



## *In vitro* evaluation of anticholinesterase, antioxidant and antibacterial activity of different fractions of *Zataria multiflora* Boiss

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### Abstract

*Zataria multiflora* has been widely used for health promotion purpose. We have studied anticholinesterase, antioxidant and antibacterial effects of different fractions of a standard extract of *Z. multiflora*. Rosmarinic acid (RA) content of plant was determined by spectrophotometric method and anticholinesterase and antioxidant activity of various fractions was studied using colorimetric method and diphenylpicrylhydrazyl (DPPH) assay respectively. Minimum inhibitory concentration (MIC) of fractions against a variety of bacteria was determined by agar dilution method. Results: RA content of the plant was 2.2% (w/w). Chloroform fraction (CHF) exhibited the greatest anticholinesterase effect (94.05± 7.6%) followed by petroleum ether fraction (PEF) (88.7±5.1%). Methanolic fraction (MF) exhibited the highest DPPH inhibition (93.3± 4.1%) followed by PEF and CHF (88.7± 3.7% and 85.4± 2.5% respectively). The least MIC was due to PEF and CHF against Gram-negative and Gram-positive bacteria respectively. Conclusion: These results indicated that PEF and CHF would be a good candidate for further studies.

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## Introduction

The loss of cholinergic neurotransmission caused by degeneration of cholinergic neurons is associated with Alzheimer's disease (AD) (Francis, 1999). Moreover, antioxidant agents may reduce the risk of AD-related neurological problems and dementia (Zhu *et al.*, 2004).

*Zataria multiflora* Boiss (known in Persian: Avishane-shirazi) is a thyme-like plant and is used in traditional medicine as antiseptic and carminative. The plant has anti-*Herpes simplex*, antimutagenicity and antibacterial effects (Mandegary *et al.*, 2013; Sharififaret *et al.*, 2007; Arabzadehet *et al.*, 2013; Dehghan-Noodeh *et al.*, 2013). This plant contains flavonoids, phenolic acids and terpenoids (Sharififaret *et al.*, 2007; Ali *et al.*, 2000; Shaiq *et al.*, 2000). Recently, we reported anticholinesterase and antioxidant effect (Sharififar *et al.*, 2012) of this plant and in the present work, intended to separate different fractions of the plant and find active fractions with anticholinesterase, antioxidant and antibacterial effect.

## Materials and methods

### Plant materials

Aerial parts containing leaves, stems and flowers of *Z. multiflora* were collected from Koochpayeh, Kerman province at altitude 2400m in July 2011. The plant was euthanized by a botanist and a voucher specimen was deposited in Herbarium center in the Department of Pharmacognosy, Kerman University of Medical Sciences, Iran (KF1127).

### Chemicals

Rosmarinic acid, 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 GF254 (35-70  $\mu\text{m}$ , Merck). Other chemicals were from analytical grade.

### Extraction and fractionation of the plant extract

Total extract was prepared from 100g dried plant using maceration method with methanol 80%. An amount of 500 g of the plant also was extracted with petroleum ether, chloroform, and methanol consecutively by maceration method. Total extract and different fractions of petroleum ether (PEF), chloroform (CHF) and methanol (MF) were concentrated in the vacuum to dryness. Dried samples were stored at  $-20^{\circ}\text{C}$  until experiment work.

### Phytochemical screening of plant fractions

Total extract and fractions of *Z. multiflora* were screened for the presence of flavonoids, alkaloids, saponins, terpenoids and tannins. Qualitative screening for the presence of flavonoids was done with reduction test in which by adding HCl-Mg indicator to each fraction, a pink to purple color was evaluated as positive reaction. For determination of alkaloids in the plant extract, three reagents of Meyer, Wagner and Dragendorff were used which induced no turbidity or precipitation with the plant fractions. One gram of the fraction was dissolved in 10 ml of distilled water and was shaken vigorously for 1-2 min. The presence of at least 1 cm height foam which persisted for 30 min was indicative for saponins. Libermann-Burchard test was used for indication of presence of terpenoids and finally ferric chloride solution was added to each fraction which gives blue-green color with tannin content of the fractions (De *et al.*, 2012; Evans *et al.*, 2002).

