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Neuropharmacological evaluation of selected Jordanian traditional herbal medicines

Sawsan Abuhamdah[1]*, Rushdie Abuhamdah[2], Ashok Shakya[3], Suleiman M. Al-Olimat and Paul Chazot[2]

[1]Department of Biopharmaceutics and Clinical Pharmacy, Faculty of Pharmacy, University of Jordan, Amman, Jordan.
[2]School of Biological and Biomedical Sciences, Durham University, Durham, United Kingdom.
[3]Faculty of Pharmacy and Medical Sciences, Al-Ahliyya Amman University, Amman, Jordan.
[4]Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Jordan, Amman, Jordan.

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Numerous medicinal plants have been described in traditional medicine for treatment of dementias, including Alzheimer's disease (AD). In this study, some of these plants were evaluated in three different types of pharmacological bioassays related to AD pathology to explore the possible mechanisms underpinning their traditional use. Six selected plants were extracted with ethanol and screened *in vitro* for acetylcholinesterase (AChE) and cycloxygenase-1 (COX-1) enzyme inhibitory activities; in addition, a range of anti-oxidant activities were evaluated. Of the tested plant extracts, *Aloysia citrodora* and *Peganum harmala* root and seeds showed inhibitory effect on AChE (IC_{50} 68, 100 and 93 µg/ml, respectively). Moreover, *A. citrodora* appeared to interact reversibly with the enzyme, while *P. harmala* appeared to show irreversible inhibition. *Asphodelus microcarpus, Inula viscosa* and *A. citrodora* displayed COX-1 enzyme inhibitory activity (IC_{50} 34.9, 3.4 and 3.2 µg/ml, respectively). DPPH radical scavenging activity was demonstrated by all tested plants. Two extracts in particular (*Arbutus andrachne* and *A. microcarpus*) exhibited potent nitric oxide (NO) scavenging activity (IC_{50} 4.5 and 5.0 ug/ml, respectively). Four extracts *A. citriodora*, *P. harmala* (Root) and (seed) and *A. microcarpus* exhibited strong metal chelating ability (IC_{50} 4.5, 6.2, 6.5 and 6.7 µg/ml, respectively). The modest reversible interaction of *A. citrodora* with AChE, potent COX-1 inhibitory and antioxidant activity, and strong metal chelating ability make this plant a promising candidate for future development in the treatment of AD, either as a whole extract or as individual bioactive constituents. *A. andrachne* and *A. microcarpus* extracts should be further evaluated since they exhibited promising NO scavenging activities.

Key words: Anti-acetylcholinesterase, anti-inflammatory, anti-oxidant, metal chelating ability, Jordanian medicinal plants, Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is the most common human neurodegenerative disorder and is characterized by a progressive decline of memory and cognition together with a range of psychological disturbances. AD arises as

*Corresponding author. E-mail: smaabuhamdah@gmail.com, s.abuhamdah@ju.edu.jo. Tel: +962 65355000/23358. Fax: +962 65300250.
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Table 1. List of plants used in the current study and respective traditional uses related to CNS.

Botanical name	Traditional uses related to the brain
P. argentea L.	Stimulant
I. viscosa L.	Anti-inflammatory
A. andrachne L.	Antioxidant and neuroprotective
A. microcarpus Salzm et Vivi	Anti-inflammatory agent
P. harmala L.	Analgesic, anti-inflammatory agent. Harmaline, an active ingredient in *P. harmala*, is a central nervous system stimulant and a reversible inhibitor of MAOA, a category of antidepressant.
A. citriodora Palau	Anxiety, depression and psychological treatments

result of progressive malfunction of different biochemical pathways, early-on characterised by reduced acetylcholine (ACh) levels, and later excessive transition metals, and oxidative stress, with pathological hallmarks including aggregated amyloid-β-peptide and hyperphosphorylated tau protein (Bolognesi et al., 2009; Jones et al., 2006).

Acetylcholinesterase (AChE), the predominant cholinesterase in the brain, hydrolyzes ACh to choline and acetate, thereby terminating the effect of this neurotransmitter within cholinergic synapses. Many natural products have already proven to be useful AChE inhibitors (Murray et al., 2013; Nordberg et al., 1998). The currently approved drugs, galantamine and rivastigmine are plant derived alkaloids which offer early symptomatic relief for AD (Bierer et al., 2002). However, there is a paucity of effective treatments for slowing or halting the progression of AD.

