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HPTLC Fingerprinting and Cholinesterase Inhibitory and Metal-Chelating Capacity of Various *Citrus* Cultivars and *Olea europaea*

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Summary

Inhibitory activity of thirty-one ethanol extracts obtained from albedo, flavedo, seed and leaf parts of 17 cultivars of *Citrus* species from Turkey, the bark and leaves of *Olea europaea* L. from two locations (Turkey and Cyprus) as well as caffeic acid and hesperidin was tested against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), related to the pathogenesis of Alzheimer's disease, using ELISA microtiter assays at 500 μg/mL. Metal-chelating capacity of the extracts was also determined. BChE inhibitory effect of the *Citrus* sp. extracts was from (7.7±0.7) to (70.3±1.1) %, whereas they did not show any inhibition against AChE. Cholinesterase inhibitory activity of the leaf and bark ethanol extracts of *O. europaea* was very weak ((10.2±3.1) to (15.0±2.3) %). The extracts had either no or low metal-chelating capacity at 500 μg/mL. HPTLC fingerprinting of the extracts, which indicated a similar phytochemical pattern, was also done using the standards of caffeic acid and hesperidin with weak cholinesterase inhibition. Among the screened extracts, the albedo extract of *C. limon* 'Interdonato', the flavedo extracts of 'Kara Limon' and 'Cyprus' cultivars and the seed extract of *C. maxima* appear to be promising as natural BChE inhibitors.

Key words: *Citrus* sp., *Olea europaea*, cholinesterase inhibition, metal-chelating activity, HPTLC

Introduction

Alzheimer's disease (AD) is a neurological disorder and the most common cause of dementia. It is a slowly progressive and degenerative disease that leads to loss of memory, decline in thinking and language skills. The pathogenesis of AD has been so far explained by cholinergic and amyloid hypotheses, and it has been stated that only drugs acting on cholinergic system have moderate, but steady effects in clinical trials (*1*). The cholinergic hypothesis is based on insufficiency of the acetylcholine (ACh) level in the brains of AD patients, which is hydrolyzed by acetylcholinesterase (AChE, EC 3.1.1.7) (*2*). On the other hand, the evidence has shown that butyrylcholinesterase (BChE, EC 3.1.1.8), also known as pseudocholinesterase, takes a slight part in hydrolyzing brain ACh levels in addition to its possible action in the etiology and progression of the disease (*3*). Thus, inhibition of both AChE and BChE is an important target for the development of new anti-Alzheimer drugs. Since AD is multi-faceted disease, it is also strongly associated with metal ion dyshomeostasis and oxidative stress (*4*). Dysregulation of some transition metals such as iron, aluminum or copper has been very important in formation of oxidative stress and cellular damage relevant to some neurodegenerative diseases including AD and Parkinson's disease (*5,6*). Therefore, it is strategically advantageous to have a drug candidate with both cholinesterase inhibition and metal-chelating properties for the treatment of AD as the need for development of novel drugs remains.

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Citrus species (Rutaceae) bearing widely consumed fruits of nutritional and industrial importance are rich in flavonoid derivatives, *i.e.* hesperidin, naringin and polymethoxylated flavonoids, whose distributions differ in *Citrus* tissues including albedo, flavedo, pericarp (peel) and seed (*7*). Earlier studies have revealed that the phenolic compounds exist in higher amounts in *Citrus* peel than in other parts of *Citrus* fruit, such as seeds, leaves or flowers (*8*). *Olea europaea* L. (olive) from Oleaceae family is an evergreen tree particularly native to the Mediterranean region. In addition to being edible, olive tree is also considered as a medicinal plant. Due to nutritional and health aspects of *Citrus* sp. and *O. europaea*, their edible tissues have been extensively investigated for their various biological activities and phytochemical content. Nevertheless, there have been relatively few reports on health-promoting values of non-edible tissues of *Citrus* fruits and *O. europaea*. Hence, in the present study, attempts have been made to investigate inhibitory effect of 31 ethanol extracts from 17 *Citrus* cultivars from Turkey as well as the bark and leaves of olive trees growing in Turkey and Cyprus against AChE and BChE using ELISA microtiter assays. Additionally, high-performance thin layer chromatography (HPTLC) fingerprinting of the extracts was done using hesperidin and caffeic acid standards, two phenolic substances commonly found in *Citrus* species.

