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

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ABSTRACT

Background: Nowadays, owing to medicinal plants as a candidate to obtain promising new medicinal agents, there is a renewed interest in the use of these natural sources for drug development. **Objective:** In the present study, we aimed to assess the anticholinesterase, antioxidant, and neuropotective effects of *Tripleurospermum disciforme* and *Dracocephalum multicaule* extracts. **Materials and Methods:** Methanolic extract of the plants was prepared by maceration method. Anticholinesterase effect of different concentrations of the plants was studied by colorimetric method and antioxidant activity was evaluated using diphenypicrylhydrazil (DPPH) assay. Protective effect of the extracts against amyloid β (Aβ)-induced toxicity in PC12 cells was determined by MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method. **Results:** Both *T. disciforme* and *D. multicaule* extracts could inhibit acetylcholinesterase (AChE) in a dose-dependent manner. The highest inhibition occurred at 5 μg/ml (71.18 ± 4.9 and 79.06 ± 3.1% inhibition respectively by *T. disciforme* and *D. multicaule*) in comparison to tacrine (86.37 ± 3.24%). The greatest DPPH inhibition of *T. disciforme* and *D. multicaule* was shown at 800 μg/ml (89.04 ± 3.9 and 78.5 ± 3.7%, respectively). None of tested extracts induced protection against βA toxicity in PC12 cell. **Conclusion:** Although the results indicated anticholinesterase and antioxidant of the *T. disciforme* and *D. multicaule*, further specific studies and scientific validity are needed.

Key words: Anticholinesterase, antioxidant, *Dracocephalum multicaule, Tripleurospermum disciforme*

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 βA peptide have been studied. These two plants have been used in folk medicine for memory enhancing.[11] *T. disciforme* (C.A. Mey) Schultz Bip.

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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: $\%I = A_{con} - A_{sam}/A_{con} \times 100$, 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.

Diphenypicrylhydrazil 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% CO_2. The cells were cultured at a concentration of 10^4 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 at concentration of 2 μg/ml). IC_{50} value of *T. disciforme* and *D. multicaule* extracts was calculated from their regression equation and determined as 1.85 ± 0.7 and 1.06 ± 0.6 μg/ml, respectively [Table 1].

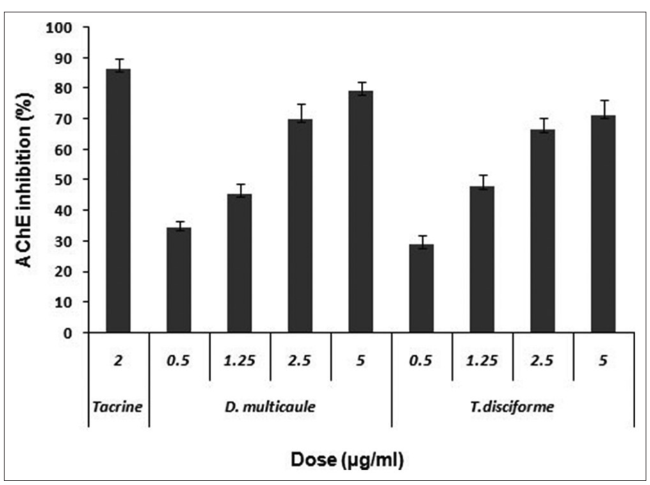

Figure 1: Anticholinesterase effect of different concentrations of *Tripleurospermum disciforme* and *Dracocephalum disciforme* extracts in comparison to tacrine (2 μg/ml)

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 βA cytotoxicity	βA 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=Diphenypicrylhydrazil, ACHE=Acetylcholinesterase, THT=Thioflavine T

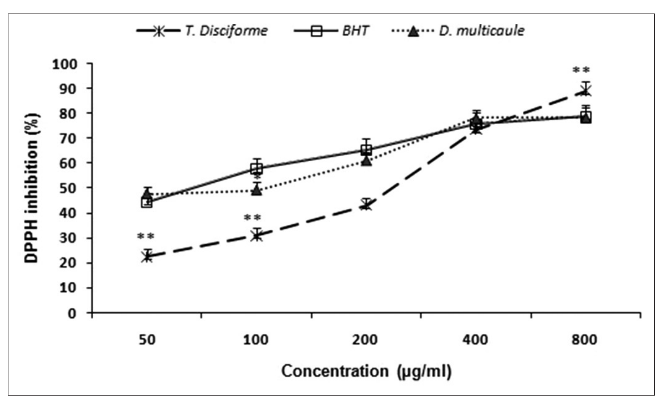

Figure 2: Diphenypicrylhydrazil radical inhibition of different concentrations of *Tripleurospermum disciforme* and *Dracocephalum multicaule* extract in comparison to butylated hydroxytoluene

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 1].

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 anticholinestease 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 monoterpenoides 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.*[25] A number of flavonoids such as quercetin and macluraxanthone possess a mild inhibitory activity against AChE.[26,27]

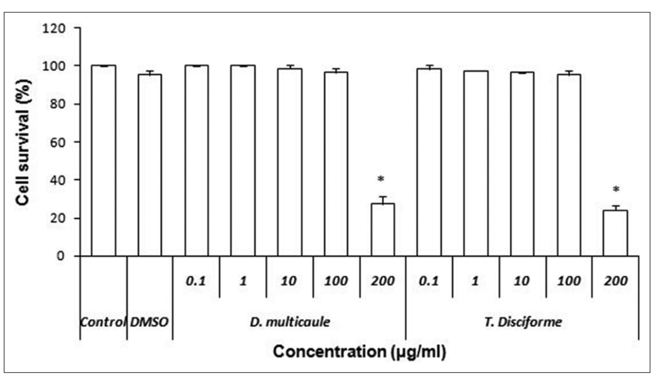

Figure 3: Cytotoxicity effect of different concentrations of *Tripleurospermum disciforme* and *Dracocephalum multicaule* extract on PC12 cells using MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. *$P < 0.05$ compared with control values. (One-way analysis of variance test)

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.

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