Journal of Medicinal Plants Research Vol. 5(30), pp. 6590-6596, 16 December, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals
DOI: 10.5897/JMPR10.289

Full Length Research Paper

Chemical composition and evaluation of acetylcholinesterase inhibition and antioxidant activity of essential oil from Dalmatian endemic species *Pinus nigra* Arnold ssp. *dalmatica* (Vis.) Franco

Olivera Politeo*, Irena Botica, Tea Bilusic, Mila Jukic, Ivana Carev, Franko Burcul and Mladen Milos

Department of Biochemistry, Faculty of Chemistry and Technology, University of Split, Teslina 10/V, 21 000 Split, Croatia.

Accepted 4 October, 2011

In the present study, the chemical composition and biological activity (acetylcholinesterase inhibition and antioxidant activity) of essential oil from endemic species *Pinus nigra* Arnold ssp. *dalmatica* (Vis.) Franco was investigated. α-Pinene, β-pinene, germacrene D and ß-caryophyllene were identified by GC-FID and GC-MS as dominant components of the oil. *P. nigra* ssp. *dalmatica* essential oil showed relatively high inhibitory activity on acetylcholinesterase (AChE), which can be in relation with good AChE inhibitory activity of pure main monoterpene components of the oil (*S*-α-pinene) and their mixtures (*S*-α-pinene:*S*-ß-pinene and *R*-α-pinene:*S*-ß-pinene, in ratio 3:2). Essential oil from *P. nigra* ssp. *dalmatica* did not show significant antioxidant activity tested by three methods: DPPH radical scavenging method, Ferric Reducing Antioxidant Potential (FRAP) and Thiobarbituric Acid Reactive Species (TBARS) assays.

Key words: Acetylcholinesterase inhibition, antioxidant activity, essential oil, *Pinus nigra* ssp. *dalmatica*, endemic species.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of age-associated memory deficit. It is a degenerative neurological disorder characterized by senile plaques containing amyloid β protein and loss of cholinergic neuromediators in the brain (Whitehouse et al., 1982; Pakaski and Kalman, 2008). The most remarkable biochemical change in the brain of AD patients is a reduction of acetylcholine (ACh) levels (Bartus et al., 1982). Inhibition of acetylcholinesterase (AChE), the enzyme responsible for hydrolysis of ACh, is one of the most established therapeutic approaches in AD treatment (Thompson et al., 2004). In recent years, the free radical hypothesis of aging has brought a better understanding of biochemical events that occur with Alzheimer's disease (Behl et al., 1997; Halliwell and Gutteridge, 1989). The brain is considered very sensitive to damage caused by oxidative stress for a variety of reasons including, high uptake of oxygen, low glutathione content and high membrane content of polyunsaturated fatty acids (Cao et al., 1988; Floyd, 1999). The fact that age is a key risk factor in AD provides considerable support for the free radical hypothesis because effects of the attacks by free radicals, particularly those produced by reactive oxygen species (ROS), can accumulate over the years (Hartman, 1995; Halliwell, 2001; Markesbery, 1997). Numerous plants and their constituents are reputed in traditional practices of medicine to enhance cognitive function and to alleviate other symptoms of AD, including depression (Howes et al., 2003; Barbosa Filho et al., 2006;

*Corresponding author. E-mail: olivera@ktf-split.hr. Tel: +385 21 329 437. Fax: +385 21 329 461.

Mukherjee et al., 2007). As a part of our continuing study into discovery of new cholinesterase inhibitors from Croatian plants (Jukic et al., 2007; Kulisic-Bilusic et al., 2008), the aim of this work is to determine inhibitory potential on acetylcholinesterase as well as antioxidant activity of the essential oil of endemic Dalmatian black pine needles, *P. nigra* Arnold ssp. *dalmatica* (Vis.) Franco and its main constituents.

MATERIALS AND METHODS

Chemicals

DPPH (2,2`-diphenyl-1-picrylhydrazyl), acetylcholinesterase (type XII-S: from bovine erythrocytes), DTNB (5-5`-dithiobis-2-nitrobenzoic acid) and physostigmine (eserin) were purchased from Sigma-Aldrich (Switzerland); (1*S*)-(-)-α-pinene, (1*R*)-(+)-α-pinene and (1*S*)-(-)-β-pinene were purchased from Merck-Schuchardt; acetylthiocholine iodide, TPTZ (2,4,6-tripyridyl-s-triazine), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), ascorbic acid, $FeCl_2x4H_2O$ and all of the applied solvents were from Fluka Chemie (Buchs, Switzerland).

