Vol. 13(5), pp. 96-103, 10 March, 2019
DOI: 10.5897/JMPR2018.6724
Article Number: 213B77160480
ISSN 1996-0875

http://www.academicjournals.org/JMPR

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Journal of Medicinal Plants Research

Full Length Research Paper

Edraianthus pumilio (Schult.) A. DC.: Phytochemical and biological profile

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Received 12 December, 2018; Accepted 5 March, 2019

***Edraianthus pumilio* is stenoendemic plant native to Dalmatia, Croatia. This paper deals with its phytochemical and biological profile. Phytochemical profile of volatile oil was performed by the gas chromatography–mass spectrometry (GC/MS), while total phenolic content of its aqueous extract was performed by Folin-Ciocalteu method. The phytochemical analysis showed that the main volatile oil compounds were nonanal (21.2%) and myristicin (16.4%). This oil could be characterized as nonanal-myristicin type. Total phenolic content of aqueous extract was 30.6 ± 1.1 mg GAE/g extract. Results of testing antioxidant potential of *E. pumilio* volatile oil and aqueous extract showed low antioxidant potentials as tested by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. Results also showed low acetylcholinesterase inhibition potential of volatile oil and low to moderate potential of aqueous extract, as tested by Ellman method.**

Key words: *Edraianthus pumilio* (Schult.) A. DC., volatile oil, aqueous extract, gas chromatography–mass spectrometry (GC/MS), total phenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), Ellman.

INTRODUCTION

In terms of biological diversity Croatia is one of the richest European countries. Croatian flora, with about 5000 species and subspecies is characterized by a markedly high level of diversity per unit of surface (Nikolic et al., 2015). Genus Edraianthus in flora Europe includes 9 species, 6 of which belong to the flora Croatica (Tutin et. al., 1980: Nikolic, 2018). *Edraianthus pumilio* (Schult.) A. DC. is stenoendemic species of flowering plant in the family Campanulaceae, native to Dalmatia in Southern Croatia. It is a strictly protected and almost endangered species. It is widespread on Mount Biokovo, near Makarska (Nikolic, 2018). The plant is highly heliophilic, thermophilic and xerophilic. It grows on limestone-rock cracks. This is a low-growing perennial plant that grows in dense pads. Linear and silver leaves grow in tufts. The underside of leaves is naked, while upper side of leaves

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is hairy. The flowers are violet-blue bells, while fruits are capsules. The plant belongs to alliance *Seslerion juncifoliae* Horvat 1930 (Nikolic et al., 2015).

Previous Edraianthus studies mainly focused on systematization of plants. The genus was the subject of cytogenetic investigations (Siljak-Yakovlev et al., 2010), molecular phylogenetic and phylogeographic studies (Park et al., 2006; Lakušić et al., 2016) as well as finding new species and determining their taxonomic status (Boštjan et al., 2009).

According to molecular phylogeny analysis the *E. pumilio* and *E. dinaricus* are two very closely related species. Both species are closely restricted to the mountains of Middle Dalmatia. These two species are quite different from other *Edraianthus* species in terms of morphological features such as leaf, inflorescence and habits (Stefanović et al., 2008).

Chemical taxonomy at this genus is poorly researched. The chemical structures of phenolics and terpenoids are often specific and restricted to taxonomically related organisms and hence useful in classification (Singh, 2016). Secondary metabolites and beneficial biological effects of *E. pumilio* have not been investigated so far. These compounds have shown to have antioxidant, antimicrobial, antiinflammatory, anticarcinogenic, antimutagenic and antiallergic properties (Roy et al., 2017; Murugesan and Deviponnuswamy, 2014; Bharti et al., 2012: Namita and Mukesh, 2012; Allesiani et al., 2010).