### Spectrophotometric determination of RA content of the plant total extract

#### Standard solutions and calibration curves of RA

An amount of 10 mg of standard RA was weighed accurately and dissolved in methanol (80%) to give 100  $\mu\text{g/ml}$  stock solutions. Serial dilutions were prepared from stock solution and the absorbance spectra of the RA were recorded in wavelength range between 200 to 400 nm with a UV-visible spectrophotometer (Lambda 25, Perkin Elmer, and USA). At the  $\lambda_{\text{max}}$  of 328 nm, absorbance of different dilutions (2, 5, 10, 15 and 20  $\mu\text{g/ml}$ ) of RA was measured. The calibration curve of standard solutions

was constructed by plotting RA concentration versus absorbance at 328 nm. The experiment was repeated three times at different days, and the mean of the absorbance was used to draw a suitable standard curve. The percent of relative standard deviation (%RSD) and error% were calculated as a measure of precision and accuracy of the method, respectively. In addition, a third derivative spectrophotometric ( $\Delta\lambda = 5\text{nm}$ ) method using the amplitude of the standard solutions at  $\lambda = 349.9\text{ nm}$  was used to construct a calibration curve to determination of RA in the extracts. This method could help to avoid interferences of accompanying constituents present in the extracts.

#### *Analysis of the extract*

A stock solution of plant extract was prepared by dissolving of 100 mg of dried extract in 100 ml methanol and then filtered using Whatman filter paper No 1. A volume of 10 ml of each sample diluted to 100 ml with methanol, UV spectrum was recorded and third derivative spectrum at  $\Delta\lambda = 5\text{nm}$  was calculated by UV-Winlab software and the amplitude was measured at 349.9nm. The RA content of the extract was determined using the calibration curve equation and amplitude of the extract preparation at 349.9nm. Each experiment was repeated three times and the results were reported as mean  $\pm$ SD.

#### *Anticholinesterase test*

##### *TLC bioautography for acetyl cholinesterase inhibition*

Total standard extract (TSE) extract and different fractions of the plant were applied on the TLC plate and developed using an appropriate solvent system. The plates were 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 plates were sprayed with 3 U/ml AChE dissolved in 50 mM Tris-HCl, pH 8 at 37 °C. Appearance of white spots in the yellow background of the plates indicated the presence of the compounds with AChE inhibitory activity. An intact TLC plate was developed in the same solvent system for fractions and sprayed with equal volume and concentration of ATCI and DTNB.

Finally after 2 min. AChE was sprayed and the appearance of white spots in a yellow background indicates false positive reactions. Eserine was used as positive control (Rhee *et al.*, 2001).

#### *In vitro evaluation AChE inhibitory activity*

The AChE inhibitory effect of different concentrations (1,10,100,1000,2000 and 5000 $\mu\text{g/ml}$ ) of the total extract and fractions of *Z. multiflora* was evaluated using Ellman based colorimetric method with some modifications(SatheeshKumara *et al.*,2010). ACh is converted to thiocholine under hydrolysis by AChE and the resulted compound reacts with the Ellman reagent, chromogenic substrate dithionitrobenzoic acid (DTNB), to form a yellow anion of 2-nitrobenzoate-5-mercaptothiocholine which shows strong absorption at 405 nm. At first, 125  $\mu\text{l}$  DTNB (3mM) was added to a mixture of 25  $\mu\text{l}$  of ATCI (15mM), 50  $\mu\text{l}$  of buffer and 25  $\mu\text{l}$  of each sample dissolved in phosphate buffer. The absorbance of mixture was measured at 405 nm for 65s every 13 s. A volume of 25  $\mu\text{l}$  of 0.22 U/ml of AChE enzyme was added and the absorbance was again read every 13 s for 104 s. 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. Eserine at the same concentrations of plant fractions was used as positive control. The percentage of inhibition was calculated as follow:

$$\%I = \frac{\text{Acon} - \text{Asam}}{\text{A con}} \times 100.$$

Where Acon is the absorbance of the control and a sample is the absorbance of the tested sample. The  $\text{IC}_{50}$  value was calculated by log-probit analysis.