Materials and Methods

Plant materials and extraction

The samples of 17 *Citrus* cultivars were obtained from The West Mediterranean Agricultural Research Institute (BATEM, Antalya Province, Turkey) in 2011, where they are cultivated in the experimental fields. The bark and leaf samples of *Olea europaea* were collected from Denizli Province (western Turkey) and the leaf samples from Mehmetcik village at Famagusta town (northern Cyprus) in 2012. The pericarps (peels) of the *Citrus* fruits were peeled and carefully separated into their albedo (whitish) and flavedo (yellow or orange) parts. All plant samples were dried at room temperature in shadow and coarsely ground prior to the extraction. The dried and powdered samples were extracted at room temperature by percolation with ethanol (80 %; Carlo Erba, Val de Reuil, France). All of the extracts were concentrated using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) *in vacuo* until a solid extract of each sample was obtained.

Microtitre assays for anticholinesterase activity

AChE and BChE inhibitory potential of the extracts was determined by modified spectrophotometric method of Ellman *et al.* (*9*) as described in our previous publication (*10*). Electric eel acetylcholinesterase (TypeVI-S, EC 3.1.1.7) and horse serum butyrylcholinesterase (EC 3.1.1.8) were used as the enzyme sources purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetylthiocholine iodide and butyrylthiocholine chloride (Sigma–Aldrich) were employed as the substrates for the reaction, while 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB; Sigma–Aldrich) was the colouring agent. A volume of 140 μL of 0.1 mM sodium phosphate buffer (pH=8.0), 20 μL of 0.2 M DTNB, 20 μL of the sample solutions and 20 μL of 0.2 M AChE/BChE solution were added with multichannel automatic pipette (Gilson S.A.S., Villiers le Bel, France) to a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 μL of 0.2 M acetylthiocholine iodide/butyrylthiocholine chloride. Formation of the yellow 5-thio-2-nitrobenzoate anion resulted from the reaction of DTNB with thiocholines after hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride, which was monitored at 412 nm utilizing a 96-well microplate reader VersaMax™ (Molecular Devices, Sunnyvale, CA, USA). Galanthamine, purchased from Sigma–Aldrich, was employed as the reference.

Data processing for enzyme inhibition assays

The measurements and calculations were evaluated by using Softmax® PRO v. 4.3.2.LS software (Molecular Devices). Percentage inhibition (I) of AChE/BChE was determined by comparison of reaction rates of test samples with the blank sample (ethanol in phosphate buffer, pH=8), and calculated using the equation given below:

$$I=((A_{blank}-A_{sample})/A_{blank})\cdot 100 \qquad /1/$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts. Data was expressed as average inhibition±standard error of the mean (SEM), which were obtained from three independent experiments.

Determination of metal-chelating capacity by Fe^{2+}-ferrozine test system

The metal-chelating capacity of the extracts was estimated with the method of Chua *et al.* (*11*) using Fe^{2+}-ferrozine test system followed by ELISA method. In brief, the samples (200 μL each) dissolved in ethanol (75 %) were incubated with 2 mM $FeCl_2$ solution (Sigma Chemical Co., Steinheim, Germany). The reaction was started after the addition of 40 μL of 5 mM ferrozine (Sigma Chemical Co.) solution, which was shaken gently and left to rest for 10 min at ambient temperature. The absorbance of the reaction mixture as well as ethylenediaminetetraacetic acid (EDTA; Sigma Chemical Co.) as the reference was measured at 562 nm using a Unico 4802 UV/Vis spectrophotometer (Dayton, NJ, USA). Metal chelation capacity was measured as a percentage inhibition of Fe^{2+}-ferrozine complex calculated using Eq. 1. The experiments were run in triplicate and the results were expressed as average values with SEM.

