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Estimation of Cholinesterase Inhibitory and Antioxidant Effects of the Leaf Extracts of Anatolian *Ficus carica* var. *domestica* and their Total Phenol and Flavonoid Contents

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Ficus carica var. *domestica* Tsch. & Rav. (common fig) is widely grown in Turkey and exported for its edible fruits. In this study, the *n*-hexane, chloroform, acetone, methanol, *n*-butanol, and water extracts of the leaves of *F. carica* var. *domestica* were screened for their cholinesterase inhibitory and antioxidant activities. Cholinesterase inhibition against acetyl- (AChE) and butyrylcholinesterase (BChE) was measured by the spectrophotometric method of Ellman at concentrations of 25, 50, and 100 μg/mL., while antioxidant activity was tested using three *in vitro* methods; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, metal-chelation capacity, and ferric-reducing antioxidant power (FRAP). Total phenol and flavonoid contents of the extracts were determined spectrophotometrically. Our results revealed that the *n*-hexane and acetone extracts exerted a notable inhibition against both AChE (62.9±0.9% and 50.8±2.1%, respectively) and BChE (76.9±2.2% and 45.6±1.3%, respectively). However, they had low activity in the antioxidant tests. The chloroform extract was found to be the richest in total flavonoid content (252.5±1.1 mg/g quercetin equivalent), while the *n*-butanol extract had the highest total phenol amount (85.9±3.2 mg/g extract gallic acid equivalent).

Keywords: *Ficus carica*, fig, Moraceae, cholinesterase inhibition, antioxidant activity, total phenol, total flavonoid.

Ficus carica L. (Moraceae) is a tree producing edible fruits, known as "figs". Dried figs are one of the main traditional export items of Turkey, 70-75% of which are exported to the European Union [1]. The genus *Ficus* L. is represented by 10 taxa in Turkey: *F. carica* L. subsp. *carica*, *F. carica* L. subsp. *rupestris* (Hausskn.) Browicz, *F. carica* var. *caprificus* Tsch. & Rav, var. *domestica* Tsch. & Rav, var. *genuina* Boiss., var. *globosa* Boiss., var. *riparum* Hausskn., var. *rupestris* Hausskn., *F. colchica* Grossh., and *F. hyrcana* Grossh [2]. Various *Ficus* species have been reported to be used ethnopharmacologically in different parts of the world [3a-3c]. Among them, *F. religiosa* was reported to be used traditionally for improving memory in India [3d]. Thus, this information prompted us to investigate cholinesterase (ChE) inhibitory activity of *F. carica* since *F. religiosa* does not grow in Turkey.

Cholinesterase inhibitors are the most prescribed drug class currently for the treatment of Alzheimer's disease (AD), which is a neurodegenerative condition with a progressive nature affecting the senior population over the age of sixty, particularly in industrialized countries [4]. However, the ChE inhibitors are not only important for their therapeutic effects for AD, but also because, due to their cholinergic action, their use extends to the treatment of glaucoma, myasthenia gravis [5a,5b], and even to the treatment of intellectual disability, such as Down's syndrome [5c]. In addition, many insecticidal agents, such as organophosphates, show their efficiency through ChE inhibition, which leads to paralysis and death in the insect [5d]. Interestingly, it is also the mechanism of action for some nerve gases used as chemical warfare agents [5e]. Based on their utilization for diverse medical cases, ChE inhibitors have become a hot topic for research worldwide.

In the present study, we have evaluated the *n*-hexane (HEX), chloroform (CHL), acetone (ACE), methanol (MET), *n*-butanol (BUT), and water (H_2O) extracts of *F. carica* var. *domestica* leaves for their *in vitro* ChE inhibitory activity using the spectrophotometric method of Ellman in ELISA microplate reader at 25, 50, and 100 μg/mL concentrations against acetyl- (AChE) and butyrylcholinesterase (BChE) enzymes. Since oxidative stress and metal transition are associated with AD [5f], we have also investigated antioxidant activity of the extracts by three *in vitro* methods; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, metal-chelation capacity, and ferric-reducing antioxidant power (FRAP). Total phenol and flavonoid contents of the extracts were determined spectrophotometrically using Folin-Ciocalteau and aluminum chloride reagents, respectively.

