Anticholinesterase, antioxidant, and neuroprotective effects of *Tripleurospermum disciforme* and *Dracocephalum multicaule*

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**INTRODUCTION**

Alzheimer’s disease (AD) is the most common form of dementia in elders worldwide, which can affect memory and the other cognitive functions.[1] The loss of cholinergic synapses has been a consistent finding in AD, so increasing the brain’s level of acetylcholine (Ach) through inhibition of acetylcholinesterase (AChE) has been one of the primary strategies for management of AD.[2] In addition, the role of oxidative stress has been proven in the pathogenesis of AD.[3,4] Neuronal systems appear to be especially sensitive to oxidation. Free radicals formation leads to inflammatory reactions which causes AD development. One of the other pathological features identified in AD is the presence of neurofibrillary tangles, amyloid plaques, and inflammations. Accumulation of amyloid β (Aβ) peptide acts as an inhibitor of certain enzyme functions. The presence of this peptide is the hallmark of the AD pathology.[5-7] There are obstacles in successful treatment of AD such as lack of full effectiveness of the current drugs in treatment of all aspects of AD, high costs, and adverse effects. In earlier studies, we have reported antioxidant and anticholinesterase effect of some medicinal plants.[8-10] There has been growing interest on traditional herbal medicines. Presently, in continuing to focus on the future-promising herbs against AD, anticholinesterase, antioxidant, and protective effect of *Tripleurospermum disciforme* and *Dracocephalum multicaule* against toxicity of Aβ peptide have been studied. These two plants have been used in folk medicine for memory enhancing.[11] *T. disciforme* (C.A. Mey) Schultz Bip.
known as “Babooneh dashti” belongs to Asteraceae family and has similar uses to Matricaria chamomilla. This plant has been used as a popular treatment for sleep disorders, inflammations, carminative, and as a hair color.[12] Anti-ulcer effect of this plant has been reported in mice.[13] D. multicaule Montbr. and Auch. known in Persian as “Palang moshk” has been widely distributed in northwestern of Iran and is from Lamiaceae family.[14] Antioxidant effect of D. moldavica has been reported previously.[15,16] Up to now, it is for the first time that the extracts of T. disciforme and D. multicaule have been studied for anticholinesterase, antioxidant, and protective effect against Aβ-induced toxicity. Primary phytochemical studies were performed for detection of classes of active constituents in tested plants which might be responsible for biological activities of the plants.

**MATERIALS AND METHODS**

**Plant materials**

Flowering tops of T. disciforme and D. multicaule were collected from Bidkhoon, Kerman province and Rasht in Gilan province, respectively in June 2011. The plants were authenticated by a botanist and a voucher specimen was deposited in Herbarium Center, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran (KF1225, KF1238).

**Chemicals**

Acetylthiocholine iodide (ATCI), AChE (EC 3.1.1.7, type VI-S from Electric Eel), and 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich, Switzerland. Tacrine was purchased from Fluka Chemie (Buchs, Switzerland). Other chemicals were from analytical grade.

**Preparation of plant extract and phytochemical screening**

About 200 g of each plant was extracted by maceration method with methanol 80% for 72 h. The extracts were concentrated under vacuum. Dried extracts were stored at −20°C until test. Phytochemical screening of the plants was performed to screen the presence of alkaloids, terpenoids, steroids, saponins, and flavonoids.[17]

**Anticholinesterase tests**

**Bioautographic method for anticholinesterase activity**

Plant extracts were applied on the thin layer chromatography (TLC) plate at concentration of 100 µg/ml and sprayed with 5 mM ATCI and 5 mM DTNB in 50 mM Tris-HCl (pH 8) until saturation of the plate. After 2 min, a solution of 3 U/ml AChE dissolved in 50 mM Tris-HCl, pH 8 was sprayed at 37°C. Appearance of white spots in yellow background indicated the presence of active compounds.[18]

**Ellman based colorimetric study of anticholinesterase activity**

AChE inhibitory effect of plant extracts was evaluated using Ellman method with some modifications.[19] Tacrine was used as positive control. The percentage of inhibition was calculated as following: %I = A_{con} - A_{sam}/A_{con} × 100, where A_{con} is the absorbance of the control and A_{sam} is the absorbance of the tested sample. The IC_{50} was calculated by log-probit analysis.