HPTLC analysis

HPTLC analysis was performed on a CAMAG (Muttenz, Switzerland) apparatus equipped with automatic TLC sampler 4, twin trough chamber (20 cm×10 cm), chromatogram immersion device III, TLC plate heater III, automatic development chamber ADC2 and visualizer. The extracts and standards, *i.e.* caffeic acid (Carl Roth GmbH, Zurich, Switzerland) and hesperidin (Acros Organics, Basel, Switzerland) at the concentrations of 5 and 0.2 mg/mL, respectively, were dissolved in methanol of ultra gradient HPLC grade (Carl Roth GmbH). The volume of each sample was 10 or 15 μL. Development conditions were as fol-

lows: relative humidity RH=33 %, solution saturation 20 min, developing distance from application position/lower edge of the plate of 62/70 mm, developing solvent ethyl acetate (Acros Organics, Basel, Switzerland)/formic acid (Acros Organics)/water (CAMAG) in a ratio of 75:15:10, developing time 20 min, and plate drying time 5 min. The plates used were HPTLC glass Si 60 F254 (20 cm×10 cm, model HX308464; Merck Co., Darmstadt, Germany), while visualization of the spots was achieved with $AlCl_3$ and natural product polyethylene glycol (PEG) 400 (Merck Co.).

Results and Discussion

Cholinesterase inhibitory and metal chelation activities of extracts

The results of enzyme inhibition tests show that *Citrus* extracts at the concentration of 500 μg/mL did not affect AChE activity, while *Olea europaea* leaf extracts slightly inhibited (below 16 %) both enzymes (Table 1). On the other hand, quite variable inhibition rates of *Citrus* extracts (between (7.72±0.71) and (70.28±1.12) %) were observed against BChE. Caffeic acid and hesperidin, tested in the same manner but at the concentration of 100 μg/mL, had (6.0±2.5) and (27.3±1.2) % of AChE inhibition as well as (24.1±0.9) and (17.7±4.2) % of BChE inhibition, respectively. The extracts had either no or very low (below 13 %) metal-chelating capacity (Table 1), while caffeic acid and hesperidin were not tested in this assay.

HPTLC profile of the extracts

According to the HPTLC profiles of the extracts screened in the current study, the flavedo extracts had more intense zones at 366 nm than the albedo extracts. Looking at the results of white light reflectance transmittance, it can be observed that the yellow zones of the albedo samples are more intense than the corresponding yellow zones of the flavedo samples. Our findings indicate that it is possible to discriminate *Citrus aurantium*, *C. deliciosa*, *C. limon*, *C. maxima*/*C. paradisi* and *C. sinensis*/*C. reticulata*. The fingerprints of *C. maxima* and *C. paradisi* look very similar, thus, these two species cannot be discriminated. This is also the case for *C. sinensis* and *C. reticulata.* As seen in Fig. 1, the fingerprints of the albedo and flavedo extracts of *C. aurantium* look very similar to each other under white light, with a strong red zone at retention factor (R_F) of approx. 0.36 and several yellow zones above it. All extracts belonging to *C. limon* species had a similar fingerprint with a characteristic red zone at R_F of approx. 0.39 under white light. The fingerprints of the flavedo and albedo samples of *C. maxima* each had a yellow zone with a higher R_F value than hesperidin. A parallel fingerprint was observed for all extracts of *C. paradisi* cultivars that contained hesperidin, while the extracts obtained from *C. reticulata* and *C. sinensis* 'Navelina' resemble each other (Fig. 2). Among the analyzed extracts in Fig. 3, all the *C. sinensis* cultivars had two yellow zones, one of them with the same R_F value as hesperidin. The leaf extracts of *O. europaea* collected from the two locations exhibited quite analogous phytochemical profiles, whereas the bark extract of this plant had a different fingerprint than those of the leaf extracts.

