



In vitro multiple pharmacological targets of *Colutea cilicica* Boiss. & Balansa against key enzymes linked to neurodegenerative diseases, diabetes, and hyperpigmentation

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ABSTRACT

Prevention and treatment of noncommunicable diseases such as neurodegenerative diseases, diabetes, and hyperpigmentation using medicinal plants has attracted increasing attention during the past few decades. In this study, *Colutea cilicica* Boiss. & Balansa extracts (ethyl acetate, methanol, and water) were evaluated against key enzymes involved in neurodegenerative diseases, diabetes, and hyperpigmentation. The antioxidant (free radical scavenging, reducing power, β -carotene/linoleic acid, and phosphomolybdenum) and metal chelation properties were also investigated. The methanol extracts of *C. cilicica* vigorously inhibited the activities of acetylcholinesterase and butyrylcholinesterase (1.33 and 0.68 mg galantamine equivalents (GALAE)/g extract, respectively). It was observed that *C. cilicica* extracts possessed a higher inhibitory potential for α -glucosidase (2.71–1.23 mmol acarbose equivalents (ACAE)/g extract) than that for α -amylase (0.57–0.12 mmol ACAE/g extract). The water extract of *C. cilicica* showed potent radical scavenging capacity against DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] [42.46 and 57.70 mg trolox equivalents (TE)/g extract, respectively). Phytochemical determination showed that *C. cilicica* water extract (17.26 mg rutin equivalents (RE)/g extract) was rich in flavonoids compared with ethyl acetate and methanol extracts (2.78 and 2.83 mg RE/g extract, for the respective extracts). These findings reveal the interesting potential of *C. cilicica* as a valuable source of phytochemicals that can be used against common noncommunicable diseases, particularly against enzymes involved in neurodegenerative diseases.

Keywords: *Colutea cilica*; Alzheimer's disease; diabetes mellitus; natural agents; phytopharmaceuticals.

INTRODUCTION

Non communicable diseases such as diabetes, and neurodegenerative diseases such as Alzheimer disease (AD) have become a major global health burden (Moreno Cervantes et al 2017). Though the exact cause of AD remains uncertain, hypotheses of the possible pathological pathways have been suggested to affecting mainly the elderly segment of the global population. Factors implicated in the pathogenesis of AD include reduced level of the neurotransmitter acetylcholine, oxidative stress, aggregation of amyloid β peptide, and tau protein, and transition metal action (Amadoruge and Barnham 2011; Butterfield et al 2007; Zhao and Zhao 2013). Based on the above mentioned evidences, scientists are aiming at finding therapeutic strategies to manage this debilitating condition. On the other hand, the prevalence of diabetes is on a sharp rise, irrespective of age, affecting younger

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adults and even children. Panoply of risk factors have been identified such as drastic rise in level of obesity, sedentary lifestyle including low levels of physical activity and familial cases of diabetes (Mutie et al 2017).

Plants have a long and well-known history in the treatment of various human ailments. The diversity of phytoconstituents which exhibit wide spectrum of pharmacological activities, make them ideal candidate for the discovery of novel lead compounds (Jambocus et al 2017). The *Colutea* genus comprises of about 28 species, forming part of the Fabaceae family (Peşin Süntar et al 2011). These deciduous flowering plants are native to southwest Asia, North Africa, and Southern Europe. *Colutea cilicica* is chiefly cultivated as an ornamental plant for its attractive yellow flowers and papery like inflated pods containing the seeds (Davis 1997). In Turkey *C. cilicica* fruits are used to treat abscesses, wounds, and inflammation (Sezik et al 2001). *C. cilicica* is used against helminthiasis and hypertension in Iraq (Molan et al 2012). D-pinitol isolated from *C. cilicica* was found to significantly reduce inflammation (Eser et al 2017).