Inflammation is a common component seen in the latter stages within neurodegenerative diseases, including AD. Prostaglandins are widely distributed in the body, and have a primary role in inflammation, and their biosynthesis has been implicated in the pathophysiology of AD (Lipsky, 1999). Metal ion homeostasis is vital for normal biological function. Homeostatic dysfunction can lead to cellular oxidative stress, through release and action of free radicals, which are highly reactive and unstable molecules. Uncontrolled release of free radicals in neurons can elicit cellular damage and result in neurotoxicity (Mariani et al., 2005; Pham-Huy et al., 2008; Willcox et al., 2004; Greenough et al., 2013; Mot et al., 2011). Antioxidant therapy has proven to have modest success in improving cognitive function and behavioural deficits in patients with mild to moderate AD (Maxwell, 1995). This has led to growing interest in evaluating potential new anti-oxidant phytochemicals (Halliwell et al., 1995). Plant products with high flavonoid and phenolic components appear to be the most promising (Mukherjee et al., 2009).

The plants of Jordan have proved a rich source of traditional medicines for many years, including for those targeting the central nervous system (Al-Quran, 2009; Abu Irmaileh and Afifi, 2000; 2008). However, many have not been pharmacological characterized to validate their optimal clinical use. In this study, selected plants were evaluated using three different types of bioassays related to the major symptoms and pathology pertinent in AD. Six selected plants (*Paronychia argentea* Lam., *Inula viscosa* L., *Arbutus andrachne* L, *Asphodelus microcarpus* Salzm et Vivi, *Peganum harmala* L and *Aloysia citriodora* Palau) were extracted with ethanol and screened *in vitro* for acetylcholinesterase (AChE), cycloxygenase-1 (COX-1) enzymes and anti-oxidant activity.

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), acetylthiocholine iodide (ATCI), AChE from electric eel (type VI-S lyophilized powder), bovine serum albumin (BSA), 5, 5-dithiobis [2-nitrobenzoic acid] (DTNB), galantamine, gallic acid and ascorbic acid were purchased from Sigma Aldrich. All other reagents used were of analytical grade.

Plant and preparation of ethanolic extracts

The source and identity verification of the plants selected for the study and extract preparation were recently described in Abuhamdah et al. (2013). Plants list and traditional use related to the brain are described as shown in Table 1.

Estimation of total polyphenolic content

Total phenolic compound contents were determined using the Folin-Ciocalteau method (Adsersen, 2005). In brief, the extract samples were mixed with Folin-Ciocalteu reagent for 5 min and aqueous Na_2CO_3 (1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetric method at 765 nm using UV-VIS spectrophotometer from SpectroScan 80D (Biotech Engineering Management Co. Ltd., UK). The standard curve was prepared by 0 to 250 mg/ml solutions of gallic acid in methanol:water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg/g of dry mass).

Yield and total polyphenol content of ethanolic extract of various plants parts are shown in Table 3.

AChE inhibitory activity assay

AChE activity was measured using the TLC bioautographic method (Orhan et al., 2008; Ellman et al., 1961). 50 mM Tris–HCl pH 8.0and microplate assay were used as a buffer throughout the experiment unless otherwise stated. In the 96-well plates, 100 ml of 3 mM DTNB, 20 ml of 0.26 U/ml of AChE, and 40 ml of buffer (50 mM Tris, pH 8.0), 20 ml of each extract in various concentrations (25, 50, 100, 250 and 500 ug/ml) dissolved in buffer containing not more than 10% ethanol were added to the wells. After mixing, the plate was incubated for 15 min (25°C) and then the absorbance was measured at 412 nm using a microplate reader (Bioteck, USA). The reaction was initiated by the addition of 20 ml of 15 mM ATCI and the hydrolysis of acetylthiocholine was monitored. Galantamine was used as positive control, and all the reactions were performed in triplicate.

Cyclooxygenase-1 assay

Determination of the inhibition of prostaglandin biosynthesis by the plant extracts was performed using a cyclooxygenase-1 assay, essentially as described in Jager (1996), where indomethacin was utilized as a standard.