A number of studies have demonstrated a potent cholinesterase inhibitory effect of various *Citrus* species, particularly against AChE, such as *C. unshiu* (*12*), *C. paradisi* (*13*), *C. junos* (*14*), *C. medica* 'Diamante' (*15*), *C. hystrix* (*16*), *C. depressa* (*17*), *C. aurantifolia*, *C. aurantium* and *C. bergamia* (*18–20*), *C. maxima*, *C. paradisi*, *C. limon*, *C. sinensis* and *C. reticulata* (*21–23*). The health effects of *Citrus* fruits have been principally attributed to the existence of phenolic compounds including flavonoids and phenolic acids. Some of the studies led to the isolation of flavonoids as the active components such as naringenin, the flavanone isolated from the peel extract of *C. junos*, which inhibited AChE at low level (IC_{50}=(621±7.8) μg/mL) as compared to that of the reference physostigmine (IC_{50}=(0.07± 0.0) μg/mL) (*15*). Naringenin also exerted antiamnesic effect *in vivo*. Similarly, nobiletin, 8-demethoxynobiletin and 6-demethoxynobiletin (60–76 % of control at 100 μM of each compound) isolated from the peel extract of *C. depressa* were concluded to be the active components for the marked anti-AChE effect of this plant (*17*).

However, in another study (*16*), furanocoumarins identified as (*R*)-(+)-6′-hydroxy-7′-methoxybergamottin, (*R*)-(+)-6′,7′-dihydroxybergamottin and (+)-isoimperatorin (IC_{50}=(11.2± 0.1), (15.4±0.3) and (23.0±0.2) μM, respectively) were suggested to be the compounds responsible for the peel hexane and dichloromethane extracts of the *C. hystrix* fruits. Other coumarin derivatives, nootkatone and auraptene isolated from the essential oil of *C. paradisi*, were revealed to inhibit only 17–24 % of AChE activity, at the concentration of 1.62 μg/mL (*13*). Fruit juices of several *Citrus* cultivars, *i.e. C. maxima* (shaddock), *C. paradisi* (grapefruit), *C. limon* (lemon), *C. sinensis* (orange) and *C. reticulata* (tangerine) and the aqueous extracts from *C. sinensis*, *C. paradisi* and *C. maxima* as well as the essential oil isolated from the peels of *C. aurantifolia*, *C. aurantium* and *C. bergamia* were reported to exert selective AChE inhibitory activity varying from moderate to remarkable levels (*18,21,22*), which seems contrary to our findings on selective BChE inhibition of the *Citrus* cultivars screened herein (Table 1). Nevertheless, *C. limon* was reported to have a weak cholinesterase inhibitory activity, dissimilar to most of our data on *C. limon* cultivars (*23*).

On the other hand, the leaves of *Citrus* cultivars have been examined in a very few studies, one of which was performed with the leaf essential oil of *C. aurantifolia*. The leaf oil was revealed to have IC_{50} values of (139±35) and (42±5) μg/mL on AChE and BChE, respectively (*19*), while Loizzo *et al.* (*20*) investigated the leaf hexane extract of *C. aurantifolia* with a marked AChE inhibitory effect. In the present study, the leaf ethanol extract of *C. aurantium* inhibited (46.37±0.54) % of BChE.

The computational studies described stronger AChE inhibitory effect of naringin than hesperidin (*24*), which inhibited (27.33±1.16) % of AChE in the current study. Without any doubt, hesperidin, present in most of the *Citrus* extracts screened in the HPTLC analysis, could be one of the compounds contributing somewhat to BChE inhibitory effects of the extracts. Caffeic acid, a dominant phenolic compound available in many *Citrus* cultivars (*25,26*), was reported earlier to exhibit either low inhibitory activity ((11.05±1.03) % at 100 μg/mL) against AChE (*27*) and