Yet, there is a lack of scientific information regarding the potential of *C. cilicica* in the management of AD. Thus in the present study, we aimed at assessing the possible inhibitory function of *C. cilicica* extracts on enzymes targeted in the management of AD, namely cholinesterases. Additionally, we studied the possible inhibitory action of *C. cilicica* extracts on enzymes related to diabetes, a condition which has been associated to AD. Finally, using a set of *in vitro* assays we determined the antioxidant potential of *C. cilicica* extracts. This study was carried out in an endeavor to provide baseline data on the biological properties of a traditionally used medicinal plant for further studies in an attempt of discovering new lead molecules to manage global health problems.

MATERIAL AND METHODS

Plant Material and extraction procedure

Aerial parts of *Colutea cilicica* L. (Soguksu national park, Kızılcahamam, Ankara) was collected during summer 2014 and allowed to air dry at the room temperature. Taxonomic identification was carried out by Dr. Murad Aydın Sanda, senior taxonomist of the Department of Biology, Selçuk University, Turkey.

To produce solvent extracts, the air-dried samples (5 g) were macerated with 100 mL of ethyl acetate and methanol at room temperature for 24 h. Further, the extracts were concentrated under vacuum at 40 °C by using a rotary evaporator. To obtain water extracts, the powdered samples were boiled with 100 mL distilled water for 20 min. The water extract was lyophilized (-80°C, 48 h). All samples were stored at +4°C in dark until use.

Total phenolics and flavonoids content

The total phenolics content was determined by Folin-Ciocalteu method (Slinkard and Singleton 1977). Sample solution (0.25

mL) was mixed with diluted Folin-Ciocalteu reagent (1 mL, 1:9, v/v) and shaken vigorously. After 3 min, Na₂CO₃ solution (0.75 mL, 1%) was added and the sample absorbance was read at 760 nm after a 2 h incubation at room temperature. The results were expressed as gallic acid equivalents (mg GAE/g extract).

For total flavonoid content: sample solution (1 mL) was mixed with one milliliter of aluminum trichloride (2%) in methanol. Blanks for each extracts were prepared by adding sample solution (1 mL) to methanol (1 mL) without AlCl₃. The sample and blank absorbances were read at 415 nm after a 10 min incubation at room temperature. The results were evaluated as milligrams of rutin equivalents (mg RE/g extract) (Zengin et al 2016).

Biological activities evaluation

Antioxidant capacity and enzyme inhibitory effects of *C. cilicica* extracts were detected for biological abilities. The assays were performed as described by our previous study (Grochowski et al. 2017), and they are summarized in the below.

Antioxidant assays

DPPH scavenging activity

After combining 1.0 mL of extract solution with 4 ml of DPPH (0.267 mM), samples were incubated for 30 minutes in room temperature in darkness. Afterwards, absorbance of samples were measured at 517 nm. Results were calculated as milligrams of trolox equivalents per gram of dry extract (TEs/g).

ABTS radical cation scavenging activity

Formation of ABTS⁺ radical cation is an effect of incubation in darkness in room temperature mixture of 7 mM ABTS with 2.45 mM potassium persulfate. Prepared solution was diluted with methanol until its absorbance reached 0.700 ± 0.02 at 734 nm. 1mL of extract solutions were combined with previously prepared 2 mL of ABTS⁺ solution and after 30 min of incubation, absorbance at 734 nm was measured. Results were expressed as milligrams of trolox equivalents per gram of dry extract.

Phosphomolybdenum method

0.3 mL of tested extract solutions were added to reagent mixture, containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and after 90 min incubation in 95 °C absorbances were read in 695 nm against blank sample (0.3 mL methanol with 3 mL reagent mixture). Millimoles of trolox per gram of dry extract were the measurement unit.