Plant material and extraction of essential oil

Dalmatian endemic species, *P. nigra* Arnold ssp. *dalmatica* (Vis.) was collected in central Dalmatia (Vidova gora, island Brac) during May 2008. Fresh needles of *P. nigra* ssp. *dalmatica* were used for the isolation of the essential oils. A voucher specimen was deposited at the Department of Biochemistry, Faculty of Chemistry and Technology, University of Split. Plant material (100 g) was hydro-distilled for 3 h in Clevenger-type apparatus using pentane/diethyl ether 1:1 (v/v) for trapping as described by Kulisic et al. (2004). After distillation, the pentane/ diethyl ether extract was separated and dried (Na_2SO_4). The essential oil was separated from the solvent by careful evaporation. The essential oil yield was determined by the gravimetric method.

Essential oil analysis

Analysis was performed on a GC-FID (Hewlett-Packard 5890 Series II gas chromatograph equipped with flame ionization detector) and GC-MS (Hewlett-Packard model 5890 with a mass selective detector model 5971A) as described by Mastelic et al. (2008). Individual peaks were identified by comparing their retention indices (RI) to those of co-injected pure standards, our homemade library and literature data (Adams, 1995), as well as by comparing their mass spectra (MS) with those of the commercial Wiley 275 mass spectral database, co-injected pure standards, our homemade library and literature data (Adams, 1995). The retention indices of the components were determined in relation to a series of homologous *n*-alkanes run at the same conditions used for the essential oils.

Evaluation of essential oil acetylcholinesterase inhibition

Evaluation of acetylcholinesterase inhibition of essential oil from *P. nigra* ssp. *dalmatica* was carried out by modified Ellman method (Nostrandt et al., 1993). Each sample was tested in triplicate over a range of concentrations. The control was also included in which 86% ethanol replaced the test inhibitor solution. Its final concentration of 2% did not inhibit the enzyme.

Evaluation of essential oil antioxidant activity

Evaluation of the antioxidant activity of *P. nigra* ssp. *dalmatica* essential oil was performed using three antioxidative methods: DPPH radical scavenging method (Brand-Williams et al., 1995), FRAP assay (Benzie and Strain, 1996) and TBARS assay (Ruberto et al., 2000). All measurements were performed in triplicate.

RESULTS AND DISCUSSION

After isolation of essential oil from *P. nigra* ssp. *Dalmatica*, α-pinene (24.4%) was identified as the major component, followed by β-pinene (16.0%), germacrene-D (14.6%) and lastly, ß-caryophyllene (9.6%) as shown in Table 1. The essential oil yield was 0.77% (w/w). Chemical composition of *P. nigra* ssp. *dalmatica* essential oil from leaf was previously investigated by Chalchat and Gorunovic (1995). They found α-pinene, borneol and ß-caryophyllene as the major compounds. But, the chemical composition from our essential oil shows similarity with those of *P. nigra* Arnold from central Italy (Macchioni et al., 2003) with α-pinene, germacrene D, ß-caryophyllene and ß-pinene as the major compounds.