DPPH radical scavenging activity

The stable DPPH radical scavenging activity was determined as described by Nabavi et al. (2009). Different concentrations of each extracts and reference were added, at an equal volume, to methanolic solution of DPPH (0.002%). After 30 min at room temperature, the absorbance was measured at 517 nm. BHA was employed as the reference.

Nitric oxide radical scavenging activity

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution reacts with oxygen to produce nitrite ions, which can be quantified by the Griess Illosvoy reaction (Prakash, 2010). In brief, the reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various doses (0 to 500 µg/ml) of the test solution in a final volume of 3 ml. After incubation for 150 min at 25°C, after the incubation period, 0.5 ml of Griess reagent was added. The product generated during diazotization of nitrite ions was measured spectrophotometrically at 546 nm versus a blank sample. All tests were performed in triplicate. Curcumin was used as a reference compound.

Ferrous ion-chelating effect

The ferrous ion-chelating effect of the samples was estimated by the method of Chua et al. (2008). Briefly, 740 µl of methanol and the samples were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 5 mM ferrozine into the mixture and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. Na_2 EDTA was used as a positive control for this assay.

Statistical analysis

The values are presented as mean ± SEM for three independent experiments (n=3). IC_{50} values were calculated via non-linear regression analysis sigmoidal fitting with variable slope using GraphPad Prism v. 5.0 (GraphPad software Inc., USA).

RESULTS AND DISCUSSION

Acetylcholinesterase inhibitory activity of the extracts

The inhibitory effect of the ethanolic extracts from the six different plants on AChE activity were evaluated using the TLC bioautographic method and microplate for active plant extracts. The screening was performed spanning a concentration range of 0 to 500 µg/ml, and the extract considered active only if they inhibited the enzyme more than 50%. The AChE inhibitory activities are shown in Table 2. Only two species, namely, *A. citrodora* and *P. harmala* root and seeds inhibited AChE effectively, by 90, 70 and 85%, respectively (Figures 1and 2) with IC_{50} values of 68, 100 and 94 µg/ml, respectively. Galantamine was used as the standard AChE inhibitor in this study and displayed an IC_{50} of 9.4 µg/ml. The inhibition type of *A. citrodora and P. harmala* was determined by assaying the change in the remaining AChE activity of the mixture of AChE and the plant extract before and after the dilution of the plant extract in the same mixture, while AChE activity increased 5-fold using 10-fold dilution of *A. citrodora*, the same dilution of *P. harmala* did not show any effect on the remaining activity of AChE after dilution. This result indicates that AChE is inhibited reversibly by *A. citrodora* and irreversibly by *P. harmala* (Cao et al., 2007; Herraiz et al., 2010; Sobhani et al., 2002).

In this study, the major biological activity demonstrated by both extracts could be attributed to the major constituents in each extracts. Little is known about *A. citrodora* active principles, except the presence of a flavonoid, luteolin-7-diglucuronide (Carnat et al., 1995; Skaltsa and Shammas, 1988), the phenolic compound verbascoside and the composition of the essential oil (from our unpublished studies and by Carnat at al. (1999)); these may be responsible for the biological activity of plant and need further investigation. The major constituents in *P. harmala* are the β-carbolines indole alkaloids. These possess diverse neuropharmacological activities (sedative, hypnotic, anxiolytic, anticonvulsant) and can interact with several enzymes and neurotransmitter systems, including topoisomerase I and monoamine oxidase-A. The activities demonstrated by both seeds and roots of the *P. harmala* plant for AChE inhibition could be attributed to the major common active constituent harmaline. The inhibition type in the study indicates that *A. citrodora* reversibly inhibits AChE and could therefore be used as a potential symptomatic AD medication, rather than *P. harmala* which appears to inhibit irreversibly AChE. This recommendation also is supported by the toxicity reports in literature. Harmaline is a toxic alkaloid and in moderate doses causes tremor and clonic convulsions (Mahmoodian and Salehian, 2002). *A. citrodora* displays a good safety profile for most people when consumed as a herbal tea, and no literature to date has reported serious adverse effects. According

