[a]	7.57±0.13[a]
Flowers	6.61±2.4[b]	5.76±0.9[b]	4.33±0.4[b]
Leaves	17.90±0.5[c]	10.79±0.07[c]	16.83±0.4[c]
Young shoots	NA	9.26±0.7[d]	6.12±1.5[d]
Thiourea	78.54±0.60[d]	78.54±0.60[e]	78.54±0.60[e]

Values are mean of triplicate determination (n=3) ± standard deviation
NA: not activity
Means with different superscripts (a-e) are significantly different, $p<0.05$

Table 5. The anticholinesterase activity of different parts of *R. ribes*

	Acetylcholinesterase inhibition (%)		
Samples	Ultrasonic bath	Maceration	Soxhlet
Radix (500 μg/mL)	45.97±1.3[a]	36.05±0.83[a]	37.43±1.53[a]
Flowers (500 μg/mL)	71.90±1.14[b]	65.39±0.25[b]	61.13±0.76[b]
Leaves (500 μg/mL)	64.90±0.35[c]	14.95±2.33[c]	55.32±1.09[c]
Young shoots (200 μg/mL)	84.19±1.82[d]	63.95±0.5[d]	87.98±1.01[d]
Galantamine (500 μg/mL)	93.35±0.06[e]		

Values are mean of triplicate determination (n=3) ± standard deviation
Means with different superscripts (a-e) are significantly different, $p<0.05$

ferent parts had lower FRAP values than BHT compound (1.10 mM Fe^{2+}/mg). The results obtained from this study showed that both maceration and Soxhlet extraction techniques (excluding ultrasonic bath young shoots extract) was the most suitable method to get the most powerful ferric reducing/antioxidant activity.

In the ultrasonic bath method, radix (0.78 mM trolox/mg extract) and leaves (0.67 mM trolox/mg extract) extracts showed a stronger cupric reducing antioxidant activity than other extracts, respectively. In the maceration method, the leaves (0.71 mM trolox/mg extract) and radix (0.62 mM trolox/mg extract) extracts had higher CUPRAC values than the other extracts, respectively. In the Soxhlet method, the radix extract (0.91 mM trolox/mg extract) exhibited the strongest cupric reducing antioxidant activity. It was also found that young shoots (0.74 mM trolox/mg extract) and leaves (0.72 mM trolox/mg extract) extracts showed very close cupric reducing antioxidant results to each other. According to the results obtained from CUPRAC experimental, the radix extracts obtained using Soxhlet and ultrasonic bath methods showed the highest cupric reducing antioxidant activity. When the antioxidant activity of all the extracts was compared to the standard compound, all extracts were found to have lower activity than the BHA (1.62 mM trolox/mg) .

Shahi et al. (2016) investigated the antioxidant activity of maceration methanol extract from *R. ribes* flowers. According to the results obtained, flowers extracted with the concentration of 200 ppm and 300 ppm showed a higher inhibitory activity of free radicals than the BHT compound (Shahi et al. 2016). When we compared our study with this study, it was found that parallel to this study, maceration methanol extract from plant's flowers (IC_{50} 4.80 μg/mL) exhibited stronger DPPH· scavenging activity than ascorbic acid (IC_{50} 6.00 μg/mL). Shafaghat et al. (2016) investigated the free radical scavenging of Soxhlet hexane extract and essential oils from plant and plant's hexane extract (IC_{50} 325.00 μg/mL) and essential oils (IC_{50} 565.00 μg/mL) showed lower DPPH radical scavenging activity compared to the synthetic antioxidant of vitamin C (IC_{50} 26.00 μg/mL). In addition, the plant's essential oils and hexan extract composition were analyzed using GC-GC/MS. The main components of the hexane extract were 9-octadecenoic acid(ω-9), 9, 12- octadecadienoic acid (linoleic acid or ω- 6), hexadecanoic acid, (palmitic acid) , 1,2-benzenedicarboxylic acid diisooctyl, dodecane and γ- linolenic acid. The germacrene-d, α-pinene, terpinolene, p-cymene, bicyclogermane and limonene compounds were analysed as major components in the essential oils of the plant (Shafaghat et al. 2014). The antioxidant activities of chloroform and methanol extracts of the roots and stems of *R.ribes* have been reported before (Öztürk et al. 2007). This study reported that both methanol extracts obtained using the maceration method showed stronger free radical scavenging capacity than the corresponding chloroform extracts, moreover, the stems's methanol extract exhibited better activity than BHT. In addition, both roots extracts exhibited more potent superoxide anion radical scavenging activity than BHT. Except for the roots's extract, the other three extracts showed better metal chelating activity than quercetin. Unlike this study, the antioxidant activity of methanol extracts from different parts of the plant were examined with DPPH, FRAP, ABTS/TEAC and CUPRAC methods and it was determined that the maceration radix extract showed lower DPPH scavenging activity than ascorbic acid.

Anti-urease inhibitory activity: The results for the assessment of urease inhibitory activity of *R. ribes m*ethanol extracts (12.50 μg/mL) obtained using the three extraction methods are shown in Table 4. In the ultrasonic bath method, the leaves extract (17.90%) showed stronger ureae inhibitory activity than the other extracts. It was also found that the radix extract (2.33%) showed the lowest anti-urease activity. In addtion, the young shoots extract didn't show any anti-urease activity. In the maceration method, the radix (12.46%), leaves (10.79%) and young shoots (9.26%) extracts exhibited a stronger anti-urease activity than the flowers extract (5.76%). In the Soxhlet method, the leaves extract (16.83%) showed the strongest anti-urease activity. It was also found that the radix (7.57%) and

young shoots (6.12%) extracts showed close anti-urease activity and that extracts exhibited stronger activity than the flowers extract (4.33%). In this study, among the extracts obtained from three different extraction methods, the leaves extracts obtained using the ultrasonic bath and Soxhlet method exhibited the strongest anti-urease activity. When the anti-urease activities of the extract and standard were compared, it was found that all the extracts from the plant had lower anti-urease activity than standard thiourea (78.54%). The anti-urease activity of the 50% methanol extract of *R.ribes* roots has been previously reported (Nabati et al. 2012). This study showed that the 50% methanol extract had a 98.93% anti-urease activity at a concentration of 10 mg/mL. In our study, the anti-urease activity of methanol extracts from the radix at a concentration of 12.5 µg/mL was investigated and found that maceration radix extract had 12.46% anti-urease activity.

Anticholinesterase activity: The results for the assessment of cholinesterase inhibitory activity of plant's different extracts are shown in Table 5. In the ultrasonic bath method, young shoots (84.19%) and flowers (71.9%) extracts exhibited stronger cholinesterase inhibitory activity than other extracts. In the maceration method, the young shoots (63.95%) and flowers (65.39%) extracts exhibited stronger cholinesterase inhibitory activity than other extracts. It was also found that the leaves extract (14.95%) had the lowest anticholinesterase activity. In the Soxhlet method, the young shoots (87.98%) and flowers (61.13%) extracts exhibited the strongest anticholinesterase activity. According to the results obtained from activity assay, the radix extract (37.43%) showed lower anticholinesterase activity than the other extracts. As a result of this experiment, it was found that the young shoots extracts obtained using the three extraction methods exhibited the strongest anticholinesterase activity. It was also found that the young shoots extracts obtained using the ultrasonic bath (84.19%) and Soxhlet (87.98%) methods showed close anticholinesterase activity to the galantamine compound (93.35%). In the present study, the Soxhlet and ultrasonic bath methods were the most extraction methods to get the strongest anticholinesterase activity. In Gholamhoseiniant et al., the *in vitro* anticholinesterase activity of the methanol extract from the rhizomes of the plant was investigated and it was found that this extract showed 72.4% activity at a concentration of 8 mg /mL (Gholamhoseiniant et al. 2009). In another study, it was clearly demonstrated that the treatment with 50% methanol extract from *R.ribes* roots and rhizomes could significantly recover the spatial and passive avoidance memory disorders caused by the destruction of the NBM nucleus in male-wistars rats (Zahedi et al. 2015). In our study, anticholinesterase activities of different parts (radix, flowers, leaves and young shoots) of the plant were investigated and it was found that these parts (especially the young shoots) showed significant activity in accordance with the literature.

CONCLUSION

Rheum ribes is mainly used in medicines and foods in Turkey. Therefore, it was very important to examine the biological activities (antioxidant, anti-urease, and anticholinesterase) of this plant. In this study, the biological activities and chemical contents of different parts of the plant were qualitatively and quantitatively determined. In this study, it was determined that the macerated extract of flowers contained higher total phenolic and tannins contents than other extracts. According to the results obtained, the macerated flowers extract showed the strongest ABTS radical scavenging and ferric reducing activity. The macerated leaves and Soxhlet radix extracts showed the highest DPPH radical scavenging and cupric reducing antioxidant activity, respectively. The young shoots extracts obtained using the Soxhlet methods showed the highest anticholinesterase activity. All extracts obtained from different parts of the plant were found to have very low anti-urease activity when compared to the anti-urease activity of standard compound. Therefore, the methanol extract from the plant's flowers, leaves and young shoots can be used as a natural antioxidant and anticholinesterase agent respectively for the pharmaceutical and food industry in the future.

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Original Article

PPARγ Pro12Ala and C161T polymorphisms, but not *PPARα* L162V, are associated with osteoporosis risk in Turkish postmenopausal women

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ABSTRACT

Stimulation of peroxisome proliferator-activated receptors (*PPARs*) causes mesenchymal stem cells of the human bone marrow differentiate into adipocytes instead of osteoblasts leading to a decreased number of osteoblasts and a decrease in bone mineral density (BMD). Thus, *PPARs* may have impacts on bone metabolism. 224 postmenopausal women (171 osteoporotic and osteopenic, 53 healthy control) were included in this study. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and agarose gel electrophoresis techniques were performed to detect *PPARα* L162V and *PPARγ* Pro12Ala/C161T polymorphisms. The distribution of *PPARγ* Pro12Ala genotype and allele frequencies was not statistically different in control and patient (osteopenic+osteoporotic) groups (p>0.05). However, in the patient group, subjects with "Pro12Pro" genotype had lower lumbar spine (L1-L4) BMD values than those with "Ala" allele (p<0.05). The frequency of *PPARγ* C161T "CC" genotype was higher in the patient group when compared with that in the control group (p<0.05). There were no significant associations between the genotype and allele frequencies of *PPARγ* C161T/ *PPARα* L162V and BMD values (p>0.05). We suggested that *PPARγ* Pro12Ala and C161T gene variants might be contributing factors in the development of osteoporosis.

Keywords: Peroxisome proliferator-activated receptor, bone mineral density, osteoporosis, polymorphism

INTRODUCTION

Peroxisome proliferator-activated receptors (*PPARs*) belong to the superfamily of nuclear receptors which are are ligand-activated transcription factors. Three different *PPAR* subtypes have been identified; *PPARα*, *PPARβ* (also called *PPARδ*), and *PPARγ* (Abbot 2009). *PPARα* is found in tissues such as heart, muscle, liver and kidney where fatty acid catabolism is important and so regulates genes involved in lipid metabolism. *PPARα* is activated by natural ligands (polyunsaturated fatty acids, lipolytic products of lipoproteins, oxidized phospholipids) and by synthetic ligands (gemfibrozil and fenofibrate) (Touyz and Schiffrin 2006). Fenofibrate which is currently used for the treatment of hypercholesterolemia and hypertriglyceridemia, also maintains bone mass. Whole body and femoral bone mineral density (BMD) values were higher in ovariectomized rats given fenofibrate, compared to controls (Stunes et al. 2011).

PPARγ is heavily expressed in adipose tissue and controls adipocyte differentiation and lipid storage. *PPARγ* regulates the action of insulin through its effects on adipose tissue and skeletal muscle (Touyz and Schiffrin 2006). Natural agonists (eicosanoids and oxidized, polyunsaturated fatty acids) and synthetic agonists (thiazolidinediones; a family of antidiabetic drugs-rosiglitazone and

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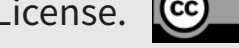

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pioglitazone) for *PPARγ* decrease peripheral insulin resistance and thereby reduce blood glucose levels in type 2 diabetic patients (Touyz and Schiffrin 2006; Harsløf et al. 2011). After activation of *PPARγ* by rosiglitazone, mesenchymal stem cells differentiate into adipocytes instead of osteoblasts leading to increased number of adipocytes and decreased number of osteoblasts and decreased BMD in mouse bone marrow (Rzonca et al. 2004; Ali et al. 2005).

To date, several polymorphisms within the human *PPARα* gene have been identified. Of these, a C→G transversion at position 484 in exon 5, leading to a substitution of valine for leucine (L162V) at codon 162, has functional effects on *PPARα* activity (Flavell et al. 2002; Do et al. 2009). Some studies have found associations between *PPARα* L162V polymorphism and plasma lipids and atherosclerosis development, however, the effects of this polymorphism on bone metabolism haven't been investigated so far.

Cytosine-guanine exchange in exon B (codon12) is the most common gene mutation in human *PPARγ* gene resulting proline (Pro) to alanine (Ala) substitution in the protein (Temelkova-Kurktschiev et al. 2004). C161T substitution in exon 6 of the *PPARγ* gene was also described (Meirhaeghe et al. 1998). *PPARγ* was found to be related with cardiovascular diseases (Takano and Komuro, 2009), diabetes mellitus (Cho et al. 2008), carcinogenesis (Elrod and Sun 2008) and inflammation (Kapoor et al. 2007a; 2007b; Szanto and Nagy 2008) in some studies. *PPARγ* was also associated with bone mineral density, osteoporosis, osteoarthritis and non-traumatic hip fracture risk in various populations (Ogawa et al. 1999; Harslof et al. 2010; Tamaki et al. 2010; Fahmi et al. 2011; Dragojevič et al. 2011; Wang et al. 2013). However, no association was found between *PPARγ* and bone mineral density variation in Chinese nuclear families (Yue at al. 2010) and in Japanese postmenopausal women (Wang et al. 2013).