AChE inhibitory activity of essential oil was dose-dependent (Figure 1). The sample of stock solution concentration of 2 g/L (90.9 mg/L in reaction system) showed highest AChE inhibitory activity (58.8 ± 11.2%), while the sample of 0.1 g/L (4.5 mg/L in reaction system) showed the lowest AChE inhibitory activity (41.4 ± 4.5%). The amount of essential oil necessary to decrease the AChE activity by 50% (EC_{50}) was 0.94 g/L (42.7 mg/L in reaction system). EC_{50} was calculated graphically (% of AChE inhibition was plotted against the essential oil concentration). In order to investigate the significance of the main essential oil monoterpene compounds in AChE inhibition, as well as their putative synergistic or antagonistic activity, the effect of pure compounds and their artificial mixtures in ratios similar to one found in the natural oil were also tested. It is known that Pinus sp. oils contain both α-pinene and β-pinene including their *R*- and *S*- enantiomeres. *R*-α-pinene and *S*-α-pinene are equally represented, while *S*-β-pinene is predominantly represented in relation to *R*-β-pinene in Pinus sp. volatile oils (Ochocka et al., 2002; Persson, 2003; Maciag et al., 2007). For this reason, we selected *R*- and *S*-α-pinene as well as *S*-β-pinene for AChE inhibition testing as shown in Figure 2. The highest AChE inhibitory activity was achieved by mixtures of α- and β- pinene enantiomers in ratio 3:2 (42.5 ± 7.5 and 40.2 ± 7.0% of inhibition, *S*-α- and *R*-α-pienene respectively). Pure *R*-α-pinene showed 35.2 ± 6.1% while S-α-pinene showed 21.2 ± 1.8% AChE inhibitory activities and pure *S*-β-pinene showed insignificant inhibitory activity. When comparing natural oil (which contains α-pinene:β-pinene~3:2, in total 40.4%

Table 1. Chemical composition of *P. nigra* ssp. *dalmatica* essential oil.

No.	Identified compounds	%	RI[a] (20M)	RI[a] (HP-101)	Mode of identification
1	α-Pinene	24.4	1030	938	Rt, MS
2	Camphene	1.5	1060	954	Rt, MS
3	ß-Pinene	16.0	1102	972	Rt, MS
4	Myrcene	1.9	1148	981	Rt, MS
5	Limonene	3.3	1180	1023	Rt, MS
6	ß-Phellandrene	1.9	1187	1001	Rt, MS
7	*Trans*-ß-ocimene	2.1	1235	1044	Rt, MS
8	Terpinolene	1.4	1257	1080	Rt, MS
9	Linalool	0.3	1518	1092	Rt, MS
10	Linalyl acetate	0.4	1531	1240	Rt, MS
11	Bornyl acetate	3.3	1555	1252	Rt, MS
12	ß-Elemene	0.6	1566	1364	Rt, MS
13	ß-Caryophyllene	9.6	1585	1385	Rt, MS
14	Terpinen-4-ol	0.6	1561	1154	Rt, MS
15	Pinocarvyl acetate	0.6	1651	1148	Rt, MS
16	*Trans*-verbenol	0.2	-	1158	Rt, MS
17	Germacrene B	0.3	1606	1400	Rt, MS
18	α-Humulene	2.5	1628	1442	Rt, MS
19	Estragole	0.3	1632	1177	Rt, MS
20	α-Terpineol	1.9	1653	1176	Rt, MS
21	α-Terpinyl acetate	2.9	-	1341	Rt, MS
22	Germacrene D	14.6	1673	1444	Rt, MS
23	α-Muurolene	0.8	1683	1506	Rt, MS
24	δ-Cadinene	2.1	1724	1486	Rt, MS
25	Phenylethyl isovalerate	2.3	1902	-	Rt, MS
26	Caryophyllene oxide	0.3	1937	1576	Rt, MS
27	Methyl eugenol	0.4	1959	1378	Rt, MS
28	α-Cadinol	0.4	2160	1614	Rt, MS
29	Tetradecanoic acid	2.8	b	1780	Rt, MS
	Total	99.6			

[a] retention indices relative to C_8-C_{22} *n*-alkanes on polar HP-20 M and apolar HP-101 column; b, retention times is outside of retention times of homologous series of C_8-C_{22} *n*-alkanes (identified by MS)-, not identified

as shown in Table 1) to artificial ones (*S*-α-pinene:*S*-β-pinene=3:2 or *R*-α-pinene:*S*-β-pinene=3:2), it can be concluded that natural oil is a better AChE inhibitor. This could also be explained by contribution of some minor components to the overall natural oil AChE inhibitory activity. Relatively high AChE inhibitory activity of monoterpene hydrocarbons can be explained by hydrophobic interactions between AChE active site and monoterpene hydrocarbon skeleton (Mukherjee et al., 2007).

Comparison of DPPH, FRAP and TBARS assays for estimating antioxidant potential shows low antioxidative activity (Figures 3 to 5). Radical scavenging activity of the oil tested by DPPH method was dose-dependent and relatively low compared with commercial antioxidants (BHT-butylated hydroxytoluene and BHA-butylated hydroxyanisole) (Politeo, 2010). This method is based on the measurement of the reducing ability of antioxidants toward DPPH stable radical. The highest inhibitory activity of the oil on DPPH was 23.6% at the concentration of 5 g/L (0.24 g/L in reaction system).

