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## Essential Oil and Methanolic Extract of *Zataria multiflora* Boiss with Anticholinesterase Effect

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**Abstract:** One of the most common strategies in the treatment of cognitive disorders is enhancing the acetylcholine level in the brain through the inhibition of acetylcholinesterase. Despite the effectiveness of current modern drugs, more attention has been paid for finding new anticholinesterase agents from medicinal plants. *Zataria multiflora* Boiss. is an endemic plant to Iran which has different uses in traditional medicine as anti-inflammatory, antimicrobial, anti spasmotic. We intended to evaluate the *in vitro* anticholinesterase and free radical scavenging activity of the essential oil and methanolic extract of *Z. multiflora*. The essential oil and methanolic extract of the plant were evaluated for anticholinesterase activity using modified Ellman method. The free radical scavenging effect of the samples were studied by using of the diphenylpicrylhydrazyl (DPPH). IC<sub>50</sub> and the percent of inhibition of acetylcholinesterase was calculated from regression equation. The results showed that both the essential oil and methanolic extract of the plant exhibited high anticholinesterase activity (95.3±3.4 and 87.9±2.2% inhibition, respectively) which was similar to eserine (96.2±1.7% inhibition). The IC<sub>50</sub> value of essential oil was determined as 0.97±0.12 µg mL<sup>-1</sup> in comparison to eserine (0.13±0.02 µg mL<sup>-1</sup>). The results of antioxidant assay showed that both the essential oil and methanolic extract potentially inhibit DPPH free radical (94.8±2.4 and 93.2±1.7% inhibition, respectively). The essential oil and methanolic extract of *Z. multiflora* have beneficial effect in health promotion and this plant would be good candidate for further studies.

**Key words:** *Zataria multiflora*; anticholinesterase, antioxidant, essential oil, extract

### INTRODUCTION

Alzheimer's Disease (AD) is a common progressive and neurodegenerative disorder in elderly which result from cholinergic system impairment caused by decreased levels of acetylcholine (ACh) in brain. The most features of this disease are memory loss, weak judgment, deterioration of language and behavioral dysfunction. The common strategy in the treatment of AD, is enhancing the ACh level in the brain through the inhibition of acetylcholinesterase (AChE) (Darvesh *et al.*, 2003). Despite the effectiveness of current modern drugs, the patients taking medicines experience serious side effects (Lahiri *et al.*, 2002). Hence nowadays more attention has been paid to natural sources that have shown great promise in the treatment of various diseases. This would be worthy if dietary plants show anticholinesterase activity. Moreover the studies show that there are some associations between the oxidative

stress and AD (Vina *et al.*, 2004) and antioxidant agents may reduce the risk of AD-related neurological problems and dementia (Zhu *et al.*, 2004). We have already reported previously the AChE inhibitory and antioxidant effects of some of medicinal plants (Gholamhossainian *et al.*, 2009; Sharififar *et al.*, 2011a,b; Sharififar *et al.*, 2007a,b; Sharififar *et al.*, 2009), in the recent work the plant of *Zataria multiflora* has been studied for anticholinesterase activity.

*Zataria multiflora* known as Avishane shirazi belongs to Lamiaceae and is native to Iran, Afghanistan and Pakistan. It's essential oil has been proposed for disorders of respiratory and gastrointestinal system (Zargari, 1990). Furthermore the plant oil is a down-regulator of MDM2 gene expression which highlights the effectiveness of this oil in malignant disease (Gohar *et al.*, 2010). The antimicrobial, antinociceptive, anticandida, antiaphthous and anti-inflammatory effects of the plant

have been reported (Nakhai *et al.*, 2007; Jafari *et al.*, 2003; Mahmoudabadi *et al.*, 2006; Ramezani *et al.*, 2004; Sharififar *et al.*, 2007a).

Previous studies indicated that among the plant constituents, alkaloids and terpenoids especially essential oils can inhibit acetylcholinesterase (Kennedy *et al.*, 2006; Perry *et al.*, 2003; Tildesley *et al.*, 2003, 2005). In the present study, we aimed to evaluate the antioxidant and anticholinesterase effect of the EO and the methanolic extract of *Zataria multiflora* Boiss.