Our results indicated that, among the extracts screened, the HEX extract was the most active against AChE, followed by the ACE extract. The HEX extract again showed the highest inhibition against BChE (Table 1).

Table 1: AChE and BChE inhibitory activities (inhibition % ± S.E.M.) of *F. carica* var. *domestica* leaf extracts (all concentrations in µg/mL).

Extracts	Inhibition % ± S.E.M.[a] against AChE			Inhibition % ± S.E.M. against BChE		
	25	**50**	**100**	**25**	**50**	**100**
HEX	-[b]	46.5 ± 0.9	62.9 ± 0.9	40.6 ± 0.1	55.4 ± 0.2	76.9 ± 2.2
CHL	-	-	28.2 ± 0.6	-	-	-
ACE	-	8.2 ± 2.2	50.8 ± 2.1	-	14.8±0.1	45.6 ± 1.3
MET	-	-	-	-	-	-
BUT	-	-	-	-	-	-
H_2O	-	-	-	-	-	-
GAL[c]	ND[d]	ND	98.9 ± 0.2	ND	ND	80.3 ± 1.1

[a]Standard error mean, [b]No activity, [c]Galanthamine, [d]Not determined.

The extracts were tested at 250, 500, and 1000 mg/mL for their antioxidant activities. However, they exerted very low scavenging activity against the DPPH radical (Table 2) and possessed unimportant metal-chelation capacity (Table 3).

Table 2: DPPH radical scavenging activity (Inhibition % ± S.E.M.) of *F. carica* var. *domestica* leaf extracts (all concentrations in µg/mL).

Extracts	Inhibition %±S.E.M.[a] against DPPH radical		
	250	**500**	**1000**
HEX	2.8 ± 0.1	3.0 ± 0.1	3.9 ± 0.2
CHL	2.8 ± 0.5	3.2 ± 0.2	3.9 ± 0.1
ACE	2.7 ± 0.3	3.3 ± 0.1	4.7 ± 0.6
MET	4.6 ± 0.1	5.8 ± 0.1	9.7 ± 0.5
BUT	4.3 ± 0.1	6.0 ± 0.2	9.6 ± 0.5
H_2O	3.2 ± 0.2	3.4 ± 0.1	3.7 ± 0.2
Gallic acid	80.9 ± 0.7	91.6 ± 0.1	92.6 ± 0.1

[a]Standard error mean

Table 3: Metal-chelation capacity (Inhibition % ± S.E.M.) of *F. carica* var. *domestica* leaf extracts (all concentrations in µg/mL).

Extracts	Metal-chelation capacity (Inhibition %±S.E.M.[a])		
	250	**500**	**1000**
HEX	6.1±0.9	10.3±0.1	11.5±1.0
CHL	-[b]	-	-
ACE	-	-	-
MET	-	-	-
BUT	-	-	-
H_2O	-	2.9±0.3	12.1±2.3
BHA[c]	ND[d]	21.7±1.1	26.9±1.5

[a]Standard error mean, [b]No activity, [c]Butylatedhydroxyanisol, [d]Not determined.

In the FRAP test, the extracts showed a moderate level of activity as compared with chlorogenic acid, as shown in Figure 1. Total phenol and flavonoid contents of the extracts were also determined. Total phenol values were calculated using the calibration equation of y = 1.022x + 0.084 (r^2 = 0.998) as gallic acid equivalents (mg/g extract). For total flavonoid content of the extracts, a calibration equation of y = 5.740x + 0.058 (r2 = 0.999) was obtained. Total flavonoid contents were calculated as quercetin equivalents (mg/g extract). The results revealed that the BUT extract had the richest total phenol content, while the CHL extract contained the highest amount of total flavonoids (Table 4).