B-carotene/linoleic acid method

A stock solution of β-carotene-linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in chloroform (1 mL). 25 µL linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 mL of oxygenated distilled water was added with vigorous shaking; 1.5 mL of this reaction mixture

was dispersed to test tubes and sample solution (0.50 mL, 2 mg/mL) were added and the emulsion system was incubated for up to 2 h at 50°C. The same procedure was repeated with the standard (Butylated hydroxytoluene (BHT)) and a blank. After this incubation period, the sample absorbance was read at 490 nm. Measurement of absorbance was continued until the color of β -carotene disappeared. The bleaching rate (R) of β -carotene was calculated according to Eq. (1).

$$R = \left[\frac{\ln(a/b)}{t} \right] \quad (1)$$

Where, ln=natural log, a=absorbance at time 0, b=absorbance at time t (30, 60, 90, 120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using Eq. (2).

$$AA = \left[\frac{(R_{\text{Control}} - R_{\text{Sample}})}{R_{\text{Control}}} \right] \times 100, \quad (2)$$

Cupric ion reducing (CUPRAC) method

Extract solutions (0.5 mL) were added to reaction mixture [CuCl₂ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM), NH₄Ac buffer (1 mL, 1 M, pH 7.0)] and the absorbance was recorded at 450 nm after 30 min of incubation at room temperature. Similarly, a blank sample (prepared in the same manner but without the extract) was prepared and analysed according to this procedure. Milligrams of trolox equivalents per gram of dry extract (TEs/g extract) were the measurement unit.

Ferric reducing antioxidant power (FRAP) method

Extract solution (0.1 mL) was added to reagent (2 mL) in acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (10 mM) in 40 mM HCl and ferric chloride (20 mM) in a final ratio of 10:1:1 (v/v/v). Then, the absorbance at 593 nm was read after 30 min of incubation at room temperature. Similarly, a blank sample (prepared in the same manner but without the extract) was prepared. Milligrams of trolox equivalents per gram of dry extract (TEs/g extract) were the measurement unit.

Metal chelating activity on ferrous ions

Extract solution (2.0 mL) was added to FeCl₂ (0.05 mL, 2 mM), and the reaction was started using 0.2 mL of 5 mM ferrozine. Similarly, a blank sample for each sample (prepared in the same manner but without ferrozine) was prepared, and all the absorbances were recorded after 10 min of incubation (room temperature) at 562 nm. Milligrams of EDTA equivalents per gram of dry extract (EDTAEs/g extract) were the measurement unit.

Enzyme inhibitory assays

Cholinesterase inhibition

After 15 min of incubation at 25 °C the reaction mixture composed by the extract solution (50 mL), DTNB (3 mM 125 mL) and enzyme solution (0.265 u/mL AChE or 0.026 u/mL BChE) solution (25 mL) in Tris-HCl buffer (pH 8.0) was added to the substrates [acetylthiocholineiodide (15 mM ATCI) or butyrylthiocholine chloride (1.5 mM BTCl, 25 mL)]. Likewise,

a blank sample (prepared in the same manner but without the extract) was prepared and all the absorbances were recorded at 405 nm after 15 min. Milligrams of galantamine equivalents per gram of dry extract (GALAEs/g extract) were the measurement unit.

α -Amylase inhibition

After 10 min of incubation at 37 °C the reaction mixture comprising the extract solution (25 mL), α -amylase solution (10 u/mL, 50 mL) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) was added to the starch solution (50 mL, 0.05%). The reaction was stopped with the addition of HCl (25 mL, 1 M), and then the iodine-potassium iodide solution was added (100 mL). Likewise, a blank sample (prepared in the same manner but without the extract) was prepared, and all the absorbances were recorded at 630 nm after 10 min of incubation at 37 °C. Millimoles of acarbose equivalents per gram of dry extract (ACAEs/g extract) were the measurement unit.

α -Glucosidase inhibition

After 15 min of incubation at 37 °C the reaction between the extract solution (50 mL) glutathione (0.5 mg/mL, 50 mL), α -glucosidase solution (0.2 u/mL 50 mL) in phosphate buffer (pH 6.8) and PNPg (10 mM, 50 mL) was stopped with sodium carbonate (50 mL, 0.2 M). Likewise, a blank sample (prepared in the same manner but without the extract) was prepared, and all the absorbances were recorded at 400 nm after 15 min of incubation at 37 °C. Millimoles of acarbose equivalents per gram of dry extract (ACAEs/g extract) were the measurement unit.