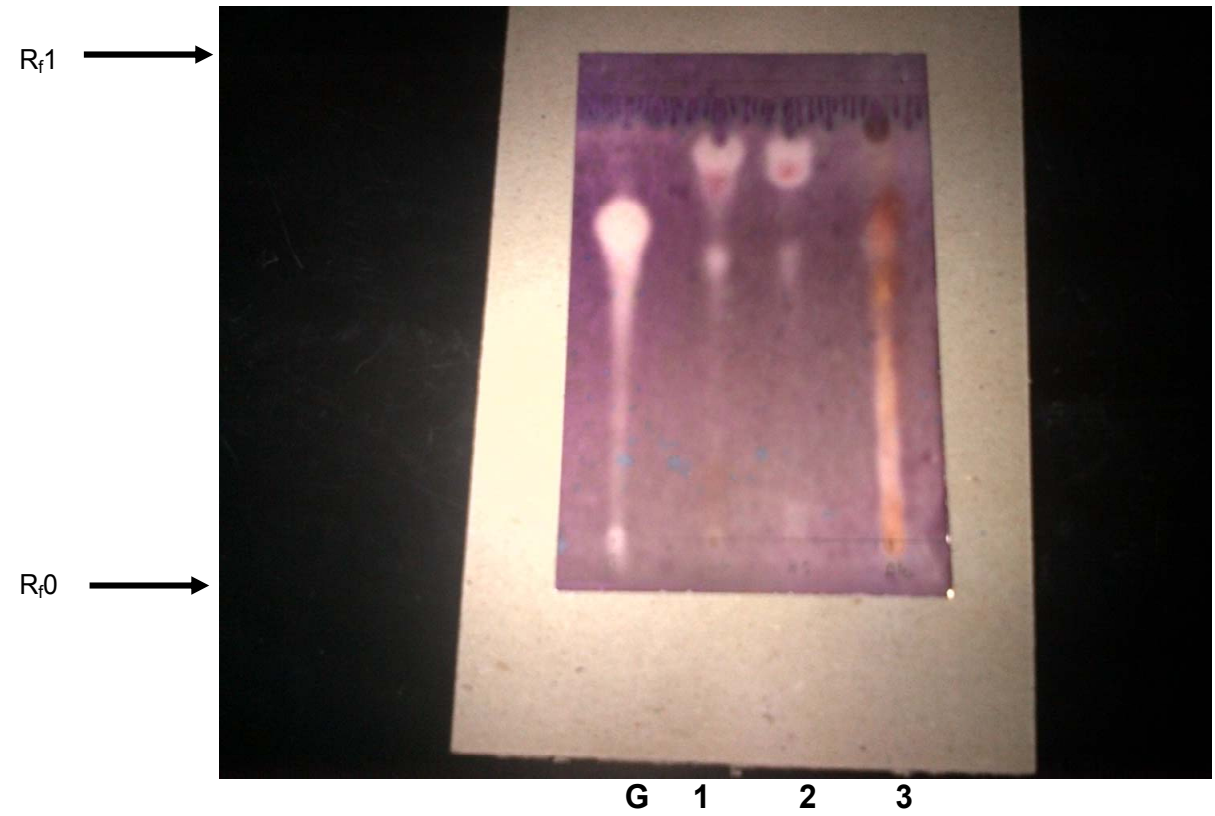

Figure 1. Bioautograph showing inhibition of acetylcholinesterase activity by Lane 1, Standard Galantamine; Lane 2, Harmal Rootl Lane 3, Harmal seed; Lane 4, Aloysia. The assay was carried out using a silica gel G60 F_{254} which had been eluted with chloroform: methanol (80:20).