Table 1. Cholinesterase inhibitory and metal-chelating activities of ethanol extracts of *Citrus* sp. and *Olea europaea* at mass concentration of 500 μg/mL (unless otherwise stated)

Species	Plant part	$\frac{Y}{\%}$	Inhibition against AChE[a] %	Inhibition against BChE[a] %	Metal-chelating capacity %
	albedo	5.87	–	7.7±0.7****	–
Citrus aurantium	flavedo	8.48	–	54.0±0.8****	–
	leaf	22.02	–[b]	46.4±0.5****	10.9±0.2****
Citrus deliciosa	pericarp	10.81	–	34.2±0.8****	–
Citrus limon 'Interdonato'	albedo	11.74	–	51.6±1.9****	–
	flavedo	13.83	–	–	–
Citrus limon 'Kara Limon'	albedo	14.17	–	19.6±0.3****	–
	flavedo	20.86	–	47.0±1.4****	–
Citrus limon 'Cyprus'	albedo	7.23	–	27.8±5.8****	–
	flavedo	14.56	–	70.3±1.1**	–
Citrus maxima	albedo	14.48	–	23.7±2.6****	–
	flavedo	15.89	–	39.5±3.0****	–
	seed	11.42	–	69.9±1.9***	–
Citrus paradisi 'Henderson'	albedo	12.25	–	28.6±2.4****	–
	flavedo	26.82	–	46.1±2.6****	–
Citrus paradisi 'Red Blush'	albedo	12.47	–	24.5±3.1****	–
	flavedo	24.61	–	42.7±1.4****	–
Citrus paradisi 'Star Ruby'	albedo	15.86	–	28.7±0.1****	–
	flavedo	33.79	–	39.9±2.4****	–
Citrus reticulata 'Lee'	pericarp	7.86	–	42.5±0.1****	–
Citrus reticulata 'Nova'	pericarp	9.37	–	40.4±1.4****	–
Citrus reticulata 'Blanco' × *Citrus paradisi*	pericarp	11.39	–	19.5±0.4****	–
Citrus sinensis 'Navelina'	albedo	9.49	–	12.3±3.3****	–
	flavedo	18.00	–	19.5±0.3****	–
Citrus sinensis 'Shamouti'	albedo	13.49	–	12.7±0.5****	–
	flavedo	16.45	–	7.5±0.6****	12.6±0.3****
Citrus sinensis 'Valencia Late'	albedo	7.56	–	25.2±2.8****	–
	flavedo	15.36	–	25.6±0.4****	–
Citrus sinensis 'Washington Navel'	albedo	13.27	–	22.6±1.4****	–
	flavedo	22.80	–	25.6±0.1****	–
Citrus japonica	pericarp	13.42	–	14.3±0.0****	–
Olea europaea (Turkey)	bark	12.53	5.7±1.5****	15.0±2.3****	7.0±0.0****
Olea europaea (Turkey)	leaf	26.77	12.0±1.4****	10.2±3.1****	4.8±2.7****
Olea europaea (Cyprus)	leaf	32.61	11.1±1.6****	11.9±2.0****	–
Caffeic acid[c]			6.0±2.5****	24.1±0.9****	–[d]
Hesperidin[c]			27.3±1.2****	17.7±4.2****	–
Galanthamine[e]			94.5±3.8	92.3±0.8	
EDTA[f]					96.5±0.9

[a]Results are expressed as mean value±standard error of the mean (N=3), [b]no inhibition/activity, [c]tested at 100 μg/mL, [d]not tested, [e]reference for AChE and BChE inhibition assays at 100 μg/mL, [f]reference for metal-chelating capacity assay at 100 μg/mL. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

IC_{50}≥200 μM (*28*) or no inhibition (*29*), which is consistent with our result ((6.01±2.50) % at 100 μg/mL).

According to our literature survey, AChE inhibitory effects of the leaf extracts of the stored and fresh samples of *O. europaea* were (69.2±6.0) and (85.4±3.4) % at 1 μg/mL, respectively (*30*). Nevertheless, the leaf and bark extracts from *O. europaea* used in this study had insignificant effect on both AChE and BChE as compared to that of the reference (galanthamine).