**Diphenylicrylhydrazil assay**

Assessment of DPPH inhibitory effect of plant extracts was performed using DPPH assay.[20] Butylated hydroxytoluene (BHT) and solvent were used as positive and negative controls, respectively.

**Toxicity on PC12 cells**

PC12 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), penicillin 100 unit, and streptomycin 100 mg and maintained at 37°C in incubator 5% CO2. The cells were cultured at a concentration of 10⁴ cells/well with poly-D-lysine (PDL). After 24 h, medium was replaced by plant extract (0.1-200 µg/ml in phosphate buffered saline (PBS)) and incubated for 24 h. Cell survival was evaluated by MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.

**Neuroprotective effect of plant extracts against Aβ peptide toxicity**

PC12 cells cultured in 96 microwells coated by PDL and treated with different plant extracts at nontoxic concentrations (0.1, 1, 10, 100, and 200 µg/ml). Aβ peptide (2 µM in distilled water) was added to wells and incubated for 24 h. A stock solution of Aβ peptide (1 mmol Aβ peptide/1 ml distilled water) incubated at 37°C for 3 days to make aggregated form. The viability of cells was checked using MTT assay.[21]

**Thioflavine T fluorescence assay**

Fluorometric method was used for determination of Aβ peptide aggregation using ThT. Aggregated Aβ peptide forms a complex with ThT which has fluorescence effect at λ exitation of 450 nm and λ extinction of 482 nm. Inhibition of Aβ peptide aggregation causes a decrease in fluorescence intensity.[22] Briefly a stock solution of lyophilized Aβ (5 mg/mL) was sonicated for 15 min. An equal volume of each extract (200 µg/ml PBS) was added to a 50 µM Aβ in PBS (pH 7.4) and incubated at 37°C for 5 days. A control sample containing Aβ with PBS was carried out in parallel. After incubation at 37°C, 50 µL of Aβ solution was added to 3 mL of ThT solution (50 µM). After 30 min, the fluorescence intensity was measured at an excitation wavelength of 450 nm and an emission wavelength...
of 482 nm with fluorometer. The fluorescence intensity was measured as the average of at least four samples.

**Statistical analysis**

Each experiment was repeated in triplicate and the results were reported as mean ± standard error of the mean (SEM).

**RESULTS**

**Extraction yield and phytochemical screening**

The yield of extraction of *T. disciforme* and *D. multicaule* was about 33.2 and 24.7% (g/g), respectively. Results of phytochemical screening indicated the presence of flavonoids, terpenoids, and tannins in both plants.

**Bioautographic and colorimetric study of anticholinesterase**

Bioautographic study of plant extracts indicated the AChE inhibitory effect of both *T. disciforme* and *D. multicaule* extracts in comparison to tacrine. These extracts caused discoloration of the yellow background of the plate as quickly as tacrine.

As shown in Figure 1, the results of colorimetric assay show that *T. disciforme* at concentrations of 1.25, 2.5, and 5 µg/ml and *D. multicaule* at concentrations of 2.5 and 5 µg/ml significantly in a concentration-dependent route inhibited AChE. (*P* < 0.005). The highest inhibition was shown at 5 µg/ml (71.18 ± 4.9 and 79.06 ± 3.1% by *T. disciforme* and *D. multicaule*, respectively) in comparison to tacrine (86.37 ± 3.2% inhibition). IC$_{50}$ value of *T. disciforme* and *D. multicaule* was calculated from their regression equation and determined as 1.85 ± 0.7 and 1.06 ± 0.6 µg/ml, respectively [Table 1].