#### *Antioxidant assay*

##### *DPPH assay*

Assessment of the DPPH inhibitory potential of TE and different fractions of *Z. multiflora* was done as explained by Sharma *et al.*, 2009. Fifty micro liters of each sample (in different concentration of 1, 5, 10, 25, and 50 $\mu\text{g/ml}$ ) in methanol was added to 5 ml of a 0.02 mM methanolic solution of DPPH. The absorbance was measured after 30 min incubation period at room temperature at 517 nm. Butylated

hydroxytoluene (BHT) at the same concentrations of plant fractions and the solvent were used as positive and negative controls respectively. The percentage of inhibition was calculated using following formula:

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

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 versus extract concentration.

#### Antibacterial effects

Minimum inhibitory concentration (MIC) of different fractions of *Z. multiflora* against eight Gram- positive and Gram-negative microorganisms was determined using agar dilution () [20]. Briefly, each fraction was diluted serially in different concentrations in dimethylsulfoxide-distilled water (10: 90), then mixed with molten Mueller-Hinton agar to give final dilutions of 0.19 to 25 $\mu$ g/ml, and immediately poured into a Petri dish. A suspension of bacteria strain ( $10^4$  cfu ml $^{-1}$ ) was spotted on the agar and inoculated plates were incubated at 37°C for 24hours. the least concentration of each fraction which exhibited no visible growth after incubation was taken as the MIC.

The solvent of fractions and gentamicin were used as negative and positive control respectively. The following strains were used: *Staphylococcus aureus* PTCC (Persian type culture collection) 1112, *S. epidermidis* PTCC 1114, *Bacillus subtilis* PTCC 1023, *Bacillus licheniformis* PTCC 1525, *Escherichia coli* PTCC 1330, *Salmonella typhi* PTCC 1639, *Pseudomonas aeruginosa* PTCC 1074, *Klebsiella pneumonia* PTCC 1525.

#### Statistical analysis

Each experiment was repeated in triplicate and the results were reported as Mean $\pm$  SEM. Data was analyzed by ANOVA followed by Tukey's multiple comparisons test for significant differences using SPSS 14.0 software.

## Results

#### Extraction yield and phytochemical screening

The highest percentage of the yield of extraction was due to MF and CHF (10% and 6.3% g/g dried total extract respectively). The results of phytochemical screening indicated that all fractions and total extract of *Z. multiflora* were terpenoid and tannins positive while alkaloid test was negative. Amongst the tested fractions, methanolic fraction and total extract were flavonoid positive (Table 1).

**Table 1.** The results of extraction and and phytochemical screening of different fractions from *Zataria multiflora*.

Sample	Percentage of extraction	Phytochemical screening				
		Terpenoids	Flavonoids	Saponins	Alkaloids	Tannins
PEF <sup>a</sup>	4.6	+++	trace	+	-	++
CHF <sup>b</sup>	6.3	+++	trace	+	-	++
MF <sup>c</sup>	10	+	+++	-	-	+++
TSE <sup>d</sup>	13.7	+++	+++	++	-	+++

<sup>a</sup>Petroleum ether fraction, <sup>b</sup>Chloroform fraction, <sup>c</sup>Methanol fraction, <sup>d</sup>Total standard extract.

#### Rosmarinic acid content of plant extract

Third derivative spectra and amplitude at 349.9 nm of the RA standard solutions and extract sample showed least interferences of accompanying constituents and depicted a good linearity, precious and accuracy. The spectrophotometric measurement of the RA showed

the best fitting line equation was  $y = 516.7x + 633$ ,  $R^2 = 0.988$ , indicated good linearity between serial RA concentration and peak area. The percentage of RA was determined as 2.2% (w/w) RA in *Z. multiflora*.

#### Bioautography for AChE inhibitory activity

The results of bioautographic assay indicated that amongst the plant fractions, PEF and CHF quickly caused appearance of white spots (with 2-2.5mm diameter) in the yellow background of the plate which indicated the inhibition of AChE by these fractions, whereas the plate remained yellow with MF which shows that this fraction could not inhibit AChE. Eserine caused discoloration of the yellow background of the plate more quickly than all of tested fractions.