Antioxidants are vital substances, which possess the ability to protect the body from damages caused by free radical-induced oxidative stress (Birben et al., 2012). A large amount of evidence has demonstrated that oxidative stress is intimately involved in age-related neurodegenerative diseases. There have been a number of studies which examined the positive benefits of antioxidants to reduce or to block neuronal death occurring in the pathophysiology of these disorders (Loizzo, 2009; Ramassamy et al., 2006).

Acetylcholinesterase (AChE) is the enzyme involved in the hydrolysis of acetylcholine neurotransmitter which plays important role in memory and cognition. Low level of AChE in brain is usually connected with neurodegenerative disease, that is, Alzheimer's disease (AD). The most promising approaches in treating AD are usually based on AChE inhibitors (Wszelaki et al., 2010; Menichini et al., 2009).

Therefore, the aim of this work was to determine the phytochemical composition of volatile oil, total phenolic content of aqueous extract as well as antioxidant and acetylcholinesterase inhibition potential of volatile oil and aqueous extract isolated from *E. pumilio*. There are no known records of this plant being used for medicinal purposes but this or another research may contribute otherwise. Also, the analysis of volatile components can contribute to a better determination of the species *E. pumilio*. To the best of our knowledge, this is the first work that shows the phytochemical and biological profile of this species.

MATERIALS AND METHODS

Plant material

Plant material (whole plants, without roots) was collected from its natural habitat, *via* St. Ilija, Biokovo Mountain, Croatia (Central Dalmatia, Makarska) during flowering in July, 2017, at 1500 m a.s.l.; *Gauss-Krüger* coordinates: X=5663111, Y=4804120. The botanical identity of the plant material was confirmed by a botanist PhD Mirko Ruscic, associate professor, Department of Biology, Faculty of Science, University of Split. Voucher specimens were deposited in herbarium at Department of Biology, Faculty of Science, University of Split, Croatia, under number EP_7/17.

Volatile oil isolation

Air-dried aerial parts of plants were hydro-distilled using Clavenger apparatus for 3 h. Obtained essential oil was dried over anhydrous Na_2SO_4 and stored in sealed vials, under - 20 ^oC before use (Nikbin et al., 2014).

Preparation of aqueous extract

Air-dried aerial parts of plants and water were placed in an *Erlenmeyer* flask and refluxed in an ultrasound bath for 2 h. The mixture was then filtered through a filter paper and evaporated under vacuum at 40°C and stored at -20°C in fridge before use.

Gas chromatography-mass spectrometry analysis

The analysis of the volatile oil was carried out using Shimadzu gas chromatography–mass spectrometry (GC/MS), QP2010 system equipped with an AOC 20i autosampler, using fused silica capillary column Inert Cap (5% diphenyl, 95% dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm). The operating conditions were as follows: injection volume: 1.0 µl of volatile oil solution (1:500 *v/v* in pentane); injection mode: splitless; injection temperature: 260 ^oC; carrier gas: helium, 1.11 ml/min; the oven temperature program: 50°C (5 min), 50 - 260°C (3°C/min); MS conditions: ion source temperature: 200°C, ionization voltage: 70 eV, mass range: m/z 40 - 400 u. GCMSolution 2.5 (Shimadzu) was used to handle data. Identification of volatile oil components was based on (a) retention indices on non-polar column relative to a homologous series of *n*-alkanes (C_8 - C_{40}), (b) on the comparison of their mass spectra and retention indices with the NIST and Wiley spectra library and with those reported in the literature (Adams, 2007; Linstrom and Mallard, 2014). GC/MS analysis was performed in triplicate and results were averaged.

Total phenolic content

Total phenolic content was measured using Folin-Cocalteu spectrophotometric method (Singleton and Rossi, 1965; Katalinic et al., 2013) and gallic acid as a standard. 15 ml water and Folin-Ciocalteu reagent (1.25 ml, diluted 1:2) were added to the sample solution (0.25 ml, 1 mg/ml). The resulting solution was mixed. After 3 min, in solution were added Na_2CO_3 (3.75 ml, 20%) and water to volume of 25 ml. The resulting mixture was then left for 2 h. The absorbance of the resulting blue color was measured at 765 nm. The concentration of the total phenolic content was calculated by using an equation obtained from gallic acid calibration curve and

expressed as mg gallic acid equivalent per gram of extract (mg GAE/g extract). The determination of total phenolic content was carried out in triplicate and the results were expressed as mean ± standard deviations.