To the best of our knowledge, there is no study regarding the association of *PPARα* polymorphisms with BMD and osteoporosis. Little is known about the association of the *PPARγ* polymorphism with the osteoporosis risk and also the results are controversial. Therefore, we aimed to investigate the relation between *PPARα* and *PPARγ* gene variants and osteoporosis in Turkish postmenopausal women.

MATERIALS AND METHODS

Subjects

224 Turkish postmenopausal women (171 osteoporotic and osteopenic, 53 healthy control), attending the Uskudar State Hospital in Istanbul were recruited in this study. World Health Organization (WHO) definitions and criteria for osteopenia and osteoporosis were used during ascertainment (World Health Organization Study Group, 1994). The patients received a standardized questionnaire including questions regarding the osteoporosis risk factors (age, menopausal status, smoking, family history of osteoporosis), medication use and other medical conditions. Demographic and morphometric characteristics were also recorded. Subjects with a clinical diagnosis of osteopenia/osteoporosis and those with normal BMD values were included in the study group. Exclusion criteria were conditions, diseases, and/or treatments known to interfere with bone metabolism, such as malignancies, severe liver or gastroenteral diseases, endocrinologic disorders (hypo-hyperparathyroidism, hyperthyroidism, Cushing's syndrome), skeletal diseases (rheumatoid arthritis, osteomalacia, osteogenesis imperfecta and Paget's disease) and current pharmacological treatment with anabolic androgenic steroids, estrogens or estrogen-related molecules, corticosteroids and anticonvulsants before enrollment. Menopause was defined as amenorrhoea of at least one year duration. The study protocol was approved by the Local Ethical Commitee of Istanbul University, Istanbul Medical Faculty (Protocol No: 2006/2145). All participants signed written, informed consent forms prior to giving their blood sample.

BMD measurement

Dual energy X-ray absorptiometry (DXA; Lunar DPX (GE Lunar Corporation, Madison, WI, USA) was used to determine BMD of the lumbar spine (L1-L4) and hip (femoral neck and total hip). All DEXA scans were analyzed according to software (encore version 2005, 9.30.044) provided by the manufacturer. BMD was expressed as grams per centimeter square (g/cm^2).

Genotype study

Genomic DNA samples were extracted from whole blood with salting out procedure (Miller et al. 1988). Polymerase chain reaction-restriction fragment lenght polymorphism (PCR-RFLP) analysis were used to detect *PPARα* L162V and *PPARγ* Pro12Ala/C161T polymorphisms as previously reported (Yen et al. 1997; Flavell et al. 2002).

Statistical analysis

The statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software package programme version 20.0 (IBM Corp., Armonk, NY, USA). Categorical variables are presented as frequencies, while continous variables are presented as means (± standard deviation-S.D.). Chi-square (χ^2) test was used for genotype and allele frequencies comparison and Hardy-Weinberg Equilibrium (HWE). BMD values of different genotypes and alleles were compared by Student's t-test. Allele frequencies were calculated by gene counting method. Values of $p<0.05$ were considered statistically significant.

RESULTS

Demographic characteristics and BMD status

The baseline characteristics of the study population were presented in Table 1. As expected, the body mass index (BMI), BMD values of lumbar spine (L1-L4), femoral neck and total hip showed significant differences between control and patient (osteopenic+osteoporotic) groups ($p<0.001$), whereas no significant differences were detected in age, age of menopause, smoking and family history of osteoporosis ($p>0.05$).

PPARγ Pro12Ala/C161T and *PPARα* L162V genotypes and allele distribution

The genotypic and allelic frequencies of *PPARγ* Pro12Ala/C161T and *PPARα* L162V polymorphisms were shown in Table 2. *PPARγ* C161T "CC" genotype frequency was found to be higher in the patient group when compared to that in the control group ($p=0.028$). No significant differences were found in the frequencies of *PPARγ* Pro12Ala and *PPARα* L162V

genotypes and alleles within two groups (p>0.05). "Ala12Ala", "TT" and "VV" genotypes were not observed in the control group.

The association of *PPARγ* and *PPARα* polymorphisms with BMD values

The association of *PPARγ* Pro12Ala/C161T and *PPARα* L162V genotypes with BMD values were presented in Table 3. In the patient group, subjects with "Pro12Pro" genotype had lower lumbar spine (L1-L4) BMD values than those with "Ala" allele (p<0.05). No significant association was found between *PPARγ* C161T and *PPARα* L162V genotypes and BMD values in the study groups (p>0.05).

DISCUSSION

The present study is the first one in Turkish population showing an association between *PPARγ* gene and the risk of the development of osteoporosis. Subjects with *PPARγ* "Pro12Pro" genotype had lower lumbar spine BMD values than those with "Ala" allele in our patient group. Similarly, in a study with β-thalassemia major patients, the risk of osteopenia was significantly higher in subjects with "Pro12Pro" genotype than the carriers of the rare alleles (Sahmani et al. 2013). In contrast to our results, postmenopausal women with "Pro12Pro" genotype had higher BMD of lumbar spine than that of subjects with "Pro12Ala" genotype (Yue et al. 2010). *PPARγ* Pro12Ala gene variants were not associated with BMD in elderly and young Swedish women (Herlin et al. 2015). Also, *PPARγ* Pro12Ala was not independently associated with BMD values in postmenopausal Japanese women, either (Wang et al. 2013). We think

Table 1. The baseline characteristics of the study population

	Control n=53	Patient n=171
Age	55.55±6.99	57.24±5.99
Age of menopause	46.47±5.17	46.50±5.13
BMI (kg/m^2)	33.22±5.11	29.52±4.83 *
Smoking (n, %)	5 (9.4 %)	17 (9.9 %)
Family history of osteoporosis (n, %)	24 (47.1 %)	74 (43.3 %)
Lumbar spine (L1-L4) BMD (g/cm^2)	1.183±0.110	0.940±0.113 *
Femoral neck BMD (g/cm^2)	0.966±0.101	0.827±0.088 *
Total hip BMD (g/cm^2)	1.044±0.099	0.882±0.093 *

n: number of subjects, BMI: Body mass index, BMD: Bone mineral density.
Values are means±SD except where noted.
* p<0.001 vs. control group.

Table 2. The distribution of *PPARγ* Pro12Ala/ C161T and *PPARα* L162V genotypes and allele frequencies in the study groups

Genotypes / Alleles	Control n, (%)	Patient n, (%)
PPARγ Pro12Ala		
Pro12Pro	42 (79.2%)	149 (87.1%)
Pro12Ala	11 (20.8%)	20 (11.7%)
Ala12Ala	0 (0 %)	2 (1.2%)
Pro	95 (89.6%)	318 (93%)
Ala	11 (10.4%)	24 (7%)
PPARγ C161T		
CC	37 (69.8%)	142 (83%)*
CT	16 (30.2%)	24 (14%)
TT	0 (0 %)	5 (3%)
C	90 (84.9%)	308 (90.1%)
T	16 (15.1%)	34 (9.9%)
PPARα L162V		
LL	39 (73.6%)	116 (67.8%)
LV	14 (26.4%)	50 (29.2%)
VV	0 (0%)	5 (2.9%)
L	92 (86.8%)	282 (82.5%)
V	14 (13.2%)	60 (17.5%)

n: number of subjects, *PPARγ*: Peroxisome proliferator activated receptor gamma, *PPARα*: Peroxisome proliferator activated receptor alpha
* p< 0.05 vs. control group.

Table 3. Association of *PPARγ* and *PPARα* genotypes with BMD values in study population

	PPARγ Pro12Ala		*PPARγ* C161T		*PPARα* L162V	
Groups/BMD	Pro12Pro	Pro12Ala +Ala12Ala	CC	CT+TT	LL	LV+VV
Control						
Lumbar spine	1.190±0.115	1.160±0.087	1.190±0.119	1.170±0.089	1.170±0.075	1.230±0.171
Femoral neck	0.974±0.110	0.934±0.049	0.976±0.116	0.943±0.053	0.968±0.103	0.960±0.101
Total hip	1.064±0.101	0.990±0.048	1.062±0.107	1.006±0.069	1.038±0.091	1.061±0.124
Patient						
Lumbar spine	0.940±0.114*	0.990±0.082	0.940±0.114	0.950±0.103	0.940±0.111	0.940±0.115
Femoral neck	0.825±0.084	0.850±0.091	0.828±0.084	0.833±0.093	0.822±0.088	0.841±0.079
Total hip	0.883±0.090	0.892±0.094	0.882±0.089	0.895±0.095	0.881±0.094	0.890±0.082

BMD: Bone mineral density, *PPARγ*: Peroxisome proliferator activated receptor gamma, *PPARα*: Peroxisome proliferator activated receptor alpha

*p<0.05 vs. ProAla+Ala12Ala genotypes

that these results differ depending on geographic background and number of subjects in the studies.

In our study, *PPARγ* C161T "CC" genotype frequency was found to be higher in the patient group when compared with the control group, however, no association was found between the C161T genotypes and BMD values. In contrast, Z scores of the lumbar and total body BMD was found to be higher in Japanese postmenopausal women with *PPARγ* C161T "CC" genotype than those in the subjects with "CT+TT" genotype. It was suggested that there is an association between *PPARγ* gene and BMD and the possible involvement of C161T polymorphism in the cause of postmenopausal osteoporosis in Japanese women (Ogawa et al. 1999). Femoral neck and total hip BMD values were significantly higher in Japanese premenopausal women with "CC" genotype than the values in subjects with "CT/TT" genotypes (Tamaki et al. 2010). Similar to our results, no association was found between *PPARγ* C161T genotypes and BMD of lumbar spine/femoral neck in healthy Korean pre- and postmenopausal women (Rhee et al. 2005).

An association was found between polymorphisms in *PPARγ*, BMD and fracture risk in Danish population indicating that the effect may be modified by environmental factors (Harsløf et al. 2011). Besides, *PPARγ* Pro12Ala and C161T polymorphisms did not have any significant relation with the non-traumatic hip fracture risk in the elderly Slovenian population (Dragojevič et al. 2011).

In the present study, the frequencies of "Ala" allele were 10.4% and 7% in the control and in the patient groups, respectively. C161T "rare allele (T)" frequency was 15.1% in the control group versus 9.9% of the patient group. "Ala12Ala" and "TT" genotypes were not observed in the control group (0%) whereas the frequencies of them were 1.2% and 3% in the patient group, respectively. The genotype populations of *PPARγ* Pro12Ala/C161T were in accordance with those in Turkish patients with inflammatory bowel disease, coronary heart disease and gastric cancer (Atug et al. 2008; Yilmaz-Aydogan et al. 2011; Canbay et al. 2012). Similar to our results, Erdogan et al. (2007) reported the frequency as 0% for "Ala12Ala" genotype in the control group and in diabetic patients with and without diabetic nefropathy. However, in their study, the frequency for "Ala" allele was found as 0% and 0.5% in control group and in diabetic group, respectively. A remarkable difference was observed in their study in terms of "Ala" allele distribution. This observed difference may be based on the different number of healthy controls and patients in these two studies. Also the different patient group of Erdogan's study consisting diabetic subjects may affect the allelic differences between the two studies. Similar to our results, "T" allele frequencies were found as 11.5 and 9.3 in control and patient group in Erdogan's study.

Pro12Ala and C161T frequencies in our study are similar to those in Slovenian population (Dragojevič et al. 2011) and those in Chinese postmenopausal women (Yue at al. 2010). The genotype and allelic frequencies of Pro12Ala was also found to be similar to our results in a meta-analysis study with European Caucasion population (Zhang et al. 2012) and in studies with Japanese postmenopausal women (Ogawa et al. 1999; Wang et al. 2013). Pro12Ala genotype and allelic frequencies in two studies with Iranian population are similar to the frequencies in the present study, however, they could not find any subjects with "Ala12Ala" genotype in their study (Namvaran et al. 2011; Sahmani et al. 2013). C161T frequencies in Japanese women (Tamaki et al. 2010) and Korean women (Rhee et al. 2005) are in accordance with our results.

As of now, there isn't any study showing a relation between *PPARα* L162V polymorphism, BMD and the risk of osteoporosis. However, no significant association was found between *PPARα* L162V genotype and allele frequencies and BMD values in our study population. The genotype and allele frequencies of *PPARα* L162V are similar to those in a previous Turkish study with coronary heart disease based on the presence of diabetes (Yilmaz-Aydogan et al. 2013) and to those in another study with Turkish subjects (Koytak et al. 2008). Besides, our frequencies differ from those found in Spanish Mediterranean, Brazilian and Croatian populations (Francès et al. 2008; Chen et al. 2010; Nadalin et al. 2014). "VV" genotype was not found in Croatian population (Nadalin et al. 2014) and in multi-ethnic Malaysian population (Chia et al. 2015). Similarly we found no subjects with "VV" genotype in our control group whereas we found only five subjects in our patient group.

The frequencies of Pro12Ala-Ala12Ala genotype, C161T-TT genotype and L162V-VV genotype are very rarely observed in the present study. Therefore, ANOVA statistical test couldn't be used for the comparison of genotypes and BMD values. Furthermore, this report was comprised of a relatively small study population. These represents the limitations of the study. We think that further studies with higher number of subjects may be necessary to conclude with greater certainty of the relation between *PPARγ* Pro12Ala/C161T polymorphisms and decreased BMD status.

CONCLUSION

The present study suggests that *PPARγ* Pro12Ala and C161T polymorphisms may contribute to the development of osteoporosis in Turkish postmenopausal women.

Ethics Committee Approval: The study protocol was approved by the Local Ethical Commitee of Istanbul University, Istanbul Medical Faculty (Protocol No: 2006/2145).

Informed Consent: All participants signed written, informed consent forms prior to giving their blood sample.

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Original Article

Deontological violations of community pharmacies in Turkey

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ABSTRACT

Deontological violations are important in terms of reputation of public health, pharmacy profession, and protection of public finance. The aim of this study is to determine types and prevalence of deontological crimes reflected in records of High Honor Court (HHC) in Turkish Pharmacists' Association (TPA), to develop proposals on corrective and preventive occupational policies. Crime types in disciplinary files were classified according to classification method of deontological crimes in 3-groups, and results were evaluated by frequency and percentage distributions.