#### Colorimetric evaluation of anticholinesterase activity

Anticholinesterase activity of different fractions of *Z. multiflora* is presented in Figure 1. As shown in this figure, PEF and CHF along with TSE potently inhibited AChE. These three samples exhibited a concentration-dependent inhibition of AChE. Although, this effect was significantly lower than that

of eserine as a standard acetylcholinesterase inhibitor ( $p \leq 0.001$ ). These results also revealed that CHF showed the highest percentage of inhibition in comparison to PEF and TSE; however, all of these fractions exhibited more than 83% inhibition at a concentration of 5000  $\mu\text{g/ml}$ . On the contrary, MF mildly inhibited AChE which was not exceeded form 48% (Figure 1).

IC<sub>50</sub> value of different fractions of *Z. multiflora* was calculated from their regression equation (Table 2). Among the tested samples, PEF and TSE with the least IC<sub>50</sub> values (0.87 $\pm$ 0.1 $\mu\text{g/ml}$  and 5.7 $\pm$ 0.5  $\mu\text{g/ml}$  respectively) caused inhibition of AChE in comparison to eserine (IC<sub>50</sub> = 0.38 $\pm$  0.06 $\mu\text{g/ml}$ ). The greatest percentage of AChE inhibition was exhibited by CHF and PEF (94.05 $\pm$  7.6% and 88.7 $\pm$ 5.1% respectively) too when compared to eserine (98.3 $\pm$  6.4% inhibition) (Table 2).

**Table 2.** IC<sub>50</sub> values and maximum percentage of inhibition of AChE and DPPH radical of different fractions of *Zataria multiflora*.

Sample	IC <sub>50</sub> ( $\mu\text{g/ml}$ ) in AChE inhibitory test	Maximum Percentage of inhibition of AChE	IC <sub>50</sub> ( $\mu\text{g/ml}$ ) in DPPH assay	Maximum Percentage of inhibition of DPPH
PEF <sup>a</sup>	0.87 $\pm$ 0.1	88.7 $\pm$ 5.1	33.5 $\pm$ 2.2	88.7 $\pm$ 3.7
CHF <sup>b</sup>	115 $\pm$ 5.2	94.05 $\pm$ 7.6	6.25 $\pm$ 1.4	85.4 $\pm$ 2.5
MF <sup>c</sup>	2013 $\pm$ 21.3	47.5 $\pm$ 2.9	2.34 $\pm$ 0.1	93.3 $\pm$ 4.1
TSE <sup>d</sup>	5.7 $\pm$ 0.5	80.67 $\pm$ 5.0	16.2 $\pm$ 3.2	80.8 $\pm$ 3.9
Eserine	0.38 $\pm$ 0.06	98.3 $\pm$ 6.4	-	-
BHT	-	-	1.67 $\pm$ 0.5	87.5 $\pm$ 5.9

<sup>a</sup>Petroleum ether fraction, <sup>b</sup>Chloroform fraction, <sup>c</sup>Methanol fraction, <sup>d</sup>Total standard extract.

Time course study of AChE inhibitory effect of the fractions demonstrated a reverse time-dependent inhibition for all tested fractions. Anticholinesterase effect of all fractions especially CHF and PEF continued to decrease with increasing time (Figure 2). The fraction of CHF showed maximum inhibition 1 minute after incubation ( 94.90%). For the TSE and MF, maximum inhibitory effect was observed at the first minute after incubation (data not shown).

#### Antioxidant assay

The results of DPPH inhibition assay of different fractions of *Z. multiflora* have given in Table 2. The highest DPPH inhibition was due to the fractions of

MF (93.3 $\pm$  4.1%) and PEF (88.7 $\pm$  3.7%) which was higher one of than BHT (87.5  $\pm$  5.9% at 5000 $\mu\text{g/ml}$ ). The least IC<sub>50</sub> value of DPPH inhibition also was due to MF ( IC<sub>50</sub>= 2.34 $\pm$ 0.1 6.25 $\pm$  1.4) followed by CHF (IC<sub>50</sub>= 6.25 $\pm$  1.4  $\mu\text{g/ml}$ ).

#### Antibacterial test

Antibacterial assessment of fractions of *Z. multiflora* indicated that PEF and CHF were active against both Gram-positive and Gram-negative strains. The least MIC was due to CHF against *S. aureus*. The results of antibacterial effects of different fractions have given in Table 3.