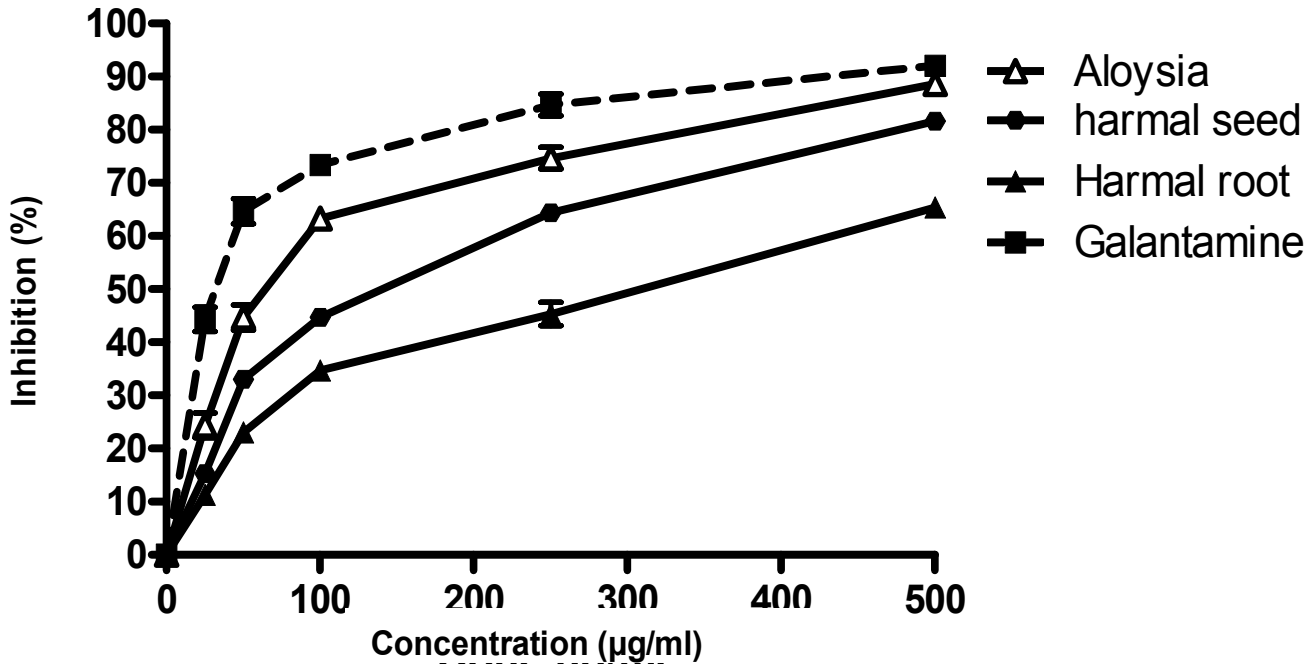

Figure 2. Inhibition of AChE activity by plant extracts: *A. citrodora*, Harmal seed, harmal root and standard galantamine. Mean (± SEM) values of three independent experiments have been plotted.

Table 2. Percent inhibition and IC_{50} values of ethanolic plant extracts biological activities using different bioassays related to AD.

Plant name	Efficacy percent inhibition					IC_{50} (µg/ml)				
	AChE[a] (%)	Cox-1[b] (%)	DPPH[b] (%)	NO[b] (%)	Fe^{2+} assay[b](%)	AChE	COX-1	DPPH	NO	Fe^{2+} assay
P. argentea	N.D	N.D	100	N.D	N.D	-	-	15.6 ± 3	-	-
I. viscosa	N.D	80	100	N.D	N.D	-	3.4	11.7 ± 2	-	-
A. andrachne	N.D	N.D	100	90	N.D	-	-	4.9 ± 0.5	4.5 ± 0.4	-
A. microcarpus	N.D	95	70	90	44	-	3.6	61± 3	5.0 ± 0.3	6.7± 0.7
P. harmala (Root)	70	N.D	80	N.D	63	93.7	-	62.5 ± 2	-	6.2 ± 0.5
P. harmala (Seed)	85	N.D	80	N.D	50	100.1	-	15.6 ± 1	-	6.5 ± 0.2
A. citriodora	90	83	100	N.D	71	68.7	6.2	4.3 ± 0.4	-	4.5 ± 0.3

[a]0-500 ug/ml, [b]100 ug/ml, N.D not detected, Results are mean ± SD (n=3). AChE inhibition: Galantamine IC_{50} = 9.4 µg ml^{-}; COX-1inhibition: Indomethacin: IC_{50} = 0.63 µg ml^{-1}; DPPH radical scavenging: BHA: IC_{50}= 3.9 µg ml^{-1}; Nitric oxide radical scavenging: curcumin: IC_{50}= 4.0 µg ml^{-1}; Fe^{2+} chelating:Quecertin: IC_{50} = 4.5 µg ml^{-1}.

the plant extracts which demonstrated potent antioxidant properties are expected to play a role in reducing oxidative stress and this may explain their use in traditional medicine for symptom improvement in AD and/or ageing related diseases.