Antioxidant capacity

Antioxidant capacity of volatile oil and aqueous extract from *E. pumilio* were tested using two different methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and ferric reducing antioxidant power (FRAP) method.

Capacity of DPPH radical scavenging by volatile oil and aqueous extract was measured according to the method of Brand-Williams et al. (1995) (Katalinic et al., 2010). DPPH method is based on the reaction between free DPPH radicals and antioxidants. As a result, a stable non-radical form of the DPPH is obtained, with simultaneous change of the violet color to pale yellow. The decrease in absorbance was measured at 517 nm. DPPH radical solution was prepared by dissolving the stock solution (4 mg of DPPH in 100 ml of ethanol). To optimize the conditions used to run the DPPH assay in microplates, 10 µl of sample (1 mg/ml) was placed in a well and 290 µl of DPPH solution was added. The mixture was shaken vigorously and left to stand at room temperature in the dark. The decrease in the absorbance was measured after 1 h, with ethanol as blank. The DPPH radical scavenging activity of the sample was calculated according to the formula:

$$\% \text{ inhibition} = [(A_0 - A_{sample})/A_0] \times 100$$

where A_0 was absorbance of the DPPH ethanol solution measured at the beginning and A_{sample} was absorbance of the sample measured after 60 min. The results were expressed as percentage inhibition of DPPH. Butylated hydroxyanysole (BHA) was used as positive control.

The reducing power of volatile oil and aqueous extract were also performed using FRAP method (ferric reducing antioxidant power) (Skroza et al., 2015; Benzie and Strain, 1996). FRAP method is based on the reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous (Fe^{2+}) complex with an intense blue color and maximum absorption at 593 nm. The method was performed in 96-well microplates, with slight modifications. The FRAP solution was freshly prepared by mixing 0.3 M acetate buffer (pH = 3.6) and 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in a ratio of 10:1:1 (by volume). The assay was carried out by placing 10 µl of the sample (1 mg/ml) and 300 µl of FRAP reagent in a well. The absorbance was measured after 4 min. The reducing power of sample was calculated by comparing with the reaction signal given by solution of Fe^{2+} ions in known concentration and expressed as µmol/l Fe^{2+}. BHA was used as positive control. The determination of antioxidant capacity, performed by both methods was carried out in triplicate and the results were expressed as mean ± standard deviations. Tested stock solution concentration was 1 mg/ml.

Acetylcholinesterase inhibition potential

Acetylcholinesterase (AChE) inhibition potential of volatile oil and aqueous extract were carried out by a slightly modified Ellman assay (Politeo et al., 2018; Ellman, 1961). A typical run consisted of 180 µl of phosphate buffer (0.1 M, pH 8), 10 µl of DTNB (at a final concentration of 0.3 mM prepared in 0.1 M phosphate buffer pH 7 with 0.12 M sodium bicarbonate added for stability), 10 µl of sample solution (dissolved in 80% EtOH), and 10 µl of AChE solution (with final concentration 0.03 U/ml). Reactants were mixed in a 96-well plate wells and reaction was initiated by adding 10 µl of acetylthiocholine iodide (ATChI to reach a final concentration of 0.5 mM). As a negative control, 80% EtOH was used instead of sample solution. Non-enzymatic hydrolysis was also monitored by measurement of two blank runs for each run. All spectrophotometric measurements were performed at 405 nm and at room temperature for 6 min. The results are expressed as percentage inhibition of enzyme activity. Eserine was used as positive control. The determination of acetylcholinesterase inhibition potential was carried out in triplicate and the results were expressed as mean ± standard deviations. Tested stock solution concentration of *E. pumilio* samples was 1 mg/mL, while stock solution concentration of eserine was 0.1 mg/ml.