In the study, 32 deontological crime types and 112 criminal cases were detected. Accordingly, it was found that deontological crimes due to competition are in the first rank with 51 cases, TPA, Drug-Pharmacy Legislation violations in the second with 50 cases and Social Security Institution protocol provisions violations in the last rank with 11 cases, and in all types of crime, "collusion" is in the first rank (30.4%).It is thought that "collusion" crime being in the first rank increasing its share from 18.6% to 30.4% in all crimes despite increasing punishments.

Persistence of crime despite punitive sanctions aggravated by recent regulations suggests that it is not possible to solve only by punitive sanctions, and it must be get to the bottom of the problem.

Keywords: Pharmacy, community pharmacies, deontological violations, deontological crimes

INTRODUCTION

Prevention of deontological violations committed in community pharmacies is important for the protection of public health, the reputation of pharmacy profession and public finance. It has been shown that these deontological violations increase during periods of liberalization policies applied in the healthcare field and economic crises (Kıran 2009; Kıran and Mandıracıoğlu 2013).

There are many laws and regulations related to pharmacy, mainly the Deontology Regulation of the Turkish Pharmacist's Association (TPA), as well as binding provisions in agreements of Social Security Institution (SSI) in order to prevent community pharmacists in Turkey to be getting involved in deontological violations. In recent years, "Regulation on the Establishment of the Collusion Evaluation Commissions and its Working Rules and Principles" issued by the Turkish Ministry of Health, Turkish Medicines and Medical Devices Agency which entered into force on 14.03.2016 is also an important legislative regulation. In Article 4/d of this Regulation; "Collusion is defined as; business activity jointly or individually by someone other than the pharmacist who appears as the owner and responsible manager of the pharmacy, whether or not he/she is in line of duty, through confidential or open, written or oral agreements as well as all such activities" (TİTCK Circular 2016).

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In addition, according to Article 48-(4) of the Regulation on Pharmacists and Pharmacies held in 2014, in case of the detection of the establishment of pharmacies opened as collusive, the license will be cancelled, and penal sanctioning have been set as these pharmacists will not be able to open a pharmacy for five years" (Official Gazette 2014). Despite these regulations that have been issued in recent years and contain severe punitive sanctions, there is little research investigating the deontological violations in Turkey.

In this context, this study was conducted in order to determine the types and prevalence of deontological violations reflected in the records of High Honor Court (HHC) in the TPA, and to develop proposals on corrective and preventive occupational policies.

Table 1. Grouped Distribution of High Honor Court Cases According to Types of Violation

* Violation Type of HHC Cases 2015-2017 Years	%	n
1. Competition-Based Deontological Violations	51	45.6
2.TPA, Drug-Pharmacy Legislation Violations	50	44.6
* 2.1. Collusion	*(34)	*(68)
3.SSI Protocol Provisions Violations	11	9.8
Total	112	100
* Collusion	*(34)	*(30,4)

*HHC: High Honor Court

MATERIALS AND METHODS

The research is cross-sectional. Crime types in disciplinary files in TPA Period-40 Working Report between 10.12.2015-30.09.2017 of HHC in TPA and submitted from 54 Regional Pharmacists Chamber in Turkey were classified according to classification method of deontological violations in 3-groups, and results were evaluated by frequency and percentage distributions (Kıran and Mandıracıoğlu 2013; Turkish Pharmacist's Association 2015; Turkish Pharmacist's Association 2017). Some files have been discussed more than once in the High Honor Court for various reasons. Therefore, although the total number of files is 99, it is reflected as 112 in the study report.

RESULTS

32 deontological violation types and a total of 112 deontological violation cases were detected from 54 Regional Pharmacists Chamber in Turkey submitted to High Honor Court in TPA between 2015-2017 years. These violations were classified in 3-groups according to classification method used in the first and only study on deontological violations committed in Turkey's community pharmacies (Kıran

Table 2. Detailed Distribution of Violations Submitted in High Honor Court Cases (2015-2017)

Violations	n=112	%
Operating collusive pharmacy	34	30.4
Prescription collection and transfer	19	17.0
Violation of night-pharmacy, failure to comply with opening and closing times	12	10.7
Over-the-counter and wholesale drug sales	10	8.9
Advertisement, Promotion	3	2.7
Not taking patient share and price difference	2	1.8
Creating a website	2	1.8
Over-the-counter and wholesale of controlled medicines and narcotic drugs	2	1.8
Invoicing of drugs to the Institution, which are not delivered to patients	2	1.8
Not delivery of drugs to patients	2	1.8
Having drugs not delivered to patients	2	1.8
Having expired drugs	1	0.9
Not giving the documents requested by the auditor pharmacists	1	0.9
Not appointing responsible manager during departures over 24 h	1	0.9
Recording a prescription to SSI Medula system to prevent drug purchase from other pharmacies	1	0.9
Failure to comply with cold chain rules	1	0.9
Having products with uncertain content and manufacturer	1	0.9
Not covering the prescription despite the institution prescription line is available	1	0.9
Absentee	1	0.9
Prescribing drugs outside patient knowledge	1	0.9
Disruptive behavior for the functioning of chamber departments	1	0.9
Not giving information and documents requested by the board of directors	1	0.9
Invoicing of fake prescription, report, drug bar code and 2-d barcode	1	0.9
Establishing business relationship that does not conform to occupational ethics	1	0.9
Lack of illuminated and current night-pharmacy boards	1	0.9
Keeping drugs with impaired original package	1	0.9
Over-the-counter sale of unlicensed and illegal drugs	1	0.9
Having drugs without complete 2-d barcode	1	0.9
Improper action against the chamber president in terms of professional norms and dignity	1	0.9
Injection in pharmacy	1	0.9
Preventing the patient's access to drugs and purchase the drug from the preferred pharmacy by recording trial prescription to the Medula Provisioning System	1	0.9
Sales via internet	1	0.9
Irregular agreement	1	0.9

Table 3. Distribution of deontological violations according to regional pharmacy chambers(2015-2017)

Regional Chamber of Pharmacists	n=99	%
1. İstanbul Chamber of Pharmacists	33	33.3
2. Ankara Chamber of Pharmacists	16	16.2
3. Adana Chamber of Pharmacists	7	7.1
4. Mersin Chamber of Pharmacists	7	7.1
5. Kocaeli Chamber of Pharmacists	6	6.1
6. Balıkesir Chamber of Pharmacists	5	5.1
7. Şanlıurfa Chamber of Pharmacists	3	3.0
8. Konya Chamber of Pharmacists	3	3.0
9. İzmir Chamber of Pharmacists	3	3.0
10. Manisa Chamber of Pharmacists	2	2.0
11. Bursa Chamber of Pharmacists	2	2.0
12. Antalya Chamber of Pharmacists	2	2.0
13. Erzurum Chamber of Pharmacists	1	1.0
14. Eskişehir Chamber of Pharmacists	1	1.0
15. Tekirdağ Chamber of Pharmacists	1	1.0
16. Isparta Chamber of Pharmacists	1	1.0
17. Trabzon Chamber of Pharmacists	1	1.0
18. Kırklareli Chamber of Pharmacists	1	1.0
19. Uşak Chamber of Pharmacists	1	1.0
20. Gaziantep Chamber of Pharmacists	1	1.0
21. Giresun Chamber of Pharmacists	1	1.0
22. Batman Chamber of Pharmacists	1	1.0

and Mandıracıoğlu 2013; Turkish Pharmacist's Association 2015; Turkish Pharmacist's Association 2017).

Accordingly, it was found that "competition-based deontological violations" are in the first rank with 51 cases (45.6%), TPA, "Drug-Pharmacy Legislation violations" in the second with 50 cases (44.6%) and "Social Security Institution (SSI) protocol provisions violations" in the last rank with 11 cases (9.8%). It was found that collusion evaluated under the scope of TPA, Drug-Pharmacy Legislation violations consist of 68% of the violations in this group with 34 cases and in all types of violation it is in the first rank with 30.4%. Groups according to the type of violation are shown in Table 1, and the detailed distribution of all violations is shown in Table 2.

The most intensive deontological violations during 2015-2017 are determined in İstanbul (33.3%), Ankara (16.2%), Adana (7.1%) and Mersin (7.1%). Distributions of deontological violations for the years of 2015-2017 according to Regional Pharmacy Chambers are shown in Table 3.

During 2015-2017, disciplinary punishments given by the High Honor Court are defined as temporary prohibit from practice (42.4%), penalty fine (21.2%), written warning (3%) and files returned with various reasons (33.4%). Distribution of punishments given to violations by the High Honor Court is shown in Table 4.

Table 4. Distribution of punishments given by the High Honor Court (2015-2017)

Punishments Given To Violations By The High Honor Court	n=99	%
Ostracized from profession for 180 days	21	21.2
Penalty fine for 15 times the yearly chamber contribution	16	16.2
Ostracized from profession for 3 days	15	15.2
Returned due to lack of evidence	12	12.1
Returned	8	8.1
Penalty fine for 10 times the yearly chamber contribution	3	3.0
Written warning	3	3.0
Returned due to finalization since it is not appealed in given time	3	3.0
Returned due to make decision according to the outcome of litigation	3	3.0
Penalty fine for 5 times the yearly chamber contribution	2	2.0
Waiting to be discussed after completing notification	2	2.0
Ostracized from profession for 5 days	2	2.0
Ostracized from profession for 30 days	2	2.0
Ostracized from profession for 15 days	1	1.0
Ostracized from profession for 20 days	1	1.0
Returned due to lack of procedure	1	1.0

Table 5. Comparative Distribution of Deontological Violation Types by Years

	1987-2010		2013-2015		2015-2017	
Violation Types	%	n	%	n	%	n
1. Competition-Based Deontological Violations	53	165	50.8	64	45.6	51
2.TPADrug-Pharmacy Legislation Violations	24.6	77	35.7	45	44.6	50
* 2.1. Collusion	76.3	*58	*48.7	*22	*68	*34
3. SSI Protocol Provisions Violations	22.4	70	13.5	17	9.8	11
Total	100	312	100	126	100	112

*Collusion rate in TPADrug-Pharmacy Legislation Violations.

Since the records of the last four years of the High Honor Court (2013-2015) and (2015-2017) were examined together with the first study records examined deontological violations in Turkey, it is understood that the order of prevalence of violation (case) types has not changed (Table 5).

DISCUSSION AND CONCLUSION

When compared, Turkey's first study based on long term records with 2015-2017 Report of HHC Period-40 with the Working Report of HHC for 2013-2015; it was seen that competition-based deontological violations in the first rank with 53% decreased to 50.8% during 2013-2015 years and to 45.6% during 2015-2017 years (Kıran and Mandıracıoğlu 2013; Turkish Pharmacist's Association 2015; Turkish Pharmacist's Association 2017). On the other hand; it was determined that TPA Drug-Pharmacy Legislation Violations with 24.6% increased to 35.7% during 2013-2015 years and this upward trend has continued during 2015-2017 years reaching up to 44.6% and one of the most important main factors in this trend was collusion violations. In addition, SSI violations with 22.4% decreased to 17% and then 9.8% within the years and it is thought that the SSI has played a role in this decrease with severe penalties imposed on community pharmacies (Kıran 2015).

The most common violation types; prescription collection and transfer (17%), violation of night-pharmacy, failure to comply with opening and closing times (10.7%), over-the-counter and wholesale drug sales (8.9%), advertisement and promotion activities (2.7%) among competition-based deontological violations, and not delivering drugs to patients and invoicing drugs to the institution (3.6%) among SSI violations.

In TPA Drug-Pharmacy Legislation Violations; it is thought-provoking that "collusion" violation in the past (76.3%) is still the most common violation (68%) and in the first rank increasing its share from 18.6% to 30.4% in all violations despite increasing punishments (Kıran and Mandıracıoğlu 2013; Turkish Pharmacist's Association 2015; Turkish Pharmacist's Association 2017).

Persistence of violation despite punitive sanctions aggravated by recent regulations suggests that it is not possible to solve only by punitive sanctions, and it must be get to the bottom of the problem (Higuchi and Kodama 2011; Panitch and Leys 2009; Wiberg 2011).

Suggestions

It is thought that it is necessary to work together with all stakeholders for solution of economic and educational problems in pharmacy, especially for the payment of sufficient salary during the retirement of community and public pharmacists and for creation of adequate and new employment areas in all fields of public, universities, pharmaceuticals and pharmacy according to the number of beds in hospitals and the production capacity of the industry, other than community pharmacy for pharmacists who have graduated from more than fifty pharmacy faculties in order to prevent collusion violation that endangers community health care, causes illegal events and harms the public economy and makes unrepairable damages to the reputation of the pharmacy profession.

Ethics Committee Approval: Ege University Scientific Research and Publication Ethics Committees does not require the application of the ethics committee for the data collected from the working reports.

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Original Article

Real time monitoring of cytotoxicity of *Callistemon citrinus* against Colo-205 cell line

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ABSTRACT

Callistemon citrinus is a member of Myrtaceae family that thrives under different ecological conditions. The leaves, flowers, stem backs and roots of the plant contain various phytochemicals that are useful in folk medicine for different remedies such as antimicrobial, anti-nociceptive, fungicide and anti-inflammatory purposes. In this study, we investigated the cytotoxic effect of *Callistemon citrinus* leaf and flower methanolic extracts against human Colo-205 Cell Line using real time cell analyzer device for monitoring in time-dependent manner. To determine the mechanism of cytotoxicity of the extracts, Western blotting assay was used for measuring evocation of Akt pathway. Extracts were found to exert cytotoxic effect at a dose dependent manner. IC_{50} values of leaves and flowers extract were 6.49 μg/mL and 5.22 μg/mL, respectively. At the early stages of the experiment, Akt pathway was triggered at high extract concentrations. Although, high extract concentrations showed proliferative effect at early stages, this effect reversed after 5 and 8 h resulting in low cell viability. Findings from this study therefore showed that extracts of leaf and flower from *Callistemon citrinus* demonstrated cytotoxic effect against Colo-205 but seems not to be related Akt signaling pathway.