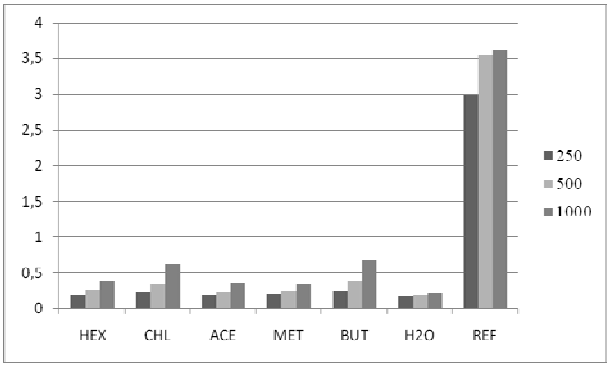


Figure 1: The FRAP graph of the extracts and the reference.

Table 4: Total phenol and flavonoid contents of *F. carica* var. *domestica* leaf extracts.

Extracts	Total phenol content ± S.E.M.[a] of the extracts[b]	Total flavonoid content ± S.E.M. of the extracts[c]
HEX	45.4 ± 4.8	56.6 ± 1.9
CHL	62.6 ± 2.9	252.5 ± 1.1
ACE	43.9 ± 3.3	83.2 ± 3.1
MET	64.5 ± 1.3	39.9 ± 2.3
BUT	85.9 ± 3.2	211.1 ± 3.0
H_2O	61.6 ± 2.8	8.0 ± 2.7

[a]Standard error mean, [b]Data expressed in mg equivalent of gallic acid (GAE) to 1 g of extract, [c]Data expressed in mg equivalent of quercetin to 1 g of extract.

Until now, there has been no study reported on ChE inhibitory activity of *F. carica*. However, another species, *F. religiosa*, was tested for its AChE inhibitory activity, due to its traditional use in India for memory enhancement [3d]. In that study, the MET extract of the stem barks of the plant showed 54.5±1.3% inhibition of AChE at 100 µg/mL. On the contrary, the MET extract of *F. carica* was completely inactive in our study against both cholinesterases, whereas the HEX and ACE extracts showed a notable inhibition against these enzymes. This could be explained by differences in the constituents of these species. In another study, the MET extract prepared from the figs of *F. religiosa* was tested for their anti-amnesic effects *in vivo* and the extract was found to be quite effective in mice [6].

F. carica contains phenolic and organic acids, flavonoids, and alpha-tocopherol in the leaves [7a,7b], anthocyanins, phenolic acids, and polysaccharide in the figs [7c-7e], as well as sesquiterpenes and organic acids in the latex [8]. In several studies on *F. carica*, its high antioxidant activity was attributed to the phenolic compounds [7a,7c,7e]. However, in our assays, the leaf extracts showed a very low antioxidant effect in two of the tests, whilst they had a relatively better antioxidant effect in the FRAP test. A correlation was observed between total phenol-flavonoid contents and the BUT extract. It displayed the highest FRAP and had the highest total phenol content, while it was the second richest total flavonoid-containing extract.

In conclusion, to the best of our knowledge, we report for the first time the ChE inhibitory activity of the *F. carica*

var. *domestica* leaf extracts. In particular, the HEX and ACE extracts, which exerted remarkable ChE inhibition, might be considered as potential sources of anticholinesterase compound(s) and worthy of further investigation. Our antioxidant results obtained from the leaf extracts of *F. carica* var. *domestica* differed from those reported previously, which might be dependent on differences in the collection sites.

Experimental

Plant materials: The leaves of *F. carica* var. *domestica* were collected from Ceyhan town in Adana Province in 2009 and identified [voucher specimen (AEF, No: 25878)] by Dr Ayse Mine Gencler, Department of Pharmaceutical Botany, Ankara University, Ankara, Turkey.

Extraction: The dried leaves of the plant were powdered and 10 g was macerated in ethanol (95%) for 6 h in a water bath at 40°C. The macerate was then filtered and this procedure was repeated 3 times. The combined filtrate was evaporated to dryness *in vacuo* using a rotary evaporator. Then, the ethanol extract was further fractionated using a separating funnel to obtain the extracts of *n*-hexane (1.25 g), chloroform (1.70 g), acetone (0.23 g), methanol (5.57 g), *n*-butanol (0.31 g), and distilled water (0.46 g), respectively. The water extract was lyophilized and all of the extracts were stored at -20^{0}C until the experiments were carried out.