RESULTS AND DISCUSSION

Phytochemical composition of *E. pumilio* volatile oil

E. pumilio volatile oil yield was 0.31% (w/w). The analysis revealed forty two compounds separated into five classes: nonterpene compounds, phenyl propanes, terpene compounds, norisoprenoids, and other compounds (Table 1). Nonterpene compounds (54.6%) were predominated compound class in *E. pumilio* volatile oil. Among them nonterpene aldehydes (41.8%) were the most common class of compounds with nonanal (21.2%) as a major one. (*E,E*)-2,4-Decadienal (3.9%), tridecanal (3.4%), decanal (2.5%), octanal (2.5%), (*E,Z*)-2,4-decadienal (2.1%) and others nonterpene aldehydes were identified in lower quantity. Nonterpene hydrocarbons (7.4%), ketones (4.0%), alcohols (1.1%) and esters (0.3%) were also identified in lower quantity. Among these compounds, the most common compound was 4-methyldecane (2.9%), while other compounds were identified in quantity lower than 2%. Second one quantitatively important compound class, phenyl propanes (17.3%), was presented with two compounds: myristicin (16.4%) as predominant compound and anisole (0.9%). Terpene compounds (15.1%) were mainly presented with monoterpene compounds. Among them the main ones were hexahydrofarnesyl acetone (5.1%), prenol (2.2%) and ß-myrcene (2.0%). Other monoterpene compounds were identified in quantity lower than 2%. Sesquiterpene compounds were present only with one compound, ß-caryophyllene (0.4%). Norisoprenoids (0.4%) were presented with two compounds: ß-ionone (0.1%) and ß-ionone epoxide (0.3%). 4-Vinyl phenol (1.6%), benzothiazole (0.7%) and indole (0.5%) were presented as other compounds (2.8%).

Total phenolic content of *E. pumilio* aqueous extract

The total phenolic content of *E. pumilio* aqueous extract (the extraction yields was 14.8%, w/w) was determined using Folin-Ciocalteu reagent and external calibration with gallic acid, according to the method previously described by Singleton and Rossi (Katalinic et al., 2013; Singleton and Rossi, 1965). The total phenolic content was 30.6 ± 1.1 mg GAE/g extract (Table 2).

Table 1. *E. pumilio*: Volatile oil constituents.