Keywords: Akt/p-Akt, *Callistemon citrinus*, colon cancer, xCELLigence

INTRODUCTION

Colon cancer is a one of the major causes of mortality and morbidity all over the World. According to a recent epidemiological report, intestinal cancers (colon and rectum) death rate was 13% of total cancer deaths from 172600 reported mortalities which was second order after the lung cancer (Malvezzi et al. 2015). Currently, therapeutic approaches for treating human colorectal include radiotherapy, chemotherapy and surgery. In addition to conventional treatment of colon carcinoma, there are a lot of alternative medicine in use from natural products. One of such natural products include phytochemicals from ornamental plants. *Callistemon citrinus* is one of the medicinal ornamental plants from Myrtaceae family (Brophy et al. 1998). Different parts of the plant contain alkaloids, polyphenols, flavonoids, tannins, steroids, aliphatic acids, monoterpenoids, triterpenoids, sesquiterpenes and several phytochemicals. The leaf oils of *C. citrinus* are known to have antimicrobial, antifungal, antinociceptive and anti-inflammatory activities (Sudhakar et al. 2004; Oyedeji et al. 2009). In folk medicine, various parts of this herb are used in making traditional pills for treating dysentery, cough, bronchitis, hemorrhoids and rheumatism (Paluri et al. 2012). In our previous study, the *in vitro* cytotoxic effects of leaf and flower extracts from *C.citrinus* has recently been reported against MCF-7 breast cancer cell line by our group (Fayemi et al. 2015).

Although, reports on bioactivities of Callistemon are available yet information on *in vitro* cytotoxicity of this plant is limited against colon cancer. *In vitro* cytotoxicity tests are prerequisites for describing intrinsic toxicodynamics of phytochemicals in any potential

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medicinal plant (Sutar et al. 2014). Therefore, monitoring cell viability and toxicity is crucial for preclinical screening of a new compound in order to ascertain its bioactivity in aiding cell proliferation or causing apoptosis. In this study however, cytotoxic effect of extracts from *Callistemon citrinus* species' flower (CF) and leaves (CL) against colon cancer cell line was investigated by monitoring its effects in a real time manner using Real Time xCELLigence system. In addition, we also investigated the mechanism of action in relation to Akt/phosphorylated Akt pathway. Because of the potential anti-apoptotic effects of plant materials, this pathway was selected which is activated by several natural products. Akt protein is one of the key proteins in Wnt signaling that evoked at the beginning of the apoptosis pathway (Koseoglu et al. 2007; Brahmachari 2012). Akt is Ser/Thr kinase which is also referred to as protein kinase B (PKB) in mammalian genomes have three Akt genes namely: Akt1 (Protein kinaseB α), Akt2 (Protein kinaseB β) and Akt3 (Protein kinaseB γ) and they are extensively expressed in various tissues. Akt1 is most abundantly expressed in the heart, lung, brain and colon. Akt2 is predominantly expressed in the skeletal muscle and embryonic brown fat and Akt3 is expressed most abundant in embryonic heart, kidney and also brain (Coffer et al. 1992; Altomare et al. 1995; Altomare et al. 1998; Brodbeck et al. 1999). Akt1, Akt2 and Akt3 genes are located on 14q32, 19q13.1-13.2 and 1q44 respectively (Staal et al. 1988; Cheng et al. 1992; Murthy et al. 2000). The phosphatidylinositol 3-kinase(PI3K)-Akt signaling pathway is evoked by many types of cell stimuli or toxic reagents and regulates fundamental cellular functions such as proliferation, transcription, translation, cell survival and growth (Datta et al. 1999; Vivanco et al. 2002).

The Akt/protein kinase B (PKB) kinase is an effector of phosphoinositide 3-kinase (PI3K) that plays important roles in the pathogenesis of human cancers (Blume-Jensen et al. 2001). Previous studies have reported that PI3K/Akt pathway plays a critical role in cell survival or apoptosis in various human cancer cells such as lung (Brognard et al. 2001), prostate (Kreisberg et al. 2004), pancreas (Grille et al. 2003), and breast carcinoma (Sun et al. 2001). Colon carcinoma is characterized by different morphological, genetic and cellular events. There are some occasion which causes to cancer formation such as over expression of Akt1 and its activation level (phosphorylated Akt) and site is link to development and progression of colon cancer (Roy et al. 2002). In order to activate Akt, it requires that the phosphorylation of Thr308 in the activation loop by the phosphoinositide-dependent kinase 1 (PDK1) and Ser473 within the carboxyl-terminal (Figure 1). Phosphorylation of Akt is promoted by phosphatidylinositol-3-OH kinase (PI3K) products, which subsequently facilitate transmembrane signaling by serving as membrane-localization molecule. It has been reported that phosphorylation of Akt was apparently detected at high level in normal colorectal cells mucosa. They also performed PI3K/Akt pathway related apoptosis mechanisms which are caspase 3, EGF (endothelial growth factor) and TNFα (Tumor necrosis factor α) (Itoh et al. 2002). To the best of our knowledge, cytotoxicity of *C. citrinus* on colon cancer cell line and its mechanism of action over Akt/p-Akt pathway was investigated. The aim of this study was investigate the dynamic monitoring of cytotoxic effect of CF and CL extract on Colo-205 cell line. In addition we existed if the Akt pathway was evoked by extracts for evaluating the mechanism of action.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO; Sigma Aldrich, Steinheim, Germany), Tris base (Sigma-Aldrich, Steinheim, Germany), Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Steinheim, Germany), Trypsin-EDTA (Sigma-Aldrich, Steinheim, Germany), Fetal Bovine Serum (FBS; Sigma-Aldrich, Steinheim, Germany) were used. Akt (Cell Signalling Technology (CST), 9272S, Leiden, Netherlands), Phospho-Akt (Ser473) antibody (CST 4060S, Leiden, Netherlands), β-Actin Antibody (CST 4967, Leiden, Netherlands) antibodies were used for western blotting.

Plant materials

Fresh leaves and flowers of *C. citrinus* were harvested, air dried and grounded into coarse powder. 40 g of plant material from leaves was diluted in 960 mL deionized water and 40 g of extract from flowers in a mixture of 480 mL methanol with 480 mL deionized water, and then distillated using Heidolph rotary evaporator (Hei-VAP HL/G3**;** Heidolph Instruments GmbH, Schwabach, Germany). The distillates from leaves and flowers were lyophilized for 24 h, then stored in plastic vials at -80°C until analysis. HPLC grade water (18.2MΩι-cm) was prepared using a Millipore Simplicity 185 Direct-Q water purification system (Millipore Corp., Bedford, USA).

Cell culture

Colo-205 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) The cells were grown in DMEM with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin and incubated in a humidified at-

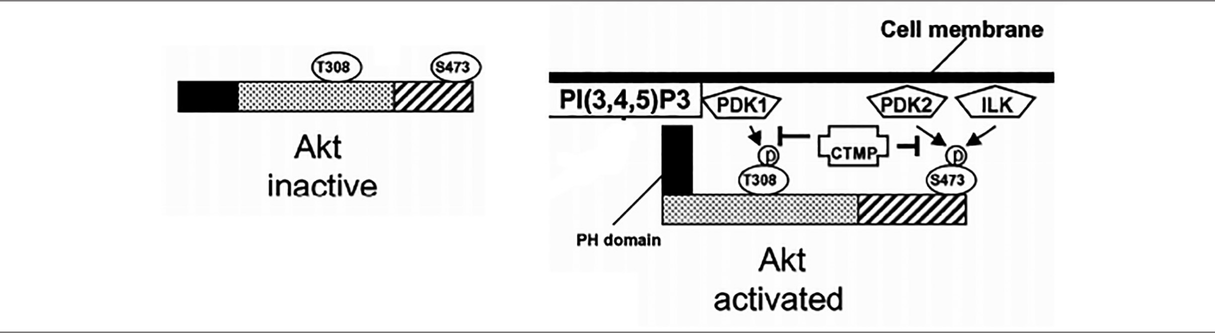

Figure 1. Akt phosphorylation sites and activating with cellular bioactive molecules

mosphere containing 5% CO_2 at 37ºC. When the confluence nearly reached 80%, the cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) solution and detached from the flasks with trypsin/EDTA. The cells were centrifuged with the Universal 320R (Hettich, Zentrifugen, 1406; Kirchlengern, Germany) at 1000rpm for 5 mins at 25ºC, seeded on 6 wells plate and 96 wells E-plate for western blot and xcelligence analysis, respectively.

Western blotting

Western blot analysis was carried out using crude lysates of Colo-205 human colon cancer cells. Cells were treated with CL and CF extract at 400, 200, 100 and 50 µg/mL concentrations for 12 h both. Cells were lysed in commercial ripa lysis buffer (SC-24948; Santa cruz; CA, USA). The lysate was centrifuged at 4 °C for 30 min at 12000 rpm. The clear supernatant was collected and the total protein amount was determined by Lowry method. 30 µg protein lysates were resolved on 8% sodium dodecyl sulphate (SDS)-polyacrylamide gels. Then electrotransferred onto polyvinylidenedifluoride (PVDF) membrane. After blocking with 5% non-fat milk in Tris-buffered saline (TBS, 0.1 M, pH 7.4). Membrane were incubated with primary antibodies anti-phospho-Akt (Ser473) (1:500 dilution, Cell Signaling Technology), anti- Akt (1:1000 dilution), anti-pAkt (1:1000 dilution), and anti-tubulin (1:2000 dilution). β-actin protein was assigned as a control for protein loading. After overnight incubation at 4 ⁰C conditions, membranes were incubated with secondary antibody, HRP-conjugated goat/rabbit anti-IgG, for 1 h at room temperature. After each step blots were washed three times with Tween (0.2%)-Tris-buffer saline (TBST). Protein bands were detected by enhanced chemiluminescence method (ECL; Santa Cruz Biotechnology, CA, USA) on XO-MAT film. The blots were scanned and analyzed using ImageJ software.

Real Time Cell Analyzer (RTCA) system for cellular monitoring against Colo-205

The xCELLigence system was used for monitoring the real time effect of extracts from *C. citrinus* against Colo-205 cells following manufacturer's instructions (Ke et al. 2011a). The xCELLigence system has got four main units: RTCA analyzer, the RTCA SP station, the RTCA computer with integrated software and disposable E-plate 96 (Figure 2). The RTCA SP station was

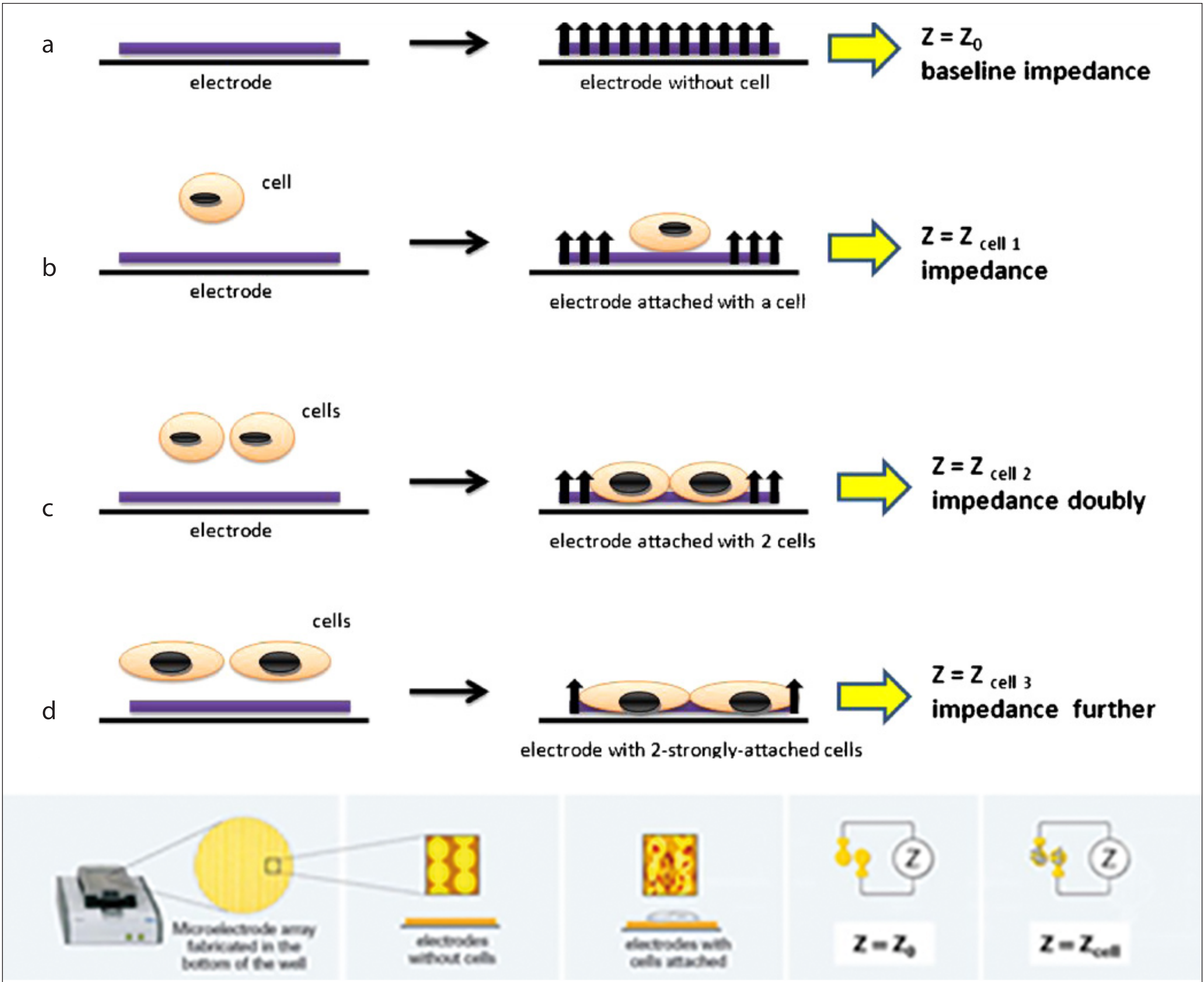

Figure 2. a-d. 96 well E- plate bottom includes golden electrodes which is sensitive to cell adhesion, proliferation and enlargement. Absence of cell (a) at the bottom of the well impedance will be 0 ($Z=Z_0$). Presence of adhesive cell can alter impedance (b) and more cells (c) increase this impedance. Also cell enlargement effects impedance (d), when cells enlarge without mitosis division, impedance alters and cell index increases.

placed inside incubator, while an analyzer and laptop computer with software are on the outside. E-plate 96 is a single use, disposable device used for performing cell-based assays on the RTCA SP instrument. However the E-plate 96 differs from standard 96-well microtiter plates vastly with its incorporated gold cell sensor arrays in the bottom, which contributes cells inside each well to measure alterations on impedance (Bird et al. 2009; Ke et al. 2011b). Electronic impedance alterations can be measured at least every 2 minutes to allow monitoring and detection of physiological changes on the cells. The impedance measured between electrodes in an individual well depends on electrode geometry, the number of the cells in the well and whether the cells are attached to the electrodes or not. In the presence of cells, cells attached to the electrode sensor surfaces and thereby alter the local ion environment at the electrode/solution interface, leading to an increase in impedance. Cell index is related with cell adhesion, cellular morphology alterations also cell detachment via cellular death. So we can evaluate the cytotoxic effect of any material with cell index alterations (Ke et al. 2011b).