Although a few studies demonstrated metal-chelating capacity of *Citrus* cultivars at various levels (*31,32*), the *Citrus* extracts studied herein exerted either very low

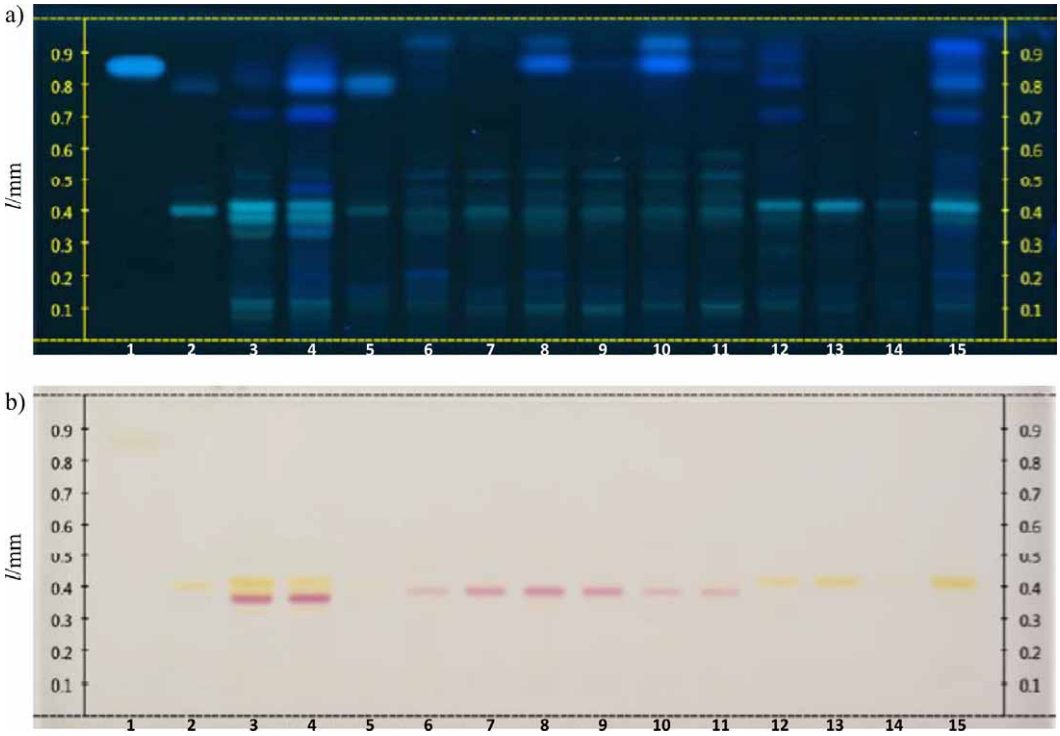

Fig. 1. HPTLC profiles of the *Citrus* extracts: a) image of the derivatized plate with $AlCl_3$ under UV light at 366 nm, and b) image of the derivatized plate under white light ($AlCl_3$, then natural product PEG 400). Lane 1=caffeic acid, lane 2=hesperidin, lanes 3 and 4=*C. aurantium* (albedo and flavedo), lane 5=*C. deliciosa* (pericarp), lanes 6 and 7=*C. limon* 'Interdonato' (flavedo and albedo), lanes 8 and 9=*C. limon* 'Kara Limon' (flavedo and albedo), lanes 10 and 11=*C. limon* 'Cyprus' (flavedo and albedo), lanes 12 and 13=*C. maxima* (flavedo and albedo), lanes 14 and 15=*C. maxima.* Volume of each sample was 10 μL, except in lane 15 (15 μL)