Tyrosinase inhibition

Extract solution (25 mL) was added to a tyrosinase solution (200 u/mL, 40 mL) and phosphate buffer (40 mM, 100 mL, pH 6.8) in a 96-well microplate and then incubated for 15 min at 25 °C. The reaction was started using L-DOPA (10 mM, 40 mL), and after 10 min of incubation at room temperature all the absorbances were recorded at 492 nm. Similarly, a blank sample (prepared in the same manner but without the extract) was prepared and analysed according to this procedure. Milligrams of kojic acid equivalents per gram of dry extract (KAE/g extract) were the measurement unit.

Statistical Analysis

All the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference post hoc test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 14.0 program.

RESULTS and DISCUSSION

Enzyme inhibitory effects

Maintaining the level of acetylcholine in the brain by inhibiting cholinesterase enzymes, is an important strategy for treating AD (Shariffar et al 2012). Cholinesterases namely, acetyl and

butyryl cholinesterase, terminate neuronal transmission by hydrolyzing acetylcholine, the main neurotransmitter involved in the cholinergic system (Samaradivakara et al 2016). Currently used medication to alleviate AD symptoms carry numerous side effects such as nausea, vomiting, diarrhea, muscle cramps, fatigue, weight loss, confusion, constipation, and dizziness (NI 2017). The need for novel molecules deprived of side effects is of utmost importance. Galantamine used in the treatment of mild to moderate AD, is an alkaloid which inhibits acetyl cholinesterase enzyme (Olin and Schneider 2002). With regards to the therapeutic potential of plants, we have studied the cholinesterase inhibition capacity of the ethyl acetate, methanol, and water extracts of *C. cilicica* using *in vitro* methods. In the present study, the methanol extract of *C. cilicica* actively inhibited AChE and BChE (1.33 and 0.68 mg GALAE/g extract, respectively), compared to a lower activity for ethyl acetate extract (0.99 and 0.09 GALAE/g extract, for respective enzymes) while no activity was recorded for the water extract (Table 1). The observed inhibitory action of *C. cilicica* methanol extract on cholinesterase enzymes might be ascribed to other phytochemicals such as alkaloids, terpenes, and sterols as previously described by Ahmed et al (2013). D-pinitol identified in the leaves of *C. cilicica* (Eser et al., 2017) was reported to interfere with the accumulation of beta amyloid, involved in Alzheimer's disease (National Library of Medicine, 2017).

Although, tyrosinase was chiefly associated to melanin synthesis and skin hyperpigmentation therapy, evidences highlight that this copper-containing enzyme is linked to Parkinson's disease (PD), the second most common neurodegenerative disorder after AD (Neagu et al 2015). PD is a chronic, progressive movement disorder which affects 6.3 million people worldwide (Coomber et al 2017). PD involves the malfunctioning and apoptosis to neurons in the substantia nigra, which controls movement and coordination (Ellis and Fell 2017). Histological data highlight the presence of neuromelanin in the substantia nigra. Indeed, the dual protective and toxic function of neuromelanin have been reported (Zucca et al 2017). Evidences of the possible role of tyrosinase in the biosynthesis of neuromelanin make its modulation a popular target for the treatment of PD (Cespedes et al 2017). From Table 1 it was observed that *C. cilicica* extracts possessed variable degree of inhibition against tyrosinase; ethyl acetate extract (54.70 mgKAE/g extract) being the most

active and water extract being the least active (42.58 mgKAE/g extract). The observed difference might be associated to the different phytochemical composition of the different extracts (Chigayo et al 2016;Thouri et al 2017).