Cholinesterase inhibition test: AChE and BChE inhibitory activities of the extracts were determined by a modified spectrophotometric method of Ellman [9]. Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum BChE (EC 3.1.1.8, Sigma) were used as enzyme sources, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as substrates for the reaction. 5,5´-Dithio-bis(2-nitrobenzoic)acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of the cholinesterase activity. All the other reagents and conditions were the same as those described in our earlier publication [10]. The hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by the enzymes at a wavelength of 412 nm, utilizing a 96-well microplate reader (VersaMax, Molecular Devices, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. Percentage of inhibition of AChE/BChE was determined by comparison of the reaction rates of the samples relative to blank sample (ethanol in phosphate buffer pH=8) using the formula *(E-S)/E x 100*, where *E* is the activity of enzyme without test sample, and *S* is the activity of enzyme with test sample. The experiments were undertaken in triplicate. Galanthamine, employed as reference, was purchased from Sigma (St. Louis, MO, USA).

DPPH radical scavenging activity: The hydrogen atom or electron donation capacity of the corresponding extracts was computed from the bleaching property of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The stable DPPH radical scavenging activity was determined by the Blois method [11]. The samples and reference dissolved in ethanol (75%) were mixed with DPPH solution (1.5 x 10^{-4} M). The remaining DPPH after 30 min reaction time was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). The results were compared with that of gallic acid employed as the reference. Inhibition of DPPH in percent (I%) was calculated as below:
I% = [($A_{control}$-A_{sample}) / $A_{control}$] x 100, where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts or reference. Experiments were run in triplicate and the results were conveyed as average values with S.E.M. (standard error mean).

Fe^{+2}-ferrozine test system for iron chelating: The ferrous ion-chelating effect of the extracts and reference was estimated by the method of Chua *et al.* [12]. Accordingly, 740 µL of the samples, dissolved in ethanol (75%), were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 40 µL of ferrozine solution into the mixture and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:
I% = [($A_{control}$-A_{sample}) / $A_{control}$] x 100. The control contained only $FeCl_2$ and ferrozine, and butylated hydroxyanisol was used as reference. Analyses were run in triplicate and expressed as average values with S.E.M. (Standard error mean).

The ferric-reducing antioxidant power (FRAP): The ferric-reducing antioxidant power (FRAP) of the extracts and reference was tested using the assay of Oyaizu [13a]. One mL of different concentrations of the extracts, as well as chlorogenic acid as reference for comparative purposes, was added to 2.5 mL of phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide. Later, the mixture was incubated at 50^{o}C for 20 min and then trichloroacetic acid (10%) was added. After the mixture had been shaken vigorously, the solution was mixed with distilled water and $FeCl_3$ (0.1%, w/v). After 30 min of incubation, absorbance was read at 700 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). Each analysis was duplicated. Increase in absorbance of the reaction indicated increase in reducing power of the extracts.

Determination of total phenol and flavonoid contents: Phenolic content of the extracts was determined using Folin-Ciocalteau's reagent (Sigma) [13b]. In brief, the samples were mixed with 750 µL of Folin-Ciocalteau's reagent and 600 µL of sodium carbonate in test tubes. The tubes were then vortexed and incubated at 40°C for 30 min. Absorption was then measured at 760 nm using a

Unico 4802 UV-visible double beam spectrophotometer (USA). Total flavonoid content was calculated by the aluminum chloride colorimetric method [14]. A number of dilutions of quercetin were obtained to prepare a calibration curve. Then, the same dilutions from the samples were also prepared and separately mixed with 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate as well as 2.8 mL of distilled water. Following incubation for 30 minutes at room temperature, absorbance of the reaction mixture was measured at a wavelength of 415 nm with a Unico 4802 UV-visible double beam spectrophotometer (USA). The total phenol and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (mg/g extract), respectively.

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