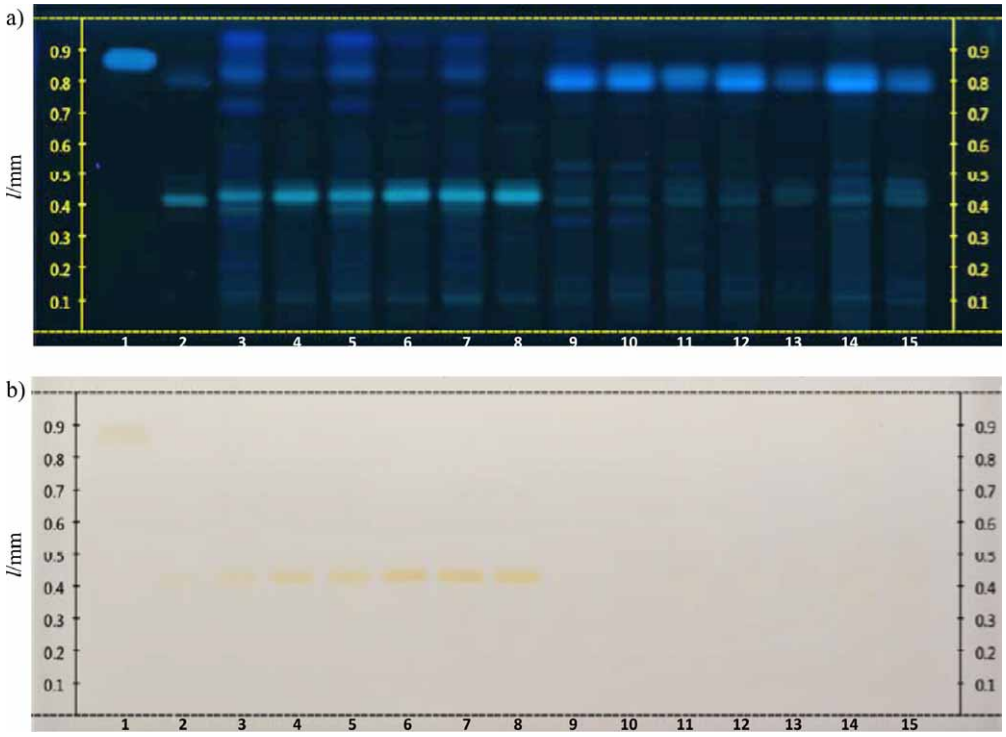

Fig. 2. HPTLC profiles of the *Citrus* extracts: a) image of the derivatized plate with $AlCl_3$ under UV light at 366 nm, and b) image of the derivatized plate under white light ($AlCl_3$, then natural product PEG 400). Lane 1=caffeic acid, lane 2=hesperidin, lanes 3 and 4=*C. paradisi* 'Handerson' (flavedo and albedo), lanes 5 and 6=*C. paradisi* 'Red Blush' (flavedo and albedo), lanes 7 and 8=*C. paradisi* 'Star Ruby' (flavedo and albedo), lane 9=*C. reticulata* 'Lee' (pericarp), lane 10=*C. reticulata* 'Nova' (pericarp), lane 11=*C. reticulata* 'Blanco' × *C. paradisi* (pericarp), lanes 12 and 13=*C. sinensis* 'Navelina' (flavedo and albedo), lanes 14 and 15=*C. sinensis* 'Navelina' (flavedo and albedo). Volume of each sample was 10 μL, except in lanes 14 and 15 (15 μL)