The lowest antioxidant activity of the oil was detected by FRAP assay, which is based on redox-colorimetric reaction of the reduction of colourless Fe^{3+} compounds into blue coloured Fe^{2+}-tripiridyltriazine in the presence of the antioxidants.

TBARS assay involves the spectrophotometric detection of thiobarbituric acid reactive species, namely malonaldehydes (MDA), which are one of the secondary lipid peroxidation products, whose quantification represents a measure of the extent of lipid degradation (Ruberto and Baratta, 2000). In TBARS assay, the

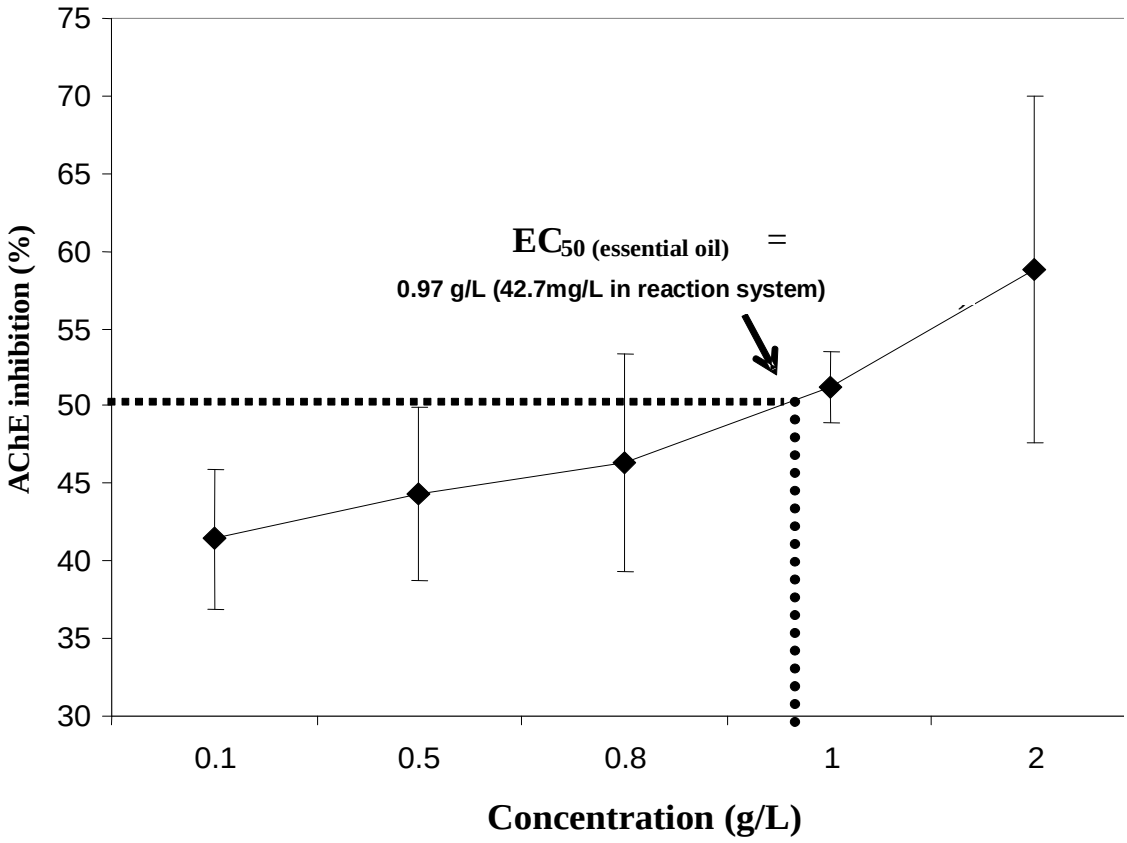

Figure 1. The Acetylcholinesterase inhibitory activity of the *P. nigra* ssp. *dalmatica* essential oil.