S/N	Compounds	%	RI[a]	Mode of identification
	Nonterpene compounds	**54.6**		
	Nonterpene aldehydes	***41.8***		
1	Benzaldehyde	1.4	961	RI, MS
2	Octanal	2.5	1001	RI, MS
3	Phenyl acetaldehyde	1.0	1044	RI, MS
4	Nonanal	21.2	1104	RI, MS
5	(*E*)-2-Nonenal*	1.1	1161	RI, MS
6	Decanal	2.5	1204	RI, MS
7	(*E*)-2-Decenal*	0.8	1263	RI, MS
8	(*E,Z*)-2,4-Decadienal*	2.1	1299	RI, MS
9	Undecanal	1.0	1305	RI, MS
10	(*E,E*)-2,4-Decadienal*	3.9	1314	RI, MS
11	(*E*)-2-Undecenal*	0.2	1360	RI, MS
12	Tridecanal	3.4	1504	RI, MS
13	Pentadecenal	0.7	b	-, MS
	Nonterpene hydrocarbons	***7.4***		
14	3,5,5-Trimethyl-2-hexene	0.8	986	RI, MS
15	4-Methyldecane	2.9	1060	RI, MS
16	2- Methyldecane	0.4	1072	RI, MS
17	1-Dodecene	1.1	1193	RI, MS
18	1-Tetradecene	1.8	1390	RI, MS
19	Pentadecane	0.4	1500	RI, MS
	Nonterpene ketones	***4.0***		
20	(*E,E*)-3,5-Octadien-2-one*	0.8	1093	RI, MS
21	(*E*)-2-Methyl-2-nonen-4-one*	1.7	1216	RI, MS
22	(*Z*)-3-Nonen-2-one*	0.6	1332	RI, MS
23	6,10-Dimethyl-undecan-2-one	0.9	1403	RI, MS
	Nonterpene alcohols	***1.1***		
24	3,5-Octadien-2-ol	1.1	1037	RI, MS
	Nonterpene esters	***0.3***		
25	*cis*-3- Hexenyl tiglate	0.3	1319	RI, MS
	Phenyl propanes	**17.3**		
26	Anisole	0.9	1235	RI, MS
27	Myristicin	16.4	1513	RI, MS
	Terpene compounds	**15.1**		
	Monoterpene ketones	***5.5***		
28	Neryl acetone	0.4	1443	RI, MS
29	Hexahydrofarnesyl acetone	5.1	b	-, MS
	Monoterpene hydrocarbons	***5.1***		
30	*α*-Thujene	1.3	925	RI, MS
31	*β*-Myrcene	2.0	991	RI, MS
32	*p*-Cymene	0.7	1022	RI, MS
33	Limonene	1.1	1028	RI, MS

Table 1. Cont.

	Monoterpene alcohols	***4.1***		
34	Prenol	2.2	778	RI, MS
35	Linalool	1.6	1101	RI, MS
36	*cis*-Sabinol	0.3	1142	RI, MS
	Sesquiterpenes	***0.4***		
37	ß-Caryophyllene	0.4	1410	RI, MS
	Norisoprenoids	**0.4**		
38	*β*-Ionone	0.1	1475	RI, MS
39	(*E*)-5,6-Epoxy-ß-ionone*	0.3	1479	RI, MS
	Other compounds	**2.8**		
40	*p*-Vinylphenol	1.6	1222	RI, MS
41	Benzothiazole	0.7	1224	RI, MS
42	Indole	0.5	1292	RI, MS
	Total	**90.2**		

* Correct isomers were not identified; RI = Kovats index determined on a Inert Cap column using the homologous series of *n*-hydrocarbons C_8-C_{40}; MS = mass spectra; b = The RI was outside of the RI interval of series of *n*-alkanes.

Table 2. Total phenolic content, antioxidant and anticholinesterase inhibition capacity of volatile oil and water extract from *E. pumilio*

Variable	Total phenolic content mg GAE/g extract	Antioxidant capacity		AChE inhibition %
		DPPH inhibition %	FRAP µmol/L Fe^{2+}	
Ep-volatile oil[a]	-	ni	15.3 ± 0.7	26.6 ± 2.1
Ep-aqueous extract[a]	30.6 ± 1.1	10.7 ± 0.7	118.7 ± 7.2	46.9 ± 4.7
BHA[a]	-	91.9 ± 2.9	5586.3 ± 72.6	-
eserine[b]	-	-	-	95.9 ± 1.9

Ep = *Edraianthus pumilio*; [a]stock solution concentration was 1 mg/ml (0.048 mg/mL for DPPH, 0.029 mg/ml for FRAP and 0.045 mg/ml for AChE in reaction system); [b]stock solution concentration was 0.1 mg/mL (4.5 µg/ml for AChE in reaction system); ni = inhibition not identified.