DPPH radical scavenging activity

It was found that the DPPH radical-scavenging activities of all the extracts increased with increasing concen-tration, and most exhibited effective (approximately 100%) free DPPH scavenging activity at the tested con-centrations. The IC_{50} values for DPPH radical-scavenging activity are reported as shown in Table 2. The most effective free radicals scavenging activity was obtained with *I. viscose* 100%, *A. andrachne* 100%, *A. citriodora* 100%, *P. argentea* 100%, while *P. harmala* (Root) 80%, *P. harmala* (seed) 80% and *A. microcarpus* 70% compared with reference antioxidant standard BHA.

Nitric oxide-scavenging activity

Nitric oxide (NO) is an essential bioregulatory molecule required for several physiological processes, including neural signal transmission (Prakash, 2010). However, excessive elevation of NO is common in several pathological conditions, including multiple sclerosis, arthritis and AD. The extracts of *A. andrachne* (IC_{50} 4.5 ug/ml) and *A. microcarpus* (IC_{50} 5.0 µg/ml) exhibited respectable NO scavenging activities, and reduced the generation of NO *in vitro* in a concentration dependent manner compared with standard reference curcumin (IC_{50} 4 µg/ml). The other plant extracts were largely ineffective.

Fe^{2+} chelating ability

Divalent transition metal ions play an important role as oxidative processes, leading to the formation of hydroxyl radicals (Chua, 2008). The transition metal iron is capable of generating free radicals from peroxides and may be implicated in many human CNS diseases (Mot et al., 2011). Because Fe^{2+} causes the production of oxyradicals and lipid peroxidation, minimizing its concentration can potentially offer protection against oxidative damage. Therefore, chelating agents can be effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Maxwell, 1995). Four plant extracts investigated herein displayed metal chelating ability with 71% inhibition for *A. citriodora* at concentration of 100 µg/ml being the most effective, followed by *P. harmala* (Root) 63%, *P. harmala* (seed) up to 50% and *A. microcarpus* 44% compared with reference antioxidant standard quercetin, with metal chelating ability up to 67%, as shown in Table 2. IC_{50} for Fe^{+2} chelating ability of these plants are reported in Table 2. All the plant extracts tested exhibited antioxidant activities in all the models studied, but to variable degrees. Extracts which contains large amounts of phenolic compounds, exhibits high antioxidant free radical scavenging activities (Table 3) as well as COX inhibitory activity. This indicates that these plant extract

Table 3. Yield and total polyphenol content of ethanolic extract of various plants parts used in the study.

Botanical name	Yield of ethanolic extract (mg/g)	Total phenol content (mg gallic acid/g)*
P. argentea	11.2	20±2
I. viscosa	23.5	153±5
A. andrachne	15.7	178±4
A. microcarpus	48.9	20±1
P. harmala (Root)	51.4	24±1
P. harmala (Seed)	12.0	27±3
A. citriodora	20.3	67±1

Values are expressed as mean 6 ± SD* (n = 3).

can provide a significant source of natural antioxidant. *A. andrachne* and *A. microcarpus* extract should be further evaluated since they exhibited promising nitric oxide (NO) scavenging activities. Historically, active components from plants have provided important sources of new drugs. Since, neurodegenerative diseases such as Alzheimer's have become an international public health burden, and the currently available drugs lack efficacy and have undesirable side effects; new treatment options based on medicinal plants may provide useful therapeutic alternatives.

Conclusions

The ethanolic extracts of plants used in Jordanian traditional medicine for improving human memory and cognitive function were screened for AChE inhibitor, anti-inflammatory and antioxidant activity. On the basis of the results obtained, *A. citriodora* showed most promise with reversible AChE inhibitory activity, anti-inflammatory activity, and considerable radical scavenging as well as iron chelating properties. Note, a number of these characteristics are shared by the corresponding essential oil (Abuhamdah et al., Unpublished). This encouraging pharmacological profile warrants further investigation. The components responsible for these activities are currently unclear, therefore, further deep phytochemical investigation is needed to isolate and identify the active compounds present in the plant extract. Furthermore, the *in vivo* anti-oxidant activity, the safety, toxicity and bioavailability of this plant extract needs to be assessed prior to clinical use.

Competing interests

All the authors declare that they have no competing interests.

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Abbreviations: AD, Alzheimer's disease; **ACh,** acetylcholine; **AChE,** acetylcholinesterase; **DTNB,** 5,5´-bisdithionitrobenzoic acid; **ATCI,** acetylthiocholine iodide; **DPPH,** diphenyl picryl hydrazine; **BHA,** butylated hydroxyanisole

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