Colo 205 cells were grown on cell culture flasks and after reaching approximately 80% confluence, the cells were detached and seeded inside E-plate and incubated for 30 min incubation at room temperature. E-plate 96 was placed into the cell culture incubator different cell numbers (50 000, 25 000, 12 500, 6250 and 3125) to assign optimal seeding concentration. We decided to seed 12500 cell/well for optimal seeding because of log growth phase achieved on this cell number. Cell proliferation, attachment and spreading were monitored every 15mins via the alterations impedance of E-plate wells.

Cytotoxicity assay using Xcelligence system

To determine the cytotoxic effect of CF and CL on Colo-205 cell line, RTCA (xcelligence) was used. A total of 12500 cells/well were seeded in the E-plate 96 wells and approximately 46h post-seeding when the cells were in the log growth phase, cells were treated with extracts and only medium control. Controls received medium and treated groups (CF and CL at 400, 200, 100, 50, 10, 1 µg/mL concentrations) were replicated 4-times and the experiments were run for 97 h. All calculations were done with the RTCA-integrated software of the xcelligence system. The RTCA software performs the curve-fitting of selected "sigmoidal dose-response equation" and calculated logarithmic half maximum effect of concentration [log (IC_{50})] values at a given time point based on log concentration producing 50% reduction of cell index (CI) value relative to the control CI value (100%). Cell index value represents the alteration of cell viability, cell attachment and cellular growth rate. Likewise, when the cell index data obtained during the experiment at the particular time points, dynamic monitoring of the cells' response can be elucidated.

Statistical analysis

For each group, data were derived from at least three independent experiments. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis was used for all the data analysis. Standard deviation (SD) was calculated with the same method. Statistical significance between the collection methods were assessed as $p<0.05$ and was calculated using the same program. Results were presented as the mean±SD.

RESULTS

Effects of *Callistemon citrinus*'s leaves and flowers on cell viability

The results of IC_{50} values calculated for CL and CF using RTCA software were 6.49 µg/mL and 5.22 µg/mL at 24 h post treatment, respectively. At higher concentrations of 400, 200 and 100 µg/mL for CL extract, cell index increased for about 5 h after the treatment time. Shortly after the treatment, it was observed that cell index decreased and continued decreasing until the end of experiment. At lower concentrations of 10 and 1 µg/mL for CL (Figure 3), cell index profiles were parallel to control and did not affect cells after the treatment time. Interestingly, 50 µg/mL dose initially increased the cell index rapidly and continued the increment gradually but was observed to decrease at 85 h. 400 and 200 µg/mL concentrations of CF increased cell index (Figure 4) after the post treatment and continued this increment about 8 h different from the CL high dose concentrations, than cell index decreased consistently. 50 µg/mL dose firstly increased cell index as the high concentrations but cell index did not decrease after 5 h,

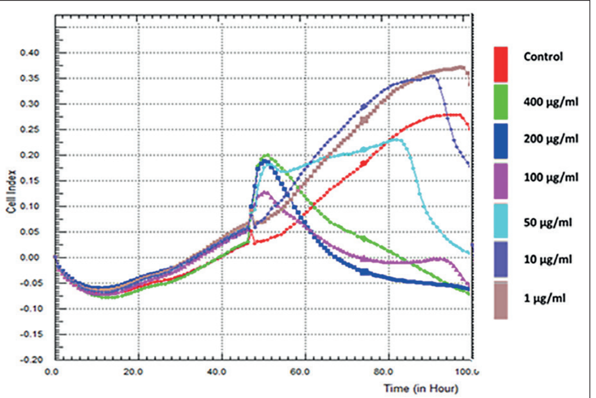

Figure 3. Cell index profiles of Colo 205 cell line treated with different concentrations of leaves extracts from *Callistemon citrinus*

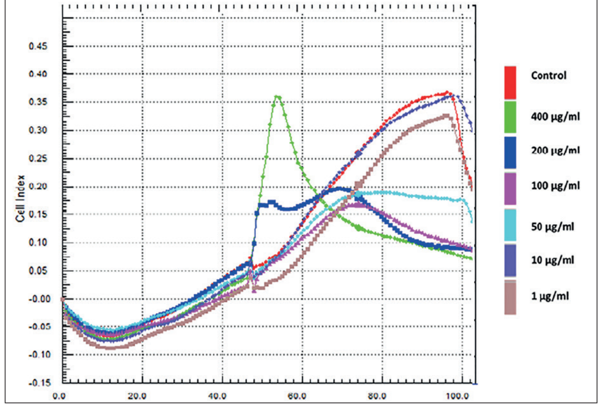

Figure 4. Cell index profiles of Colo 205 cell line treated with different concentrations of flower extracts from *Callistemon citrinus*

it showed cytostatic effect after 14 h post treatment, than it decreased cell index rapidly after about 32 h post treatment interestingly its cell response profile was similar CL. When we look at other concentrations (10, 1 µg/mL) there was no apparent difference in comparison with control (Figure 3).

Effects of *Callistemon citrinus'*s leaves and flowers on the Cell Index Alterations

All the statistical analysis were performed with two way ANOVA and groups were compared to control as presented in Table 1.The cell index alterations (CIA) of the groups that were treated with CL extract gave an idea about cell proliferation, adhesion and enlargement getting increase or decrease. Cell index alterations were calculated as difference at 6th, 12th, 24th and 48th time points compared to the first treatment point (time 0). High dose of 400, 200 and 100 µg/mL, respectively for CL extract, CIA levels decreased significantly ($p<0.05$) at 24th and 48th h in a time dependent manner (Figure 5). On the contrary, the CIA for at 50 µg/mL dose decreased but it was slower in comparison to high concentrations and only 48th h value was significant (Table 1). Control and low concentrations (10, 1 µg/mL) CIA increased depending on experimental time schedule. But only 10 µg/mL decreased CIA level at 48th h. It was an expected situation for control group because cell index of healthy cell increases due to proliferation and culture medium efficiency.

CF extract treated groups showed different CIA profiles comparing to CL. 400 and 200 µg/mL concentrations CIA was higher than control because of high cell index at 6 h but this values decreased and get lower than control at 24 h. 100 and 50 µg/mL concentrations CIA was increased slightly and consistently (Table 2). 10, 1 µg/mL concentrations were similar profiles to control (Figure 6).

Western blot results

The role of PI3K-Akt pathway in initiating cell survival and proliferation has been extensively reported in scientific literatures. Although from previous studies, a lot of substrates have been discovered for manipulating Akt activity and yet some questions are still waiting to be answered. In our study, Akt and pAkt protein expression levels were investigated to understand Akt activity. When we look at activated Akt level at 12th h for CL extracts, p-Akt levels were increased in higher concentrations particularly for 400, 200 and 100 µg/mL concentrations in a dose dependent manner but none of those increments were statistically significant ($p>0.05$). In comparison with the control, the value of p-Akt was low for 50 µg/mL dose for CL extract but did not have significant difference ($p>0.05$) (Figure 7). Results obtained from CF at 400, 200 and 100 µg/ml concentrations also increased p-Akt slightly but those increments were not significant ($p>0.05$) in comparison to the control. Akt protein expression were increased by 200 and 100 µg/mL concentrations of CL and 400

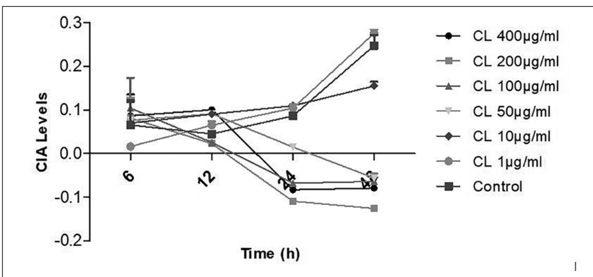

Figure 5. CIA of *Callistemon citrinus*' Leaves at 6th, 12th, 24th and 48th h compared to the first treatment time point. CIA values were given mean±SD.

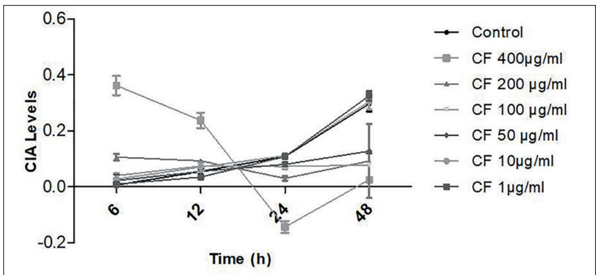

Figure 6. CIA of *Callistemon citrinus*' flowers at 6th, 12th, 24th and 48th h compared to the first treatment time point. CIA values were given mean±SD.

Table 1. CIA values of CL were analyzed comparing to control with two way ANOVA.

	6th h	12th h	24th h	48th h
CL 400 µg/mL vs Control	2.1±1.1	5.5±3.1 * p=0.0174	16.9±9.1 *** p=0.0001	32.7±17.7 *** p=0.0001
CL 200 µg/mL vs Control	1.6±0.8	2.2±1.2	19.5±10.6 *** p=0.0001	34.3±20.3 *** p=0.0001
CL 100 µg/mL vs Control	3.9±2.1	1.9±1.1	15.3±8.3 *** p=0.0001	31.1±16.9 *** p=0.0001
CL 50 µg/mL vs Control	1.1±0.5	4.6±2.5	7.09±3.8	30.3±16.5 *** p=0.0001
CL 10 µg/mL vs Control	0.4±0.2	4.5±2.4	2.3±1.2	9.2±5.1 *** p=0.0001
CL 1 µg/mL vs Control	4.9±2.6 * p=0.0456	2.1±1.1	1.4±0.9	2.8±1.5

statistically significance assigned p<0.05 (*), p<0.01 (**), p<0.001 (***).

Percent alterations±SD values given in the table.

Table 2. CIA values of CF were analyzed comparing to control with two way ANOVA.

	6th h	12th h	24th h	48th h
CF 400 µg/mL vs Control	35.6±7.45	18.2±8.9	25.1±1.2	27.1±1.3
	*** p=0.0001	*** p=0,0001	*** p=0,0001	*** p=0,0001
CF 200 µg/mL vs Control	10.1±2.9	3.8±1.8	7.7±0.3	20.4±1.5
	*** p=0.0001		** p=0,0015	*** p=0,0001
CF 100 µg/mL vs Control	3.4±1.6	1.9±0.9	3.5±0.7	21.6±4.5
				*** p=0,0001
CF 50 µg/mL vs Control	1.6±0.7	0.6±0.1	2.7±1.1	16.9±0.8
				*** p=0,0001
CF 10 µg/mL vs Control	2.1±1	16.1±0.78	0.3±0.1	0.7±0.1
CF 1 µg/mL vs Control	0.3±0.1	2.1±0.2	0.2±0.1	2.8±0.6

Statistically significance assigned p<0.05 (*), p<0.01 (**), p<0.001 (***). Percent alterations±SD values given in the table.

Figure 7. Expressed levels of Akt1 and β-actin by western blot analysis after 12 h treatment with flowers and leaves extract of *Callistemon citrinus*

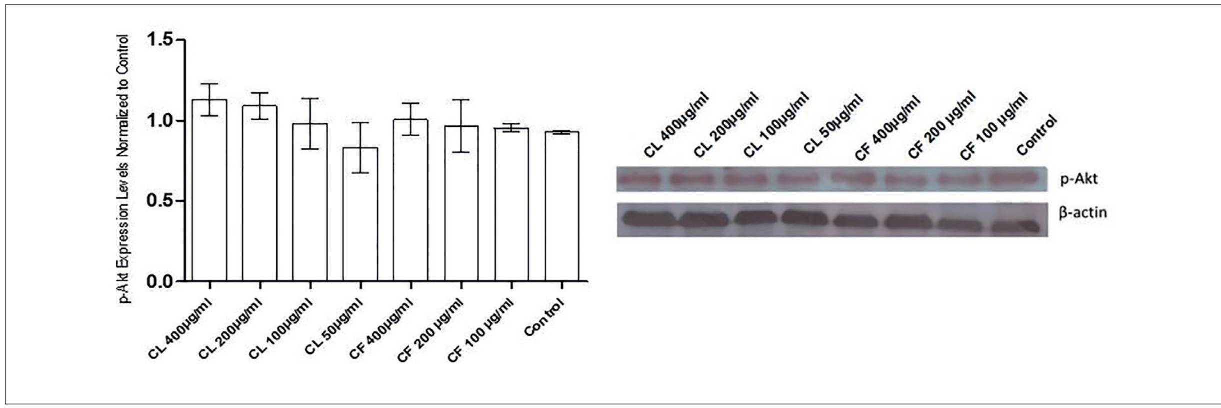

Figure 8. Expressed levels of p-Akt1 (Ser473) and β-actin by western blot analysis after 12 h treatment with flowers and leaves extract of *Callistemon citrinus*

µg/mL dose of CF. The other concentrations of CL and CF extracts decreased Akt comparing to control (Figure 8).