Magnetic resonance imaging of the brain of elderly suffering from diabetes showed a decrease in the hippocampus size, resulting from reduced neurogenesis and enhanced neuronal death (Pugazhenthil et al 2017). In fact, epidemiological data have revealed that elevated glucose level, the hallmark of diabetes, increases the risk of developing dementia and prompts the exacerbation of mild cognitive impairment of AD. Hyperglycemia was reported to increase amyloid- β levels in the brain by altering neuronal activity via K_{ATP} channels (Macauley et al 2015). Epidemiological data also suggest that insulin resistance impaired glucose absorption by neurons, impairing neuronal transmission and cognition (Kandimalla et al 2017). Thus, controlling glucose level in elderly diabetic patients might prevent the onset and/or worsening of cognitive impairments. Alpha-Amylase and α -glucosidase are two enzymes which have been extensively studied for the management of diabetes. α -Amylase is responsible for the hydrolysis of polysaccharides at the early stage of digestion, while α -glucosidase cleaves disaccharides, producing glucose which is absorbed by the enterocytes of the intestinal villi (Zhang et al 2017). From Table 1, it was observed that *C. cilicica* extracts possessed higher α -glucosidase (2.71-1.23 mmol ACAE/g extract inhibitory potential compared to α -amylase (0.57-0.12 mmol ACAE/g extract). The order of inhibition against α -amylase was as follows ethyl acetate>methanol>water. The same trend was observed against α -glucosidase. From Table 2, it was noted that the ethyl acetate extract (24.77 mgGAE/g extract) of *C. cilicica* contained the highest amount of phenolic. Indeed, Ademiluyi and Oboh (2013) and Jiang et al (2017) also reported the potent inhibitory effect of phenolic rich extracts on α -amylase and α -glucosidase.

Antioxidant properties

Oxidative stress is known to play a key role in AD. Oxidative stress refers to the increased production of reactive oxygen species accompanied by the reduced efficiency of the innate antioxidant defense mechanism (Tramutola et al 2017). Reactive oxygen species participate in cellular signaling pathways *in vivo*, but overproduction induces oxidation of membrane lipids, inactivate enzymes and normal cellular

Table 1. Enzyme inhibitory effects of *C. cilicica* extracts.

Extracts	AChE inhibition (mgGALAE/g extract)	BChE inhibition (mgGALAE/g extract)	Tyrosinase (mgKAE/g extract)	α -amylase (mmolACAE/g extract)	α -glucosidase (mmolACAE/g extract)
Ethyl acetate	0.99±0.01*	0.09±0.01	54.7±2.28	0.57±0.076	2.71±0.05
Methanol	1.33±0.03	0.68±0.02	52.28±0.38	0.38±0.03	2.60±0.09
Water	na	na	42.58±0.72	0.12±0.01	1.23±0.10

* Values expressed are means ± S.D. of three parallel measurements. AChE: acetylcholinesterase; BChE: butyrylcholinesterase; GALAE: galantamine equivalents; ACAE: acarbose equivalents; KAE: kojic acid equivalents; na: not active.

Table 2. Total phenolic and flavonoid contents and total antioxidant, and metal chelating abilities of *C. cilicica* extracts

Extracts	TPC (mgGAE/g extract)	TFC (mgRE/g extract)	DPPH scavenging (mgTE/g extract)	ABTS scavenging (mgTE/g extract)	FRAP (mgTE/g extract)	CUPRAC (mgTE/g extract)	β -carotene/ linoleic acid assay (inhibition [%] at 2 mg/ml concentration)	Phospho- molybdenum (mmolTE/g extract)	Metal chelating activity (mgEDTAE/g extract)
Ethyl acetate	24.77 \pm 1.88*	2.78 \pm 0.21	34.26 \pm 1.09*	22.67 \pm 0.34	32.00 \pm 1.92	56.21 \pm 4.25	91.96 \pm 0.20	1.88 \pm 0.01	6.78 \pm 0.03
Methanol	17.82 \pm 1.87	2.83 \pm 0.10	12.89 \pm 2.75	16.22 \pm 1.25	21.36 \pm 1.26	49.97 \pm 1.99	91.44 \pm 0.50	1.61 \pm 0.01	0.65 \pm 0.01
Water	20.25 \pm 0.73	17.26 \pm 0.16	42.46 \pm 0.08	57.70 \pm 1.22	58.48 \pm 0.34	74.68 \pm 0.66	86.12 \pm 0.40	0.91 \pm 0.04	2.72 \pm 0.02
BHT	nt	nt	nt	nt	nt	nt	91.70 \pm 0.39	nt	nt