Antioxidant capacity of volatile oil and aqueous extract from *E. pumilio*

Antioxidant potential of volatile oil and aqueous extract from *E. pumilio* (Ep) are presented in Table 2 and Figure 1. Results obtained were compared with those of well-known antioxidant BHA. Result showed no antioxidant potential of *Ep*-volatile oil and low antioxidant potential of Ep-aqueous extract (10.7±0.7%) tested by DPPH method as well as low antioxidant capacity of Ep-volatile oil (15.3±0.7 ek. µmol/L Fe^{2+}) and Ep-aqueous extract (118.7±7.2 ek. µmol/L Fe^{2+}) tested by FRAP method for tested stock solution concentration of 1 mg/ml (0.048 mg/ml for DPPH and 0.029 mg/ml for FRAP in reaction system). In comparison, well-known antioxidant compound BHA inhibited DPPH with 91.9±2.9%, while antioxidant capacity tested by FRAP method was eq.. 5586.3 ± 72.6 µmol/L Fe^{2+} for the same tested concentration. Low antioxidant capacity of Ep-volatile oil and slightly better, but still low, antioxidant capacity of Ep-aqueous extract could be connected to chemical composition of these extracts. Namely, tested Ep-volatile oil does not contain components responsible for antioxidant potential such as phenolic or monoterpenoid compounds (eugenol, carvacrol, thymol, menthol) (Brewer, 2011; Bakkali et al., 2008), while low antioxidant capacity of Ep-aqueous extract is in correlation with low total phenolic content of tested extract (30.6±1.1 mg GAE/g extract). Therefore, Ep-aqueous extract probably contains low quantity of phenolic components with good antioxidant capacity such as phenolic acids (gallic, protochatechuic, caffeic and rosmarinic acids), phenolic

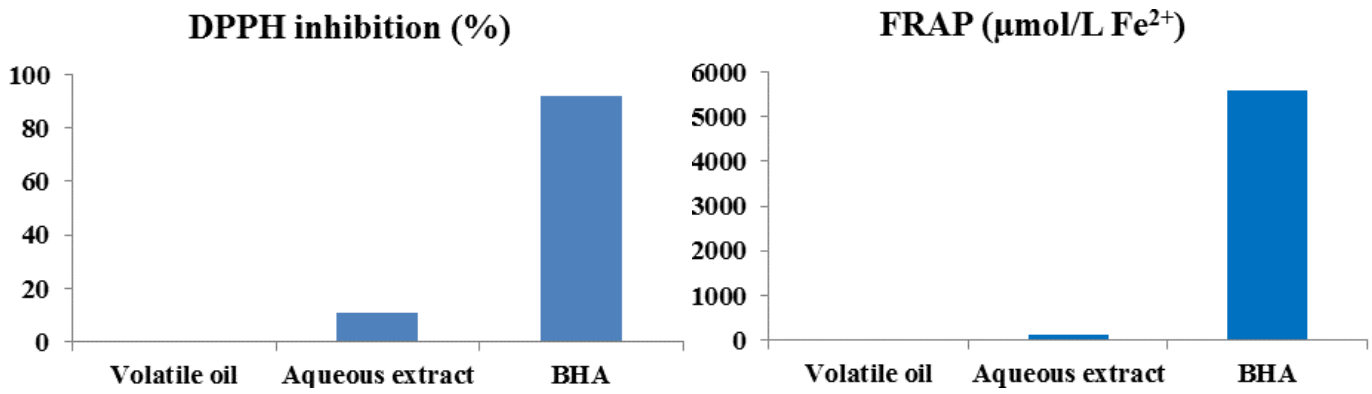

Figure 1. Antioxidant capacity of volatile oil and aqueous extract from *E. pumilio* (tested concentrations were 1 mg/ml).