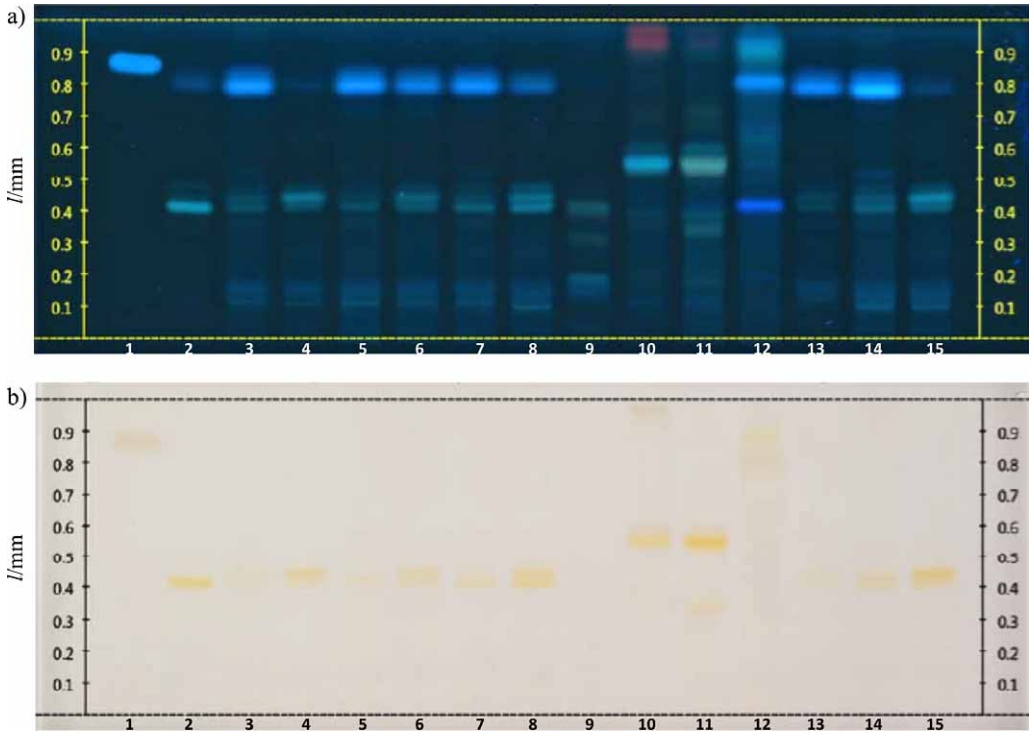

Fig. 3. HPTLC profiles of the *Citrus* and *Olea europaea* extracts: a) image of the derivatized plate with $AlCl_3$ under UV light at 366 nm, and b) image of the derivatized plate under white light ($AlCl_3$, then natural product PEG 400). Lane 1=caffeic acid, lane 2=hesperidin, lanes 3 and 4=*C. sinensis* 'Shamouti' (flavedo and albedo), lanes 5 and 6=*C. sinensis* 'Valencia' (flavedo and albedo), lanes 7 and 8=*C. sinensis* 'Washington Navel' (flavedo and albedo), lane 9=*C. japonica* (pericarp), lane 10=*Olea europaea* (Turkey) (leaf), lane 11=*Olea europaea* (Cyprus) (leaf), lane 12=*Olea europaea* (Turkey) (bark), lanes 13 and 14=*C. sinensis* 'Shamouti' (flavedo), lane 15=*C. sinensis* 'Shamouti' (albedo). Volume of each sample was 10 μL, except in lane 13 (5 μL) and lanes 14 and 15 (15 μL)

or no chelating capacity, which might be related to their phytochemical content.

Conclusions

The present study demonstrated that, among the seventeen cultivars of *Citrus* and the leaf and bark samples of *Olea europaea*, the maximum BChE inhibition was caused by the flavedo extract of *C. limon* 'Cyprus', followed closely by the seed extract of *C. maxima*. Our results indicated that the albedo and flavedo extracts showed diverse levels of BChE inhibition, which might mainly depend on the difference in their phytochemical content. The screened *Citrus* extracts had a selective BChE inhibitory and no AChE inhibitory activity at all. The extracts had either no or low metal-chelating capacity. To the best of our knowledge, this is the first study disclosing cholinesterase inhibitory and metal-chelating activities of the *Citrus* species cultivated in Turkey as well as of *O. europaea* growing in Turkey and Cyprus, and also the first HPTLC fingerprinting of these plants. Overall, the aforementioned *Citrus* extracts could be considered as BChE inhibitors rather than AChE inhibitors.

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