**Antioxidant assay**

The results of DPPH inhibition assay show that *T. disciforme* and *D. multicaule* extracts inhibited DPPH radical in concentration-dependent manner [Figure 2]. The greatest inhibition occurred at 800 µg/ml (89.04 ± 3.9 and 78.5 ± 3.7%, respectively by *T. disciforme* and *D. multicaule*) in comparison to BHT (78.9 ± 4.8% inhibition). IC$_{50}$ value of *T. disciforme* and *D. multicaule* was equal to 262.1 ± 8.5 and 156.5 ± 6.2 µg/ml, respectively [Table 1].

**Effect of *T. disciforme* and *D. multicaule* on cell viability of PC12 cells**

The results of toxicity against PC12 cells shows that *T. disciforme* and *D. multicaule* exhibited no cytotoxicity under normal condition after 24 h up to 100 µg/ml [Figure 3].

**Neuroprotective effect of *T. disciforme* and *D. multicaule* on PC12 cells**

The incubation of PC12 cells with different concentrations of *T. disciforme* and *D. multicaule* (0-100 µg/ml) indicated no protection against Aβ peptide toxicity by these plant extracts. Tacrine at concentration of 1 µM exhibited 100% protection [Table 1].

**Table 1: IC$_{50}$ values of extracts in acetylcholinesterase and diphenypicrylhydrazil inhibition and cytotoxicity and Thioflavine T assay**

| Sample       | IC$_{50}$ (µg/ml) for AChE inhibition | IC$_{50}$ (µg/ml) for DPPH inhibition | Protection against Aβ cytotoxicity (%) in ThT assay | Aβ aggregation inhibition (%) in ThT assay |
|--------------|-------------------------------------|--------------------------------------|-------------------------------------------|------------------------------------------|
| *T. disciforme* | 1.85±0.7                           | 262.1±8.5*                          | -                                         | 10.73±2.8                                |
| *D. multicaule* | 1.06±0.6                           | 156.5±6.2*                          | -                                         | 19.07±1.6                                |
| BHT          | -                                   | 33.75±2.6                           | -                                         | -                                        |
| Tacrine      | -                                   | -                                   | 100                                       | -                                        |

*P<0.05 compared with control values. (One-way analysis of variance test).

BHT=Butylated hydroxytoluene, DPPH=Diphenylpicrylhydrazil,
ACHE=Acetylcholinesterase, ThT=Thioflavine T

![Figure 1: Anticholinesterase effect of different concentrations of *Tripleurospermum disciforme* and *Dracocephalum disciforme* extracts in comparison to tacrine (2 µg/ml)](image)

![Figure 2: Diphenypicrylhydrazil radical inhibition of different concentrations of *Tripleurospermum disciforme* and *Dracocephalum multicaule* extract in comparison to butylated hydroxytoluene](image)
Fluorometric evaluation of inhibitory effect on Ab peptide aggregation

The results of Tht fluorescence assay of *T. disciforme* and *D. multicaule* extracts indicated that these plant extracts could not inhibit aggregation of Aβ peptide at used concentrations. The greatest of inhibition was shown at concentration of 200 µg/ml (10.73 ± 2.8 and 19.07 ± 1.6% by *T. disciforme* and *D. multicaule*, respectively) [Table I].

**DISCUSSION**

TLC bioautographic study indicated the presence of cholinesterase inhibitory activity of *T. disciforme* and *D. multicaule* by formation of well-defined white spots made visible by spraying with DTNB, which gave a yellow background. Even though the TLC assay is a qualitative method, the extracts exhibited white spots at different R$_f$ values which indicated the presence of different compounds with anticholinesterase effect in these plants. In colorimetric method, under our study, the highest activity was appeared to be present at 5 µg/ml (71.18 ± 4.9 and 79.06 ± 3.1% AChE inhibition by *T. disciforme* and *D. multicaule*, respectively). Phytochemical screening showed the positive results for terpenoids, flavonoids, and tannins in both tested plants, so each or a combination of these metabolites might be responsible for anticholinesterase effect of these plants. Anticholinesterase effect of terpenoids has been reported previously. Terpenoids in tea tree oil were found to possess AChE inhibitory effect individually as well as in the mixed form.[23] The promising anticholinesterase effects have also been reported for some bicyclic monoterpenoids such as α-pinene, and 3-sabinene.[24] Moreover in a study, the presence of hydroxy flavones and methoxy flavonoids has been reported in *D. multicaule*. A number of flavonoids such as quercetin and macluraxanthone possess a mild inhibitory activity against AChE.[26,27]