DISCUSSION

The aim of this study was to investigate the cytotoxic effects of CF and CL extracts on COLO-205 cell line.

Akt activity mainly plays a role in apoptosis pathway on cancer and normal physiological conditions. Therefore we investigated Akt and p-Akt levels for understanding if Akt pathway evoked or not evoked by CL and CF extracts. In this study we captured a statistically significant increment after 5 h of treatment for CL and 8 h of treatment for CF at the highest concentrations. In 2015 Kumar et al. investigated essential oils of this plant and they found that essential oils induced apoptosis on human lung cancer (A549 cell line) and rat glioma (C-6 cell line) cells. Also they reported growth inhibition effect of essential oils on Colo-205 cell line. Hydrodistillation product of *Callistemon citrinus*

contains essential oils which is response for its major effect (Kumar et al. 2015). However extraction method is so important for evaluating the effect of plant material. In addition to our results on Colo-205 cell line, this data give us an idea about extracts of CF and CL essential oils might be a hope for alternative or supplement cancer therapy in addition to conventional cancer therapy. Supporting this increase in cell proliferation, the Akt levels decreased and p-Akt levels increased at 400 µg/mL for CL, revealing that the Akt is phosphorylated triggered the cell proliferation at this dose. In addition, real time monitoring made it possible to catch relevant time points for the induction of cell proliferation. For the colon cancer cell line it was important for us to show when this cytotoxic effect starts and which dose would be the best dose for treatment. Although this increase in proliferation at high concentrations followed by a decrease, this dose was decided not to be relevant concentration for treatment, since it was triggering Akt and p-Akt pathway at the beginning. For the following concentrations 200, 100 and 50 µg/mL Akt/p-Akt both decreased reflecting this pathway inactivated at these concentrations. Our findings also supported real time monitoring results for CL extracts. For the CL extracts the IC_{50} level was 6.49 µg/mL. For the CF extracts, the real time monitoring results were approximately similar to CL results at all concentrations. CF extracts did not affect the Akt phosphorylation significantly, revealing that the action of mechanism might depend on another pathway of extract of CF. IC_{50} level for extract of CF were 5.22 µg/mL and the relevant treatment time should be at least 24 h to see its effect.

CONCLUSION

There is limited report on potential cytotoxic effect of *Callistemon citrinus* plant material. Compared to conventional end-point cell-based assays, dynamic monitoring of cell response, such as cell adhesion, proliferation, and cell survival is one of the advantages of the xcelligence system to optimize the cell concentration for *in vitro* and *in vivo* assays and also allows both cell and assay conditions to be constantly obtained before and during the time of the experimentation.

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Review

Plants used in traditional treatment against diarrhea in Turkey

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ABSTRACT

Turkey is one of the richest countries in the world in terms of flora with its extraordinary plant diversity. Its flora consists of about 10,000 vascular plants and approximately one third of them (34.4 %) are endemic to the country. In recent years, the use of ethnobotanical information obtained from medicinal plant research has gained attention all around the world. For this reason, numerous ethnobotanical studies have recently been published and much has been written about medicinal plants in our country. This study deals with 133 taxa used in traditional treatments against diarrhea in Turkey and it aims to give information about scientific and local names of these taxa, families, used parts and usage in diarrhea.

Keywords: Diarrhea, medicinal plants, traditional treatment, Turkey

INTRODUCTION

Diarrhea is defined as loose stools, increased stool frequency, or urgency by patients. Although most patients use this term to describe changes in consistency (loose or watery stool), diarrhea can be considered as urgency or high stool frequency. In fact, with normal consistency, frequent defecation is often referred to as pseudodiarrhea; for this reason, an abnormal stool form and frequency should not be used to describe diarrhea. Most diarrheal episodes in developed countries are acute and self-limited and are usually caused by infections. In immunocompetent patients, acute contagious diarrhea typically resolves within 4 weeks (most often within 1 week). Therefore, chronic diarrhea is defined as that lasting longer than 4 weeks. It is estimated that 1%-5% of adults suffer from chronic diarrhea. In immunocompetent patients in developed countries, chronic diarrhea is not usually contagious (Lawrence et al. 2017).

Acute bloody diarrhea in children and adults is a difficult diagnostic problem. Acute bloody stools have different spectra between adults and children, but there are overlapping causes (infectious colitis and less frequently intussusception). Identification of patients with infectious causes is mandatory, so that they are suitably treated with antimicrobials and so that infection control measures can be fulfilled (Lori et al. 2009).

Detection of the cause of this disorder may be a problem, because there are many enteric pathogens that cause acute bloody diarrhea and several noninfectious gastrointestinal disorders; these are interpreted as loose, bloody stools, mostly diarrhea (Lori et al. 2009).

There is a battle between the host microbiology of normal flora and the exterminating microbes. Symptomatic infections for the host when invaded can alter the bowel barrier and absorptive functions, or can quickly cause a number of problems that can

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lead to lethal dehydration, diarrhea, toxic megacolon or shock. Asymptomatic infections may go unnoticed, but they have durable results for children's growth and development. Most are acquired through contaminated food or water; however, only few pathogens (such as Shigella, Cryptosporidium, Giardia, rotaviruses, or noroviruses) can cause infection (Pawlowski et al. 2009).

Rotavirus is the leading cause of diarrhea hospitalization among children in the world. In 2003, a world-wide estimate of rotavirus-related deaths was published, based on a review of published literature on deaths from diarrhea and rotavirus hospitalizations in children from 1986 to 1999. Studies published between 1986 and 1999 showed that rotavirus causes ≈22% (range 17%-28%) of diarrheal hospitalizations in childhood. From 2000 to 2004, this rate increased to 39% (range 29%-45%). Application of this ratio to the recent World Health Organization estimates of diarrhea-related childhood deaths gave a predicted 611,000 (range 454,000-705,000) rotavirus-related deaths (Parashar et al. 2006).

In mass tourism, traveler's diarrhea is one of the most common health problems in long-distance journeys. Globally, there are 40 million cases per year. For this reason, travelers to risky areas should be informed in advance of what measures they should take in case of acute diarrhea and which medicines to include in the first aid kit (Jelinek et al. 2017).

The first choice of treatment of acute uncomplicated traveler's diarrhea - more than 90% of all cases - is the secretion inhibitor racecadotril. Usual practice, which recommends the antimotility drug loperamide as the first option, should be rethought in favor of the last active ingredient racecadotril. Antibiotics should be used only in complicated cases. Generally, anticipation of a large number of passengers demanding antibiotic treatment should be impaired. Other therapeutic measures currently available for the treatment of acute diarrhea while traveling play a minor role (Jelinek et al. 2017).

Several studies have shown that antibiotics can reduce the rates of diarrhea in travelers to resource-limited countries. However, preventive antibiotic therapy is not recommended because of its side effects and so, if necessary, rapid-acting, single-dose antimicrobial therapy is used. In some studies, probiotics have shown benefit (Pawlowski et al. 2009).

There are a lot of kinds of diarrhea shown in this study and the drugs used for the treatment of diarrhea have many side effects. Medicinal plants have traditionally been used for the treatment of diarrhea for many decades.

In this study, we have compiled 133 taxa used in traditional treatments of diarrhea in Turkey. The aim is to give information about scientific and local names of these taxa, families, used parts and usage in diarrhea (Table 1).

Table 1. List of the plants used in traditional treatment against diarrhea in Turkey

Botanical name	Family	Local name	Plant parts used	Preparation, administration and use	Reference
Achillea aleppica DC. subsp. *aleppica*	Asteraceae	Civanperçemi	Aerial parts	Dec., Int.	(Doğan 2014)
[a] *Achillea wilhelmsii* C. Koch.	Asteraceae	Ayvadere, Kedicırnağı, Kedi Tırnağı, Tilki otu	Aerial parts	+Milk, Dec. or Inf., Int Crushed, Ext. Dec., Int. Inf., Int.	(Oral 2007) (Şenkardeş 2014) (Tuzlacı 2016)
Ailanthus altissima (P. Mill.) Swingle	Simaroubaceae	Ossuruk ağacı	Branches	Dec., Int.	(Güneş et al. 2017)
Alchemilla sp.	Rosaceae	Aslanpençesi, Dutya	Aerial parts, Flower	Inf., Int.	(Karagöz and Serteser 2017)
Alhagi maurorum Medik.	Fabaceae	Xirnuf	Fruit	Raw, Int.	(Dalar et al. 2018)
Alhagi pseudoalhagi (Bieb.) Desv.	Fabaceae	Hurnif, Çeti, Çoban çalısı	Fruit	Dried Fruits Eaten Crushed, Int.	(Korkut 2006; Gençay 2007) (Tuzlacı 2016)
Allium sativum L.	Liliaceae	Sarımsak	Bulb	+Yoghurt, Fac.	(Uysal 2008)
Alyssum pateri Nyár. subsp. *pateri*	Brassicaceae	Keselmahmut	Aerial parts	Dec., Int.	(Kaval et al. 2014; Tuzlacı 2016)
Anacamptis pyramidalis (L.) Rich.	Orchidaceae	Sahlep, Salep çiçeği	Root	Dec., Int.	(Doğan 2014; Tuzlacı 2016)
Anthemis cretica L. subsp. *pontica* (Willd.) Grierson	Asteraceae	Papatya	Capitulum	Inf., Int.	(Tütenocaklı 2014)
Artemisia absinthium L.	Asteraceae	Acı yavşan	Leaf	Take as a pill, Int.	(Yıldırım 2015)

Table 1. List of the plants used in traditional treatment against diarrhea in Turkey (continued)

Botanical name	Family	Local name	Plant parts used	Preparation, administration and use	Reference
Arum conophalloides Kotschy ex Schott	Araceae	Yılan bıçağı	Seed	Raw, Swallowed	(Güneş et al. 2017)
Arum dioscoridis SM.	Araceae	İlan purçulağı, Kabargan, Ölüm körü, Yılan purçulağı		Cooked, Int.	(Yıldırım 2015)
Berberis crataegina DC.	Berberidaceae	Karamuk, Karamık, Kızılcık	Flowering and Fruity Branches Fruit	Dec., Int. Raw, Int.	(Özkan 2002; Tuzlacı 2016) (Yeşil and Akalın 2009; Vural 2008)
Berberis vulgaris L.	Berberidaceae	Karamuk, Kadıntuzluğu	Fruit	Raw, Int.	(Korkmaz and Karakuş 2013)
Camellia sinensis(L.) Kuntze	Theaceae	Siyah çay	Leaf	Inf., Int. Raw or Inf., Int.	(Saraç et al. 2013; Tuzlacı 2016) (Uysal 2008)
Capparis spinosa L. var. *aegyptia* (Lam.)Boiss.	Capparaceae	Gebere, Kebere	Gemma	Swallowed	(Kazan 2007)
Cardiospermum helicacabum L.	Sapindaceae	Balon sarmaşığı, Japon feneri	Young Shoots	Dec., Int.	(Güzel et al 2015)
Celtis tournefortii Lam.	Ulmaceae	Derdoğan	Fruit	Raw, Int.	(Doğan 2014; Tuzlacı 2016)
Centaurea pterocaula Trautv.	Asteraceae	Şermnik	Leaf	Dec., Int.	(Kaval et al. 2014)
Centaurea solstitialis L. subsp. *solstitialis*	Asteraceae	İshal dikeni	Aerial parts	Inf., Int.	(Şenkardeş 2014; Tuzlacı 2016)
Cerasus avium (L.) Moench	Rosaceae	Kiraz	Stem and Branch Bark	Dec., Int.	(Kural 2012; Tuzlacı 2016)
Ceratonia siliqua L.	Fabaceae	Buynuz, Harıp, Harnup, Keçiboynuzu, Keçibuynuzu	Fruit	Raw, Int. Jam, Int. Mac., Int.	(Bulut 2006; Tuzlacı 2016; Gürdal and Kültür 2013; Yıldırım 2015)
Ceterach officinarum DC.	Aspleniaceae	Altın otu, Mayasıl otu	Aerial parts	Cooked, Int. Dec., Int.	(Güzel et al. 2015) (Bulut 2006; Tuzlacı 2016)
Chenopodium foliosum Aschers	Chenopodiaceae	Kedi üzümü, Kuş üzümü	Aerial parts	Inf., Int.	(Doğan 2014; Şenkardeş 2014; Tuzlaci and Şenkardeş 2011; Tuzlacı 2016)
Cicer arietinum L.	Fabaceae	Nohut	Seed	Cooked, Int.	(Uysal 2008; Tuzlacı 2016)
Cistus creticus L.	Cistaceae	Pamuk otu	Leaf	Dec., Int.	(Onar 2006; Tuzlacı 2016)
*Citrus limon*L.	Rutaceae	Limon, Limon ağacı	Fruit	Juice, Int.	(Sargın et al. 2013)
Convolvulus arvensis L.	Convolvulaceae	Basırık (Bağırsak) otu, Mahmude otu	Branches and Leaves	Dec., Int.	(Güneş et al. 2017)
Cornus mas L.	Cornaceae	Kızılcık, Kiren, Zaye	Fruit	Raw, Int. Syrup, Int. Dec., Int. Inf., Int.	(Koçyiğit and Özhatay 2006; Ayan 2015; Tuzlacı 2016) (Polat 2010; Korkmaz and Karakurt 2014) (Karcı 2013) (Güler et al. 2015)

Table 1. List of the plants used in traditional treatment against diarrhea in Turkey (continued)