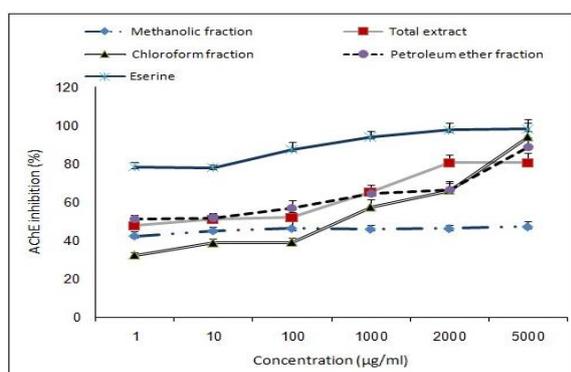
**Table 3.** Minimum inhibitory concentration (MIC) ( $\mu\text{g/ml}$ ) of different fractions of *Zataria multiflora* against eight standard bacterial strains.

Sample	Microorganism							
	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumonia</i>	<i>B. subtilis</i>	<i>S. typhi</i>	<i>B. licheniformis</i>
PEF <sup>a</sup>	0.78	0.39	1.56	1.56	0.78	0.78	0.78	0.78
CHF <sup>b</sup>	0.39	0.19	1.56	3.12	0.39	1.56	0.39	1.56
MF <sup>c</sup>	6.25	6.25	6.25	12.5	6.25	0.78	12.5	25
TSE <sup>d</sup>	0.39	0.39	3.12	3.12	0.39	1.56	0.39	6.25
Gentamicin	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39

<sup>a</sup>Petroleum ether fraction, <sup>b</sup>Chloroform fraction, <sup>c</sup>Methanol fraction, <sup>d</sup>Total standard extract.

### Discussion

Rosmarinic acid is a phenolic acid in *Z. multiflora* and some of the other Lamiaceae plants with anti oxidant effect. So standardization of *Z. multiflora* was performed on the basis of rosmarinic acid content by spectrophotometric method. The content of RA was determined about 2.2% (w/w). In continuing of our studies for anticholinesterase effect of different medicinal plants (Shariffar *et al.*, 2011, 2012) and especially this plant<sup>[9]</sup>, we intended to find what classes of compounds of *Z. multiflora* could be responsible for antioxidant and anti-cholinesterase effect of the plant.

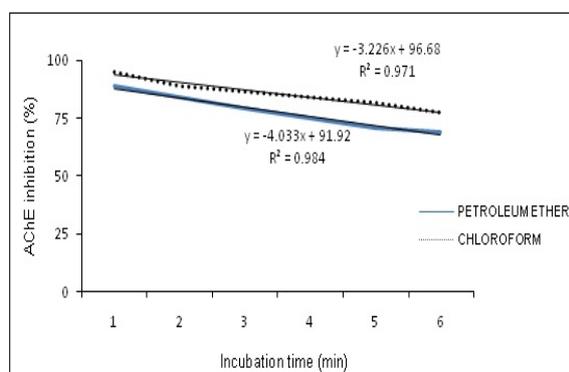


**Fig. 1.** Anticholinesterase effect of different fractions of *Zataria multiflora* in comparison to serine in various concentrations. Each test was performed in triplicate and results were exhibited as mean  $\pm$  SD.

Anticholinesterase activity of the plant fractions was evaluated using bioautography and colorimetric method on the basis of Ellman method. Spotting of the fractions on TLC plate confirmed that PEF and CHF potentially inhibited AChE which was visualized