## MATERIALS AND METHODS

**Plant materials:** The aerial parts of *Z. multiflora* were collected from Kerman province at altitude 3300 m in June 2010. The plant was euthanatized by a botanist and a voucher specimen was deposited in Herbarium center in the Department of Pharmacognosy, Kerman University of Medical Sciences and Kerman, Iran (KF1241).

**Chemicals:** Acetylthiocholine iodide (ATCI), acetylcholinesterase (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. Eserine was prepared from Fluka Chemie (Buchs, Switzerland). Analytical thin-layer chromatography was carried out on silica gel GF<sub>254</sub> (35-70 µm, Merck). Other chemicals were from analytical grade.

**Essential oil isolation:** One hundred grams of the dried aerial parts of the plant was placed in a Clevenger apparatus and the essential oil was isolated after 3 h hydro distillation of the plant. The separated oil, dried with anhydrous sulphate sodium and stored at -20°C until experiment.

**Extraction and fractionation of the plant extract:** The total extract was prepared using maceration method for 72 h in room temperature with methanol from 100 g dried aerial parts of *Z. multiflora*. The obtained extract was concentrated in the vacuum to dryness. Dried extract were stored in -20°C until experiment work.

**Phytochemical screening of plant fractions:** The methanolic extract of the the plant was screened for the presence of alkaloids, terpenoids, steroids, saponins and flavonoids as explained by Trease and Evans (*et al.*, 2002).

### Anticholinesterase test

**TLC bioautography for acetyl cholinesterase inhibition:** The essential oil and methanolic extract of the plant were applied on the TLC plate and then developed using an

appropriate solvent system. The plate was dried 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 the plate was sprayed with 3 U mL<sup>-1</sup> AChE dissolved in 50 mM Tris-HCl, pH 8 at 37°C. Appearance of white spots in the yellow background of the plate indicates the presence of the compounds with AChE inhibitory activity. Another plate was done similarly for removing false positive reactions (Rhee *et al.*, 2001).

**In vitro evaluation of inhibition of AChE activity:** The AChE inhibitory effect of the essential oil and methanolic extract of *Z. multiflora* was evaluated using Ellman method with some modifications (SatheeshKumar *et al.*, 2010). ATCI is converted to thiocholine under hydrolysis by AChE and the resulted thiocholine reacts with the Ellman reagent, chromogenic substrate dithionitrobenzoic acid (DTNB), to form a yellow anion, 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, which show strong absorption at 405 nm. One hundred twenty five µL DTNB (3 mM) was added to a mixture of 25 µL of ATCI (15 mM), 50 µL of buffer and twenty five µL of each sample dissolved in phosphate buffer. The absorbance of mixture was measured at 405 nm for 65 sec every 13 sec. 25 µL of 0.22 U mL<sup>-1</sup> of AChE enzyme was added and the absorbance was again read every 13 sec for 104 sec. The same mixture without AChE was used as blank. By plotting the absorbance versus the time of incubation, enzyme activity was calculated from the slope of the line and expressed as a percentage of. Eserine was used as positive control. The percent of inhibition was calculated as follows:

$$I\% = \frac{A_{con} - A_{sam}}{A_{con}} \times 100$$

where, A<sub>con</sub> is the absorbance of the control and A<sub>sample</sub> is the absorbance of the test sample. The IC<sub>50</sub> was calculated by log-probit analysis.

### Antioxidant assay

**DPPH assay :** The inhibitory potential of DPPH stable radical of the essential oil and methanolic extract of *Z. multiflora* was determined as explained by Burits *et al.*, in triplicate (Bruits and Bucar, 2000). Fifty micro liters of each sample (indifferent concentration) in methanol was added to 5 mL of a 0.004% methanol solution of DPPH. The absorbance was read after 30 min incubation period at room temperature against a blank at 517 nm. Butylated Hydroxy Toluene (BHT) and the solvent were used as positive and negative controls respectively. The percent of inhibition was calculated in follows:

$$I\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

where,  $A_{\text{blank}}$  is the absorbance of the control mixture (containing all reagents except the test compound) and  $A_{\text{sample}}$  is the absorbance of the test compound. Sample concentration providing 50% inhibition ( $IC_{50}$ ) was calculated from the graph plotting inhibition percentage against extract concentration.