* Values expressed are means \pm S.D. of three parallel measurements. TPC: Total phenolic content; TFC: Total flavonoid content; GAE: gallic acid equivalents; RE: rutin equivalents; TE: trolox equivalents; EDTAE: EDTA equivalents; nt: no tested.

functioning, damage proteins and DNA, thus contributing to AD (García-Blanco et al 2017). In the present study, we assessed the antioxidant potential of *C. cilicica* extracts by using a set of antioxidant assays. Free radicals are unsw molecules containing one or more unpaired electrons, making them highly reactive (Poprac et al 2017). ABTS and DPPH radicals have been extensively used *in vitro* to provide an insight of the radical scavenging capacity of antioxidant molecules. The water extract of *C. cilicica* showed potent radical scavenging capacity against DPPH and ABTS (42.46 and 57.70 mgTE/g extract). Phytochemical determination showed that *C. cilicica* water extract (17.26 mg RE/g extract) was rich in flavonoids as compared to ethyl acetate and methanol extracts (2.78 and 2.8326 mg RE/g extract, for respective extracts). This finding is in accordance with the statements of Pizzino et al (2017) and Kumar and Pandey (2013). The FRAP and CUPRAC assays were employed to evaluate the reducing potential of *C. cilicica* extracts. The redox potential of phytochemicals makes them potent reducing agents (Al-Rimawi et al 2016). As noted from Table 2, water extract of *C. cilicica* was a potential reducing agent (58.48 and 74.68 mg TE/g extract, for FRAP and CUPRAC assays respectively). This extract contained the highest amount of flavonoids, which was previously reported to act as a potent reducing agent (Ghasemzadeh and Ghasemzadeh 2011). β -carotene/linoleic acid and phosphomolybdenum assays are also currently used to provide an insight of the oxidant scavenging potential of plant phytochemicals. From Table 2, it was observed that the extracts of *C. cilicica* (value ranging from 91.96 to 89.12% inhibition as compared to 91.70% inhibition for BHT) prevented β -carotene discoloration induced by linoleic acid oxidation in the following order ethyl acetate>methanol>water. The same trend was observed for the phosphomolybdenum assay.

Metal ions such as copper, zinc, and iron, are involved in neuron signaling, apoptosis, cell proliferation, inflammation, and oxidative stress control (Kepp 2017). Disruption of the homeostasis of metal ions was associated to amyloid- β deposits and tau phosphorylation, resulting in the formation of neurofibrillary tangles and senile plaque formation, which

fuel AD (Wang and Wang 2017). Finding molecules possessing metal chelating abilities might serve in the management of AD. From the present study ethyl acetate extract (6.78 mg EDTAE/g extract) of *C. cilicica* showed the most potent metal chelating activity followed by water extract (2.72 mg EDTAE/g extract) and methanol extract (0.65 mg EDTAE/g extract). Total phenolic content determination followed the same trend (Table 2), suggesting that phenolic composition of the extracts obtained from solvents of different polarities affected the observed chelating potential.

CONCLUSION

AD is a complex, multifactorial disorder affecting the elderly segment of the world's population. While existing treatments are palliative and offer no improvement of the disease conditions, the major concern is the discovery of new effective agents. This study provides an insight on the potential of *C. cilicica* in the management of AD. *C. cilicica* acted as cholinesterase inhibitor. *C. cilicica* was also found to modulate the action of other enzymes namely α -amylase and α -glucosidase, which are the main targets of glycaemic control. Indeed, chronic dyshomeostasis of blood glucose level was linked to AD. Data collected from the present study showed that *C. cilicica* water extract was rich in flavonoids and possessed potent reducing potential. *C. cilicica* previously reported to be used in traditional medicine, might also be used as a natural source of antioxidants. Therefore, *C. cilicica* merits further investigation as it is a source of valuable multi-target compounds for the management of AD.

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