by appearance of white spots in a yellow background. Through colorimetric method, results were expressed as  $\text{IC}_{50}$  values of AChE inhibition. The  $\text{IC}_{50}$  value of fractions was calculated from the regression equations of absorbance versus concentration of each sample. The least  $\text{IC}_{50}$  values were due to PEF and TSE ( $0.87 \pm 0.1$  and  $5.7 \pm 0.5 \mu\text{g/ml}$  respectively) which was close to one of eserine ( $0.38 \pm 0.06 \mu\text{g/ml}$ ). Amongst the tested fractions, CHF showed highest percentage of AChE inhibitory activity ( $94.05 \pm 7.6\%$  at  $5000 \mu\text{g/ml}$ ) followed by PEF ( $88.7 \pm 5.1\%$ ) and TSE ( $80.67 \pm 5.0\%$ ) in comparison to eserine ( $98.3 \pm 6.4\%$ ). All samples inhibited AChE in a concentration-dependent route. The fraction of MF exhibited a weak AChE inhibition. On whole, it can be postulated that PEF and CHF have strong AChE inhibitory effect. Phytochemical screening results indicated the presence of tannins and especially terpenoids in these two fractions (Table 1). Previous studies have reported the presence of the terpenoid of paracymene and its derivatives such as multiflorol and multiflotriol in *Z. multiflora* extract.<sup>[7]</sup> In the other hand, some of monoterpenes with high concentration in the plant oil like para-cymene and /or gamma-terpinene could be responsible for the observed AChE inhibitory effect of the active fractions. Terpenoids account for more than 25% of this essential oil.<sup>[4]</sup> The anticholinesterase activity of monoterpenes and some of diterpenes have been reviewed (Perry *et al.*, 2002; Ren *et al.*, 2004; Svelev *et al.*, 2003; 2006). Orhan *et al.* (2007) reported that in most cases, when solvent polarity increases, AChE inhibitory effect of the plant

extract gradually decreases. This might most likely be due to the presence of high percentage of non polar anticholinesterase compounds such as terpenoids and/or alkaloids in the plant extracts. In contrast, polar extracts containing phenolic compounds exert less inhibitory activity. In a study on the essential oil of *Thymus vulgaris*, the anticholinesterase activity of the principle constituents of essential oil including carvacrol has been reported. Even though, carvacrol, exists in high concentration in the essential oil of *Z. multiflora*, its presence in the hydroalcoholic extracts of the plant is highly questionable. One might attribute the higher anticholinesterase activity of non polar fractions of PEF and CHF to the presence of this compound. Boskabady *et al.*, (2011) reported the possible inhibitory effect of carvacrol on the muscarinic receptors. In another study conducted by Boskabady, anticholinergic effect of hydroalcoholic extract of *Z. multiflora* has been shown (Boskabady *et al.*, 2012) Although this antagonistic effect needs to be confirmed by using accurate methods such as receptor binding assay, presence of carvacrol in the extract used in this study is negligible.



**Fig. 2.** Time course anticholinesterase effect of petroleum ether and chloroform fractions of *Zataria multiflora*.

The results of DPPH inhibition assay also underlined the strong and dose –dependent antioxidant activity of MF (93.3± 4.1% inhibition). The other fractions exhibited moderate effect. The results of antioxidant test reinforce the hypothesis that this activity of the fractions could be ascribed to the other compounds with antioxidant effect. Taheri *et al.*, (2011) have reported that the essential oil of *Z. multiflora* could inhibit the peroxidation of fatty acids in fillet of Cobia

fish during frozen storage at concentration of 500ppm. *In vivo* and *in vitro* antioxidant activity of the essential oil and its preservation effect of cake lipids have been reported too (Karimian *et al.*, 2011; Kordsardouei *et al.*, 2013; Sharififar *et al.*, 2012). However, few studies have compared the antioxidant effect of the plant extract (Sharififar *et al.*, 2007). It seems that there is a general trend that tannins and flavonoids are mostly responsible for radical scavenging effect. This thesis is in accordance with our results of phytochemical screening which confirmed the presence of flavonoids and tannins in MF.

Apart from the antioxidant and anticholinesterase effect of the separated fractions, their antibacterial properties have been studied. Amongst the tested fractions, the least MIC was due to CHF against *S. aureus* (MIC=0.39). The fraction of PEF exhibited promising activity against Gram-negative microorganisms while the Gram-positive strains were sensitive to CHF. The least sensitivity was exhibited to MF. In previous study, antibacterial effect of total extract and essential oil of this plant was reported [4]. In general, considering the obtained results can conclude that PEF and CHF of the plant would be good candidates for further studies for finding anticholinesterase agents. These fractions also exhibited antioxidant and antibacterial effect. *In vivo* studies of AChE inhibitory activity, toxicological evaluation and protection against toxicity of beta amyloid peptides of *Z. multiflora* is being carried out.

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