In DPPH scavenging assay, *T. disciforme* as well as *D. multicaule* exhibited a concentration-dependent DPPH inhibition. The greatest inhibition was 89.04 ± 3.9 and 78.5 ± 3.7% at concentration of 800 µg/ml by *T. disciforme* and *D. multicaule*, respectively. Recently antioxidant effect of *D. moldavica*, has been reported. This plant demonstrated the ability to reduce iron (III) to iron (II) ions. *D. moldavica* also could scavenge 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium cation free radical (ABTS$^-$) too. This plant potentially inhibited the bleaching of β-carotene by scavenging of ROO radical. These reports show that *D. moldavica* protected 2-deoxy-D-ribose from oxidative degradation by scavenging OH radical. This plant extract exhibited the ability to scavenge DPPH radical at 1 mg/mL, while the extract of *D. multicaule* exhibited 50% inhibition of DPPH at 156.5 µg/ml[15,16]

The results of neuroprotective effect of *T. disciforme* and *D. multicaule* extracts showed that none of the plant concentrations was active against Aβ toxicity. These extracts also were inactive in fluorometric evaluation of inhibition of Aβ peptide aggregation. With increasing concentrations of 1~100 µg/ml, the protective role of the plants exhibited no protection enhancement. The inactivity of the extracts to inhibit Aβ aggregation and to protect the PC12 cell lines from Aβ does not rule out them as candidates for further studies as anti-AD. Because PC12 is an immortalized cell line and its responses to the therapeutic agents might be different from primary cultures. Furthermore, there is some evidence indicating that therapeutic effect of AChEIs in AD is not direct inhibition of Aβ aggregation. According to the “Cholinergic hypothesis of AD”,[2] it is believed that AChE inhibitors reduce the breakdown of endogenously released ACh, leads to greater activation of postsynaptic ACh receptors that would result to reduction of tau phosphorylation; returning towards normal the secretion of secreted amyloid precursor protein sAPP; reduction of β-amyloid production and returning towards normal glutamatergic neurotransmission. This shows that secretion of Aβ in reduced by AChE inhibitors (AChEIs). There is a hypothesis about the activation of an ‘anti-inflammatory cholinergic pathway’ in response to Aβ.[28] From this one may concluded that AChEIs would attenuate the inflammatory response evoked by Aβ.

**CONCLUSION**

Although our in vitro experiments are preliminary, from the view of preventive medicine, the results demonstrated the importance of future studies of these plants. Obtained results indicated the antioxidant and anticholinesterase effect of *T. disciforme* and *D. multicaule* extracts. There is a need to do further scientific and specific studies and
investigate the efficacy of the plants in different AD models. In addition, it is needed to do more studies to find the possibility of a correlation existing between antioxidant and AChE inhibitory activity.

ACKNOWLEDGEMENT

We would like to thank for financial support of Vice Chancellor for Research, Kerman University of Medical Sciences, Kerman, Iran. This article also has been derived from the thesis of Pharm. D student in Kerman University of Medical Sciences, Faculty of Pharmacy, Kerman, Iran.

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How to cite this article: Mandegary A, Soodi M, Sharififar F, Ahmadi S. Anticholinesterase, antioxidant, and neuroprotective effects of Tripleurospermum disciforme and Draccocephalum multicaule. J Ayurveda Integr Med 2014;5:162-6.

Source of Support: Vice Chancellor of Research, Kerman University of Medical Sciences, Kerman, Iran. Conflict of Interest: None declared.