Botanical name	Family	Local name	Plant parts used	Preparation, administration and use	Reference
Crataegus monogyna Jacq. subsp. *monogyna*	Rosaceae	Alıç, Ekşi muşmula, Keçi alıcı, Kız elması, Kocakarı hurması	Fruit Leaf, Flower	Eaten Inf., Int.	(Sargın et al. 2013) (Furkan 2016)
Cupressus sempervirens L.	Cupressaceae	Mezarlık selvisi, Selvi	Cone	Dec., Int.	(Uysal 2008; Tuzlacı 2016)
Cydonia oblonga Miller	Rosaceae	Ayva	Leaf Seed Flower	Inf., Int. Dec., Int. Dec., Int. Inf., Int.	(Sargın et al. 2013) (Güneş et al. 2017; Uysal 2008; Metin 2009; Kayabaşı 2011; Deniz 2008; Kıncal 2018; Tuzlacı 2016) (Uysal 2008; Yüzbaşıoğlu 2010; Tuzlacı 2016) (Yıldırım 2015)
Cyperus rotundus L.	Cyperaceae	Topalak otu	Root	Crushed, Ext.	(Uysal 2008)
Diospyros lotus L.	Ebenaceae	Laz hurması, Kara hurma	Leaf	Inf., Int.	(Saraç et al.2013; Tuzlacı 2016)
Elaeagnus angustifolia L.	Elaeagnaceae	İğde	Seed Fruit Leaf	Dec., Int. Eaten Inf., Int.	(Altundağ 2009) (Güzel et al 2015; Korkmaz and Karakurt 2014) (Polat 2010)
Erica manipuliflora Salisb.	Ericaceae	Funda, Püren, Süpürge otu	Young Shoots	Dec., Int.	(Bulut 2006)
Eriobotrya japonica (Thunb.) Lindly	Rosaceae	Malta eriği, Muşmula	Leaf	Inf., Int. Dec., Int.	(Uysal 2008) (Tuzlacı 2016)
Euphorbia denticulata Lam.	Euphorbiaceae	Hekletis, Sütleğen otu	Latex	Int.	(Kaval et al. 2014; Tuzlacı 2016)
Fagus orientalis Lipsky	Fagaceae	Doğu kayını	Stem Bark	Dec., Int.	(Kural 2012)
Ficus carica L. subsp. *carica*	Moraceae	İncir	Leaf	Raw, Int. Dec., Int.	(Alkaç 2013) (Tuzlacı 2016)
Glaucium leiocarpum Boiss.	Papaveraceae	Gelincik	Flower	Inf., Int. Dec., Int.	(Doğan 2014) (Tuzlacı 2016)
Gundelia tournefortii L.	Asteraceae	Kenger	Root	Latex, Eaten	(Hayta et al. 2014; Çakılcıoğlu and Türkoğlu 2010)
Helichrysum arenarium (L.) Moench.	Asteraceae	Altın otu Leaf	Capitulum,	Inf., Int. 2015)	(Akan and Sade
Helichrysum plicatum DC. subsp. *plicatum*	Asteraceae	Arı Çiçeği, Ölmez Çiçek, Yayla Çiçeği	Aerial parts Flowering Branches	Inf., Int. Inf.,Int.	(Arısan 2010) (Altundağ 2009)
[b]*Helleborus orientalis* L.	Ranunculaceae	Bohça, Bohça otu, Çöp otu, Çöpleme	Leaf, Rhizome	Eaten	(Kızılarslan and Özhatay 2012)
Hordeum vulgare L.	Poaceae	Arpa	Spike Whole plant	Inf., Int. Inf., Int.	(Sargın et al. 2013) (Korkmaz and Karakurt 2014)
Hypericum cerastoides (Spach) N. Robson	Hypericaceae	Kantaron, Küçük Kantaron	Aerial parts	Dec., Int.	(Kızılarslan and Özhatay 2012; Tuzlacı 2016)
Hypericum scabrum L.	Hypericaceae	Mide otu, Yara otu	Aerial parts	Inf., Int.	(Doğan 2014; Tuzlacı 2016)

Table 1. List of the plants used in traditional treatment against diarrhea in Turkey (continued)

Botanical name	Family	Local name	Plant parts used	Preparation, administration and use	Reference
[b]*Inula viscosa* (L.) Aiton	Asteraceae	Khişkeş, Mıcve, Yerce, Zimbit	Leaf	Eaten	(Güzel et al. 2015)
Juglans regia L.	Juglandaceae	Ceviz	Seed Leaf Fruit Bark	Inf., Int. Dec., Int. Dec., Int.	(Aktan 2011) (Şenkardeş 2014; Tuzlacı 2016) (Sargın et al. 2013)
Juniperus drupacea L.	Cupressaceae	Andız	Tar	+Water, Int.	(Orhan 2011)
Juniperus oxycedrus L.	Cupressaceae	Ardıç	Leaf, Branches		(Orhan 2011)
Jurinella moschus (Habl.) Bobrov subsp. *pinnatisecta* (Boiss.) Danin and P.H.Davis	Asteraceae	Gazangulpu, Kazankulpu	Whole plant	Dec., Int.	(Altundağ 2009)
Lysimachia vulgaris (L.) Pohl	Primulaceae	Giya baluk	Leaf	Dec., Int.	(Dalar et al. 2018)
Malva neglecta Wallr.	Malvaceae	Ebegümeci, Ebemkömeci	Aerial parts	Dec., Int.	(Altundağ 2009)
Matricaria chamomilla L. var. *recutita* (L.) Grierson	Asteraceae	Papatya	Aerial parts (Without Flower)	Inf., Int.	(Sargın et al. 2013)
Melissa officinalis L.	Lamiaceae	Oğul otu	Leaf and Young Shoots	Inf., Int.	(Bulut 2006; Tuzlacı 2016)
Mentha longifolia (L.) Hudson subsp. *longifolia*	Lamiaceae	Bung, Pung, Yarpız, Yarpuz	Leaf	Inf., Int	(Altundağ 2009; Tuzlacı 2016)
Mentha x piperita L.	Lamiaceae	Bünk, Mentol nane, Kedi nanesi, Tıbbi nane	Aerial parts	Dec., Int.	(Akan and Sade 2015)
Mespilus germanica L.	Rosaceae	Beşbıyık, Döngel, Muşmula, Töngel	Leaf Fruit	Inf., Int. Dec., Int. Eaten	(Karcı 2013) (Onar 2006; Tuzlacı 2016) (Bulut 2006; Kural 2012; Güler et al. 2015; Tuzlacı 2016)
Morus alba L.	Moraceae	Akdut, Beyaz dut, Dut	Leaf	Inf., Int.	(Sargın et al. 2013)
Musa sapientum L.	Musaceae	Muz	Fruit	Eaten	(Uysal 2008; Tuzlacı 2016)
Myrtus communis L.	Myrtaceae	Mersin, Murt	Leaf	Dec., Int. Inf., Int.	(Güneş et al. 2017) (Tuzlacı 2016)
Olea europaea L.var. *sylvestris* (Mill.) Lehr.	Oleaceae	Zeytin	Leaf and Stem Bark	Inf., Int.	(Bulut 2006)
Opuntia ficus-indica (L.) Mill.	Cactaceae	Frenk inciri, Kaynana dili, Tin sabır	Fruit	Eaten	(Güzel et al 2015; Güler et al. 2015)
Orchis coriophora L.	Orchidaceae	Sahlep	Root	Dec., Int.	(Doğan 2014; Tuzlacı 2016)
Orchis palustris Jacq.	Orchidaceae	Sahlep	Root	Dec., Int.	(Doğan 2014; Tuzlacı 2016)
Orchis punctulata Steven ex Lindley	Orchidaceae	Sahlep	Root	Dec., Int.	(Doğan 2014; Tuzlacı 2016)
Origanum onites L.	Lamiaceae	Beyaz kekik, Deli kekik, Eşek kekiği, Güve otu, Karakekik, Kekik	Aerial parts Leaf	Cooked with +Monk's pepper, Thyme, Flour, Water, Ext. Inf. or Dec., Int. Aromatic Water, Int. Inf., Int.	(Uysal 2008) (Gürdal and Kültür 2013; Tuzlacı 2016) (Tütenocaklı 2014)

Table 1. List of the plants used in traditional treatment against diarrhea in Turkey (continued)

Botanical name	Family	Local name	Plant parts used	Preparation, administration and use	Reference
Paliurus spina-christi Miller	Rhamnaceae	Çaltı	Fruit	Inf., Int.	(Bulut and Tuzlacı 2009)
Papaver somniferum L.var. *somniferum*	Papaveraceae	Afyon, Afyon çiçeği, Haşhaş, Haşhaş kozağı, Yeleğen mavi	Fruit Bark	Dec., Int.	(Sargın et al. 2013; Tuzlacı 2016)
[a]*Parietaria judaica* L.	Urticaceae	Duvar reyhanı, Yapışık ot	Aerial parts	Inf., Int. Eaten	(Tuzlaci and Şenkardeş 2011)
[b]*Phlomis pungens* Willd.var. *hirta Velen*	Lamiaceae	Ayıkulağı, Calba	Aerial parts	Eaten	(Vural 2008)
[b]Pinus brutia Ten.	Pinaceae	Çam, Kızılçam	Dried Stem Bark and Mastic	Crushed, Int.	(Güneş et al. 2017)
Pinus nigra Aitonsubsp. *pallasiana* (Lamb.) Holmboe	Pinaceae	Çam gıdısı, Karaçam	Tar	Ext.	(Arısan 2010)
Pinus pinea L.	Pinaceae	Fıstık çamı	Branches	Dec., Int.	(Kökçü 2015)
Pistacia eurycarpa Yalt.	Anacardiaceae	Menengeç	Fruit	Eaten	(Doğan 2014; Tuzlacı 2016)
Pistacia terebinthus L.	Anacardiaceae	Menengiç	Fruit		(Akan and Sade 2015)
Pistacia vera L.	Anacardiaceae	Kaliki-fıstığa	Fruit Bark	Dec., Int.	(Dağlı 2015)
Plantago lanceolata L.	Plantaginaceae	Sinir otu, Sinirli ot	Seed	Int. Dec., Int.	(Genç and Özhatay 2006) (Kolaç 2018)
Plantago major L. subsp. *major*	Plantaginaceae	Damar otu, Kara kabarcık, Siğilli ot, Sinir otu, Sinirli ot	Flower Seed	Dec., Int. Dec., Int. +Yoghurt, Int.	(Genç and Özhatay 2006) (Karataş 2007) (Kızılarslan and Özhatay 2012)
Platanus orientalis L.	Platanaceae	Çınar, Kavak	Fruit Leaf Stem Bark	Dec., Int. Inf., Int. Dec., Int. Dec., Int.	(Bulut and Tuzlacı 2009; Kökçü 2015; Tuzlacı 2016) (Koçyiğit and Özhatay 2006) (Vural 2008) (Polat 2010)
Populus tremula L.	Salicaceae	Bodur kavak	Stem Bark	Dec.,Int.	(Doğan 2014; Tuzlacı 2016)
Potentilla recta L.	Rosaceae	Acı hayıt, Beşparmak otu	Root	Dec., Int.	(Deniz 2008)
Potentilla reptans L.	Rosaceae	Beşparmak otu	Leaf	Dec., Int.	(Yılmaz 2011; Öztürk 2006; Tuzlacı 2016)
Prosopis farcta (Banks and Sol.) J.F.Macbr.	Fabaceae	Çeti, Hışhaş	Fruit Root	Eaten Dec., Int.	(Gençay 2007; Tuzlacı 2016) (Balos and Akan 2007)
Prunus cerasus L.	Rosaceae	Vişne	Fruit	Juice, Int. Eaten	(Korkmaz and Karakurt 2014) (Metin 2009)
Prunus divaricata Ledeb.	Rosaceae	Dağ eriği, Gakka, Kuş eriği	Fruit	Juice, Int.	(Korkmaz and Karakurt 2014)
Prunus x domestica L.	Rosaceae	Erik	Fruit	Boiled with Turkish Coffee, Int.	(Kolaç 2018)

Table 1. List of the plants used in traditional treatment against diarrhea in Turkey (continued)

Botanical name	Family	Local name	Plant parts used	Preparation, administration and use	Reference
Prunus persica (L.) Batsch.	Rosaceae	Şeftali	Fruit Flower	Eaten Inf., Int.	(Korkmaz and Karakuş 2013; Metin 2009; Kolaç 2018) (Kolaç 2018)
Prunus spinosa L.	Rosaceae	Dağ eriği	Fruit	Eaten	(Eşen 2008)
[a]*Punica granatum* L.	Punicaceae	Hennar, Hınar, Nar	Fruit Seed Fruit Bark	Juice, Int. Cooked in Cinder, Ext. Eaten Crushed, Int.	(Gençay 2007) (Uysal 2008) (Güzel et al 2015) (Balos and Akan 2007)
Pyrus amygdaliformis Vill. subsp. *amygdaliformis*	Rosaceae	Ahlat, Çakal armudu, Çördük armudu, Deli armut, Yaban armudu	Leaf Fruit	Dec., Int. Eaten	(Uysal 2008) (Deniz 2008; Tuzlacı 2016)
Pyrus elaeagnifolia Pall. subsp. *elaeagnifolia*	Rosaceae	Dağ armudu, Yabani armut	Fruit	Eaten	(Tuzlaci and Şenkardeş 2011; Tsetsekos 2006; Güldaş 2009; Keskin 2011)
Pyrus elaeagnifolia Pall. subsp. *kotschyana* (Boiss.) Browicz.	Rosaceae	Ahlat, Ahlet, Deli armut, Taş armut	ImmatureFruit Flower	Raw, Eaten Inf., Int.	(Korkmaz and Karakurt 2014; Tuzlacı 2016) (Yıldırım 2015)
Pyrus syriaca Boiss. var. *syriaca*	Rosaceae	Çakal armut, Dağ armudu, Yaban armudu	Fruit	Eaten	(Furkan 2016; Tuzlacı 2016)
Quercus coccifera L.	Fagaceae	Kermes meşesi, Piynar	Gall	Dec., Int.	(Metin 2009; Çilden 2011)
Quercus ithaburensis subsp. *macrolepis* (Kotschy) Hedge and Yalt.	Fagaceae	Meşe palamudu	Gall	Eaten	(Akan and Sade 2015; Tuzlacı 2016)
Rhus coriaria L.	Anacardiaceae	Sımak, Sumak	Fruit Seed Leaf	Eaten Inf., Int. Dec., Int. Dec., Int. Mac., Int.	(Güldaş 2009) (Balos and Akan 2007) (Metin 2009) (Metin 2009) (Furkan 2016)
Rosa canina L.	Rosaceae	İtburnu, İtgülü, Kuşburnu, Öküzgözü	Fruit	Dec., Int. Inf., Int. Eaten Marmalade, Int.	(Sargın et al. 2013; Karataş 2007) (Güneş et al. 2017) (Karagöz and Serteser 2017) (Karagöz and Serteser 2017; Keskin 2011; Tuzlacı 2016)
*Rosa damascena*Mill.	Rosaceae	Gül	Flower	Inf., Int.	(Güler et al. 2015; Tuzlacı 2016)
[a]*Rubus canescens* DC. var. *glabratus* (Gordon) Davis and Meikle	Rosaceae	Böğürtlen, Kapina, Karamık	Root	Dec., Int. Eaten	(Genç and Özhatay 2006; Tuzlacı 2016) (Kayabaşı 2011)
Rubus sanctus Schreb.	Rosaceae	Böğürtlen	Root	Dec., Int.	(Uysal 2008)
Rumex acetosella L.	Polygonaceae	Ekşilik, Kuzukulağı	Leaf	Eaten	(Güler et al. 2015)