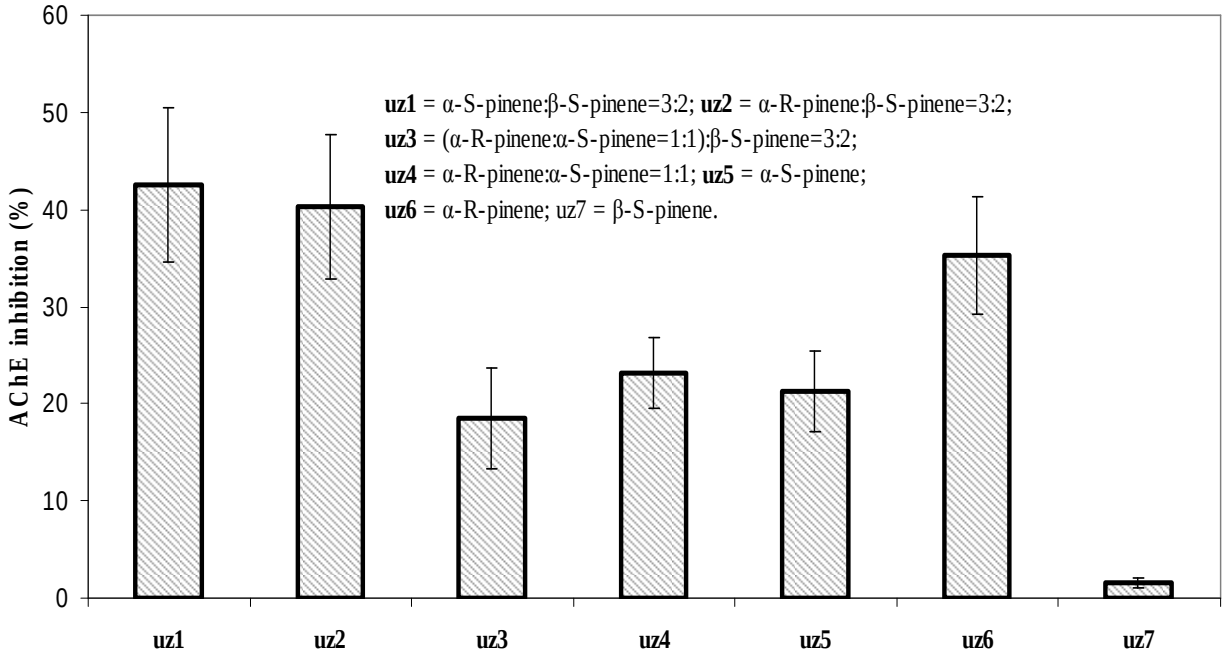

Figure 2. The Acetylcholinesterase inhibitory activity of α- and ß- pinene enantiomers mixtures.