**Statistical analysis:** Each experiment was repeated in triplicate and the results were reported as Mean±SEM.

## RESULTS

**The results of phytochemical screening:** The results of phytochemical screening methanolic extract of *Z. multiflora* show positive reaction for flavonoids, terpenoids and tannins. Alkaloids and saponins were not detectable in the plant extract.

**Results of bioautography for AChE inhibitory activity:** The results of bioautographic assay indicated that both the essential oil and methanolic extract of the plant caused discoloration of the yellow background of the plate. The essential oil exhibited more quickly discoloration of the plate.

**Results of *in vitro* evaluation of inhibition of AChE activity:**  $IC_{50}$  value of the essential oil and methanolic extract of *Z. multiflora* was calculated from their regression equation (Table 1). The essential oil showed less  $IC_{50}$  value in comparison to methanol extract ( $0.97 \pm 0.12$  and  $3.2 \pm 0.7 \mu\text{g mL}^{-1}$ , respectively) which was similar to ones of eserine ( $IC_{50} = 0.13 \pm 0.02 \mu\text{g mL}^{-1}$ ). The essential oil and methanolic extract of the plant have shown  $95.3 \pm 3.4$  and  $87.9 \pm 2.2\%$  inhibition of AChE, respectively compared with eserine ( $96.2 \pm 1.7\%$  inhibition) (Table 1). The kinetic study of AChE in the presence of essential oil and methanolic extract of *Z. multiflora* indicated that these samples inhibited AChE in both concentration-dependent and time-dependent manner (Fig. 1-2).

**Results of antioxidant assay:** The essential oil and methanolic extract of the plant could inhibit potentially the DPPH radical with  $IC_{50}$  values of  $0.67 \pm 0.03$  and  $0.92 \pm 0.02 \text{ mg mL}^{-1}$ , respectively in comparison to BHT ( $IC_{50} = 0.53 \pm 0.07 \text{ mg mL}^{-1}$ ). The highest percentage of inhibition of essential oil and methanolic extract was determined as  $94.8 \pm 2.4$  and  $93.2 \pm 1.7\%$ , respectively. The maximum DPPH inhibition by BHT was  $87.5 \pm 5.9\%$  (Table 1).

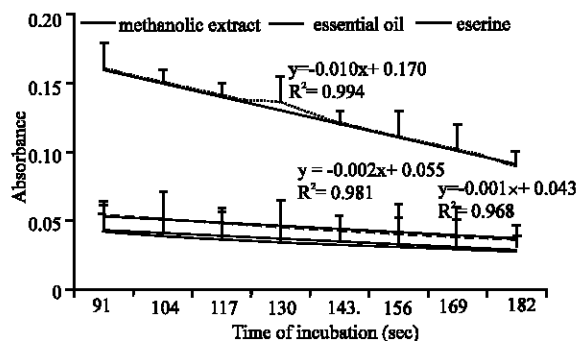


Fig. 1: Time-dependent inhibition of acetylcholinesterase (AChE) in the presence essential oil and methanolic extract of *Z. multiflora* in comparison to eserine. The experiment repeated in triplicate and data was demonstrated as Mean±SD

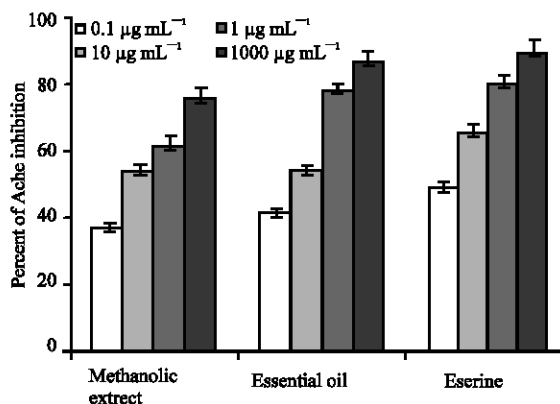


Fig. 2: The inhibitory effect of different concentration of essential oil and methanolic extract of *Z. multiflora* against acetylcholinesterase (AChE) in comparison to serine. The experiment repeated in triplicate and data was demonstrated as Mean±SD