Table 1. List of the plants used in traditional treatment against diarrhea in Turkey (continued)

Botanical name	Family	Local name	Plant parts used	Preparation, administration and use	Reference
Rumex crispus L.	Polygonaceae	Ekşi ot, Gıcırgıcır, Labada	Root Leaf Seed	Dec., Int. Inf., Int. Eaten	(Oral 2007) (Oral 2007) (Kökçü 2015)
Rumex patientia L.	Polygonaceae	Ebelik, Kalmuk çayı, Labada, Tırşıka karan, Yılkıkulak	Leaf Root	Dec., Int. Inf., Int.	(Uysal 2008) (Yeşil and Akalın 2009)
Salvia tomentosa Mill.	Lamiaceae	Boğaç otu, Boşçapula, Ellik otu, Sancı otu, Şabıla, Şalpa, Yakı otu	Aerial parts	Inf., Int.	(Sargın et al. 2013)
Sanguisorba minor Scop. subsp. *magnolii* (Spach) Briq.	Rosaceae	Amel otu, Çayır düğmesi, Kara gömdürme, Kelek otu	Aerial parts	Raw, Eaten	(Furkan 2016)
Scolymus hispanicus L.	Asteraceae	Altın dikeni, Sarıdiken	Leaf, Capitulum	Dec. Int.	(Metin 2009)
Secale cereale L.	Poaceae	Çavdar	Seed	Inf., Int.	(Yıldırım 2015)
Sideritis tmolea P.H. Davis	Lamiaceae	Sarı çiçekli yakı otu	Aerial parts with Flower	Inf., Int.	(Sargın et al. 2013)
Silene vulgaris (Moench) Garcke. var. *vulgaris*	Caryophyllaceae	Cırvınık	Aerial parts	Dec., Int.	(Doğan 2014; Tuzlacı 2016)
Solanum nigrum L.	Solanaceae	Köpek sirkeni	Fruit	Cooked with +Thyme, Monk's pepper, Flour, Water, Ext.	(Uysal 2008)
Solanum tuberosum L.	Solanaceae	Gumpir, Kıtola, Kumpir, Patates, Patati	Tuber	Cooked, Int.	(Uysal 2008; Korkmaz and Karakuş 2013; Karakurt 2014; Ayandın 2010; Saday 2009; Yıldırım 2015; Tuzlacı 2016; Kıncal 2018; Kolaç 2018)
Sorbus aucuparia L.	Rosaceae	Kuş üvezi	Fruit	Jam	(Kural 2012)
Sorbus domestica L.	Rosaceae	Hüvez	Fruit	Eaten	(Kökçü 2015)
[b]*Taraxacum androssovii* Schischk.	Asteraceae	Acıgıcı, Hapşuruk otu, Zeze	Capitulum	Dec., Int.	(Altundağ 2009)
Taraxacum farinosum Hausskn. and Bornm. ex Hand.-Mazz.	Asteraceae	Hindiba, Karahindiba	Leaf	Inf., Int.	(Güneş et al. 2017)
[b]*Taraxacum fedtschenkoi* Hand.-Mazz.	Asteraceae	Acıgıcı, Hapşuruk otu, Zeze	Capitulum	Dec., Int.	(Altundağ 2009)
[b]*Taraxacum macrolepium* Schischk.	Asteraceae	Acıgıcı, Hapşuruk otu, Zeze	Capitulum	Dec., Int.	(Altundağ 2009)
Teucrium chamaedrys L. subsp. *chamaedrys*	Lamiaceae	Bodur Mahmut, Bodurca Mahmut, Cüce Mahmut	Aerial parts Leaf	Dec., Int. Inf., Int. Dec., Int.	(Oral 2007) (Oral 2007) (Alkaç 2013)

Table 1. List of the plants used in traditional treatment against diarrhea in Turkey (continued)

Botanical name	Family	Local name	Plant parts used	Preparation, administration and use	Reference
Teucrium polium L.	Lamiaceae	Acı Yavşan, Egzama otu, Kırmızı ballıbaba, Mayasıl otu, Meryemkot, Oğlan otu	Aerial parts Leaf and Flower Leaf	Dec., Int. Inf., Int. Boiled with Molasses, Ext. Inf., Int. Inf., Int.	(Han and Bulut 2015) (Akan and Sade 2015; Tuzlacı 2016) (Metin 2009) (Bulut 2006) (Tetik et al. 2013)
Tribulus terrestris L.	Zygophyllaceae	Çakır dikeni, Çoban çökerten, Demir diken, Deve çökerten	Fruit	Dec., Int. Inf., Int.	(Doğan 2014; Tuzlacı 2016) (Tetik et al. 2013)
Trifoliumpratense L.	Fabaceae	Yonca	Aerial Parts	Dec., Int.	(Hayta et al. 2014; Tuzlacı 2016)
Triticum sp.	Poaceae	Buğday	Seed	Flour, Int.	(Günbatan et al. 2016)
Urtica dioica L.	Urticaceae	Isırgan otu	Leaf with Seed	Dec., Int.	(Uysal 2008)
Vaccinium arctostaphylos L.	Ericaceae	Ayı üzümü, Lifos, Likarba	Leaf	Dec., Int.	(Kural 2012)
Viburnum lantana L.	Caprifoliaceae	Germeşo, Germişek	Fruit	Dec., Int.	(Altundağ 2009; Tuzlacı 2016)
Vicia faba L.	Fabaceae	Bakla, Pakla	Seed	Eaten	(Yıldırım 2015)
[b]*Viscum album* L.	Loranthaceae	Ökse otu	Leaf	Dec., Int.	(Saraç et al.2013)
[a]*Vitex agnus-castus* L.	Verbenaceae	Hayıt	Seed Branches	Dec., Int. Swallowed, Int. Crushed, Int. Poultice, Ext.	(Sargın et al. 2013) (Polat 2010; Tuzlacı 2016) (Uysal 2008) (Gürdal and Kültür 2013)
Vitis vinifera L.	Vitaceae	Üzüm, Asma, Tevek	Fruit	Juice, Int.	(Furkan 2016)

Int: Internal, Dec: Decoction, Ext: External, Fac: Fruits are crushed, Inf: Infusion, Mac: Maceration

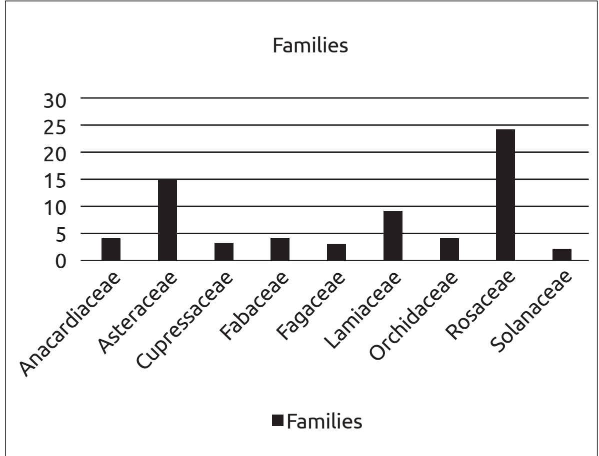

Figure 1. Graph of main families used in traditional treatment against diarrhea in Turkey

MATERIALS AND METHODS

In this study, a thesis search was carried ot at the National Higher Education Center alongside an analysis of ethnobotanical studies conducted in various parts of Turkey with selecting regional plants used for the treatment of diarrhea.

RESULTS AND DISCUSSION

Plants have always been an important source for not only nutrition but also therapeutic use against a considerable number of human diseases. Recent phytochemical studies on medical plants have supported the effectiveness of folkloric medicines. Since ancient times, plants have been used for curing various diseases and infections (Singh et al. 2017).

Turkey has an extraordinary plant diversity and varies by region. Its flora consists of about 10,000 vascular plants and approximately one third of its flora (34.4 %) is endemic to the country (Demirci and Özhatay 2012; Gürdal and Kültür 2013). Recently, the use of ethnobotanical information obtained from medicinal plant research has gained attention all around the world. For this reason, numerous ethnobotanical studies have recently been published and much has been written about medicinal plants in our country (Gürdal and Kültür 2013). Since these medicinal plants have been used in folk medicine by the public for many years, the information about how to use these plants in the treatment of illnesses has been passed down for generations.

In this study, we compiled 133 plant species used in folk medicine for the treatment of diarrhea in Turkey from the ethnobo-

tanical studies and the theses published at the National Higher Education Center between the years of 2002-2018.

Accordingly, this study reveals that *Achillea wilhelmsii* C. Koch., *Parietaria judaica* L., *Punica granatum* L., *Rubus canescens* DC. var. *glabratus* (Godron) Davis and Meikle, *Vitex agnus-castus* L, shown as *a* in the table, are used for the treatment against diarrhea in both humans and animals.

Moreover, some species such as *Helleborus orientalis* L., *Inula viscosa*(L.) Aiton, *Phlomis pungens* Willd. var. *hirta* Velen, *Pinus brutia* Ten., *Taraxacum androssovii* Schischk.,*Taraxacum fedtschenkoi* Hand.-Mazz., *Taraxacum macrolepium* Schischk., *Viscum album* L. which are marked as *b* in the Table 1 are only used for the treatment of diarrhea in animals.

In Figure 1, there is a graph of the main families used in the treatment of diarrhea in Turkey. The plants used for the treatment of diarrhea are mainly from Rosaceae, Asteraceae, Lamiaceae, Fabaceae, Anacardiaceae, Orchidaceae, Cupressaceae, Fagaceae and Solanaceae families (Figure 1). The plants of these families mainly take bioactive molecule groups in their different parts such as fruit, seed, root, aerial parts (Bilaloğlu and Harmanar 1999).

Phytomedicines have a significant role, both as traditional home remedies and as galenic preparations, in the symptomatic treatment of diarrhea. Three groups of preparations are particularly important: tannin-containing herbs, pectins, and a special strain of live dried yeast (Schulz et al. 2004).

Since diarrhea may occur because of fungal, bacterial, viral, and non-infectious causes and many of the plants reported in this study contain pharmaceutically bioactive compounds, including flavones, flavonoids, phenolic acids, tannins, anthocyanin compounds, volatile oil, minerals, vitamins, and polysaccharides (Bilaloğlu and Harmandar 1999). In these molecule groups, tannins especially are medicinally significant because of their astringent properties. Inwardly tannins are administered in cases of diarrhea, intestinal catarrh and as an antidote in cases of heavy metal poisoning (Adhikari and Kundu 2017). They can provide short-term healing and anti-inflammatory effects on the gut wall, though they are likely to rapidly reduce in transit through the tract unless they are in a slowly dispersing solid form. Effects on the bowel, can be significant if the symptom is a reflex consequence of irritation in the gastric or upper enteric passages. The use of tannins is not to be recommended as a long-term solution. Because when they are used as long-term therapy, they can cause constipation, iron deficiency anemia and malnutrition. Therefore long-term therapy with high doses of tannins is to be avoided (Bone and Mills 2013). In this study, *Potentilla*, *Quercus*, *Camellia*, *Vaccinium* and *Alchemilla* sp. are known as plant remedies traditionally used for tannin constituents (Schulz et al. 2004; Bone and Mills 2013).

For centuries, physicians have used preparations containing flavonoids as basic physiologically active components and lay healers attempt to treat human diseases (Cushnie and Lamb 2005). Up till today, plant-derived flavonoids have showed numerous biological activities, including antiallergic, antibacterial, antidiabetic, antiinflammatory, antiviral, anti-proliferative, antimutagenic, antithrombotic, anticarcinogenic, hepatoprotective, oestrogenic, insecticidal, and antioxidant activities (Cushnie and Lamb 2005; Orhan et al. 2010). Flavonoid containing poultices, infusions, spices and balms have been used in many cultures based on ethnomedicinal use for centuries (Cushnie and Lamb 2011). As stated above, there are many kinds of diarrhea and some are caused by infections. In this way, flavonoids in terms of having antimicrobial activities might show a strong ability to cure the pathogenesis. As a result, flavonoids can also be used as a drug to treat diarrhea.

Consequently, these compounds should be investigated in order to determine the main component which is effective against diarrhea and produce natural-based and effective drugs used for this common disease with fewer side effects than chemical drugs. We assume that this study would lead to the development and optimization of new antidiarrheal drugs with no side effects.

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