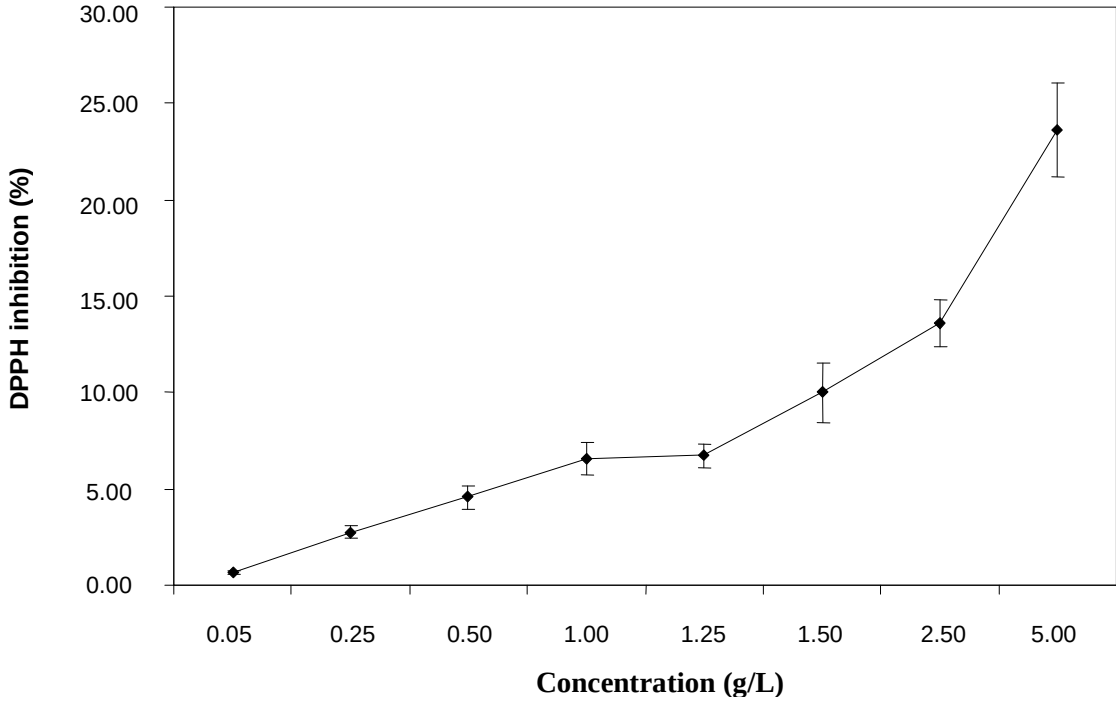

Figure 3. The DPPH radical scavenging activity in the presence of the *P. nigra* ssp. *dalmatica* essential oil.

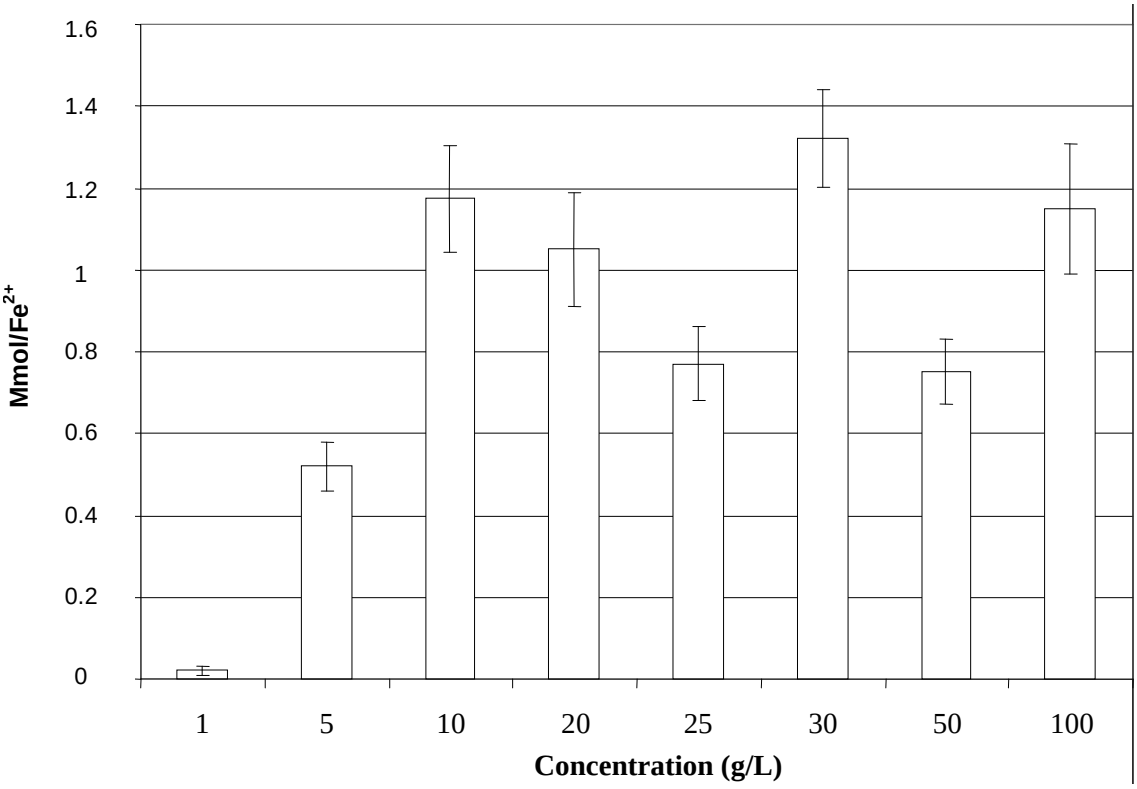

Figure 4. Antioxidant activity of the *P. nigra* ssp. *dalmatica* essential oil tested by FRAP method.