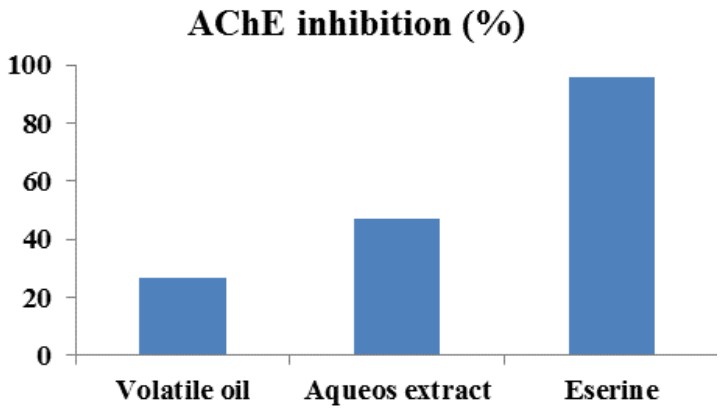

Figure 2. Acetylcholinesterase inhibition of volatile oil and aqueous extract from *E. pumilio* (tested concentrations were 1 mg/ml and 0.1 mg/ml for eserine).

diterpenes (carnosol and carnosic acid) and flavonoids (quercetin and catechin) (Brewer, 2011) but these compounds cannot significantly contribute to antioxidant capacity of Ep-aqueous extract.

Acetylcholinesterase inhibition potential of volatile oil and aqueous extract from *E. pumilio*

Acetylcholinesterase inhibition potential of Ep-volatile oil and Ep-aqueous extract is presented in Table 2 and Figure 2. Results obtained were compared with those of well-known AChE inhibition agent, eserine. Results showed low AChE inhibition potential of Ep-volatile oil (26.6±2.1%) and low to moderate AChE inhibition potential for Ep-aqueous extract (46.9±4.7%) in tested concentration of 1 mg/ml (0.045 mg/ml in reaction system). In comparison, well-known AChE inhibitor eserine showed 95.9±1.9% AChE inhibition in tested concentration of 0.1 mg/ml (4.5 µg/ml in reaction system).

Low AChE inhibition potential of Ep-volatile oil and low to moderate AChE inhibition potential for Ep-aqueous extract could also be connected to chemical composition of tested extracts. Namely, tested volatile oil probably does not contain compounds responsible for AChE inhibition potential, such as *α*-pinene, *δ*-3-carene, 1,8-cineole, *α*- and *β*-asarone (Burcul et al., 2018), while Ep-aqueous extract probably contains low quantity of compounds responsible for AChE inhibition, such as compounds with catechol moiety in their structure (Ji and Zhang, 2006), polymers of resveratrol (Jang et al., 2007), stilbene oligomers (Sung et al., 2002) or others (Szwajgier, 2014; Suganthy et al., 2009; Mukherjee, 2007).

Conclusions

The phytochemical analysis of *E. pumilio* volatile oil revealed forty two compounds separated into five

classes: nonterpene compounds, phenyl propanes, terpene compounds, norisoprenoids, and other compounds. Among identified compounds, the main *E. pumilio* volatile oil compounds were nonanal (21.2%) and myristicin (16.4%) and this oil could be characterized as nonanal-myristicin type. The total phenolic content of *E. pumilio* aqueous extract was 30.6 ± 1.1 mg GAE/g extract.

Results of testing antioxidant potential of *E. pumilio* volatile oil and aqueous extract showed no antioxidant potential of volatile oil and low antioxidant potential of aqueous extract tested by DPPH method as well as low antioxidant capacities of volatile oil and aqueous extract tested by FRAP method, in comparison with BHA. Results of acetylcholinesterase inhibition potential test showed low potential of volatile oil and low to moderate potential of aqueous extract in comparison with eserine. The volatile oils rich in nonanal as well as nonanal isolated from volatile oil may have other useful biological properties such as antidiarrhoeal (Zavala-Sanchez et al., 2002). Myristicin (a phenylpropanoid) was reported to have cytotoxic effects (Lee et al., 2005), anti-cholinergic, antibacterial, hepatoprotective effects and anti-inflammatory properties (Lee and Park, 2011) and also can inhibit tumorigenosis in mice (Zheng et al., 1992).

Future studies of phytochemical compound should focus on other possible biological effects such as anti-inflammatory, antidiarrheal, and anticancer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This work was supported by the Croatian Science Foundation; project IP-2014-09-6897.

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