## DISCUSSION

In the present study the essential oil and methanolic extract of *Z. multiflora* were evaluated for AChE inhibitory activity. In the bioautography assay, the essential oil demonstrated inhibitory effect against AChE activity more quickly than the other samples. The methanolic extract also strongly inhibited AChE and caused the appearance of white spots in yellow background on TLC plate. It could be postulated that both the essential oil and methanolic extract have potential activity against of AChE activity. The results of colorimetric method for the evaluation of AChE inhibitory effect of samples were expressed as percentage of inhibition and  $IC_{50}$  values calculated from the regression equation of each sample in comparison to

Table 1: The results of determination of IC<sub>50</sub> and percent of inhibition of AChE and DPPH scavenging effect of the essential oil and methanolic extract *Z. multiflora* Boiss

Samples	IC <sub>50</sub> (µg mL <sup>-1</sup> ) in AchE inhibitory test	Maximum percent of inhibition of AchE	IC <sub>50</sub> (µg mL <sup>-1</sup> ) in DPPH assay	Maximum percent of inhibition of DPPH
Essential oil	0.97±0.12	95.3±3.4	0.67±0.03	94.8±2.4
Methanolic extract	3.20±0.7	87.9±2.2	0.92±0.02	93.2±1.7
Eserine	0.13±0.02	96.2±1.7	-	-
BHT	-	-	0.53±0.07	87.5±5.9

Each experiment was done in triplicate and data are Mean±SD

eserine (Table 1, Fig. 2). Methanolic extract as well as the essential oil exerted noticeable AChE inhibitory activity. Essential oil of the plant could inhibit more than 95.3±3.4% of AChE activity which was comparable to eserine for that (96.2±1.7% inhibition). Methanolic extract of the plant also potentially inhibited cholinesterase (87.9±2.2% inhibition). The IC<sub>50</sub> value of essential oil was determined as 0.97±0.12 µg mL<sup>-1</sup> in comparison to eserine (0.13±0.02 µg mL<sup>-1</sup>). The IC<sub>50</sub> value of the methanolic extract was 3.2±0.7 µg mL<sup>-1</sup> which was comparable to one of eserine (IC<sub>50</sub>= 0.13±0.02 µg mL<sup>-1</sup>). Regarding a variety of compounds in the essential oil and methanolic extract of *Z. multiflora* in comparison to pure eserine, this IC<sub>50</sub> indicate the high inhibitory potency of the plant. As we have reported, the GC/MS analysis indicated the presence of thymol, carvacrol and a variety of monoterpenes in the plant essential oil (Sharififar *et al.*, 2007a). Our results of phytochemical screening also indicated the presence of terpenoids in the methanolic extract (Table 1). These compounds especially monoterpenes might be responsible for the observed inhibitory effect of the methanolic extract. Anticholinesterase activity of monoterpenes and some of diterpenes have been reported elsewhere (Perry *et al.*, 2002; Ren *et al.*, 2004; Savelev *et al.*, 2003). Both the essential oil and methanolic extract exhibited concentration-inhibition and time-inhibition relationships (Fig. 1-2).

In DPPH assay, the highest activity was due to the essential oil of the plant (94.8±2.4% DPPH inhibition), while the methanolic extract also exerted strong antioxidant effect (Table 1). This shows that the essential oil and methanolic extract of *Z. multiflora* might be effective in AD through two different mechanisms of AChE inhibition and antioxidant effect. There are a few studies about *Z. multiflora* in the literature and it is for the first time that the AChE inhibitory activity of this plant has been reported. In general, considering the obtained results can conclude that both the essential oil and methanolic extract of the plant would be good candidates for further studies. *In vivo* studies of AChE inhibitory activity, toxicological studies and toxicity against of beta amyloids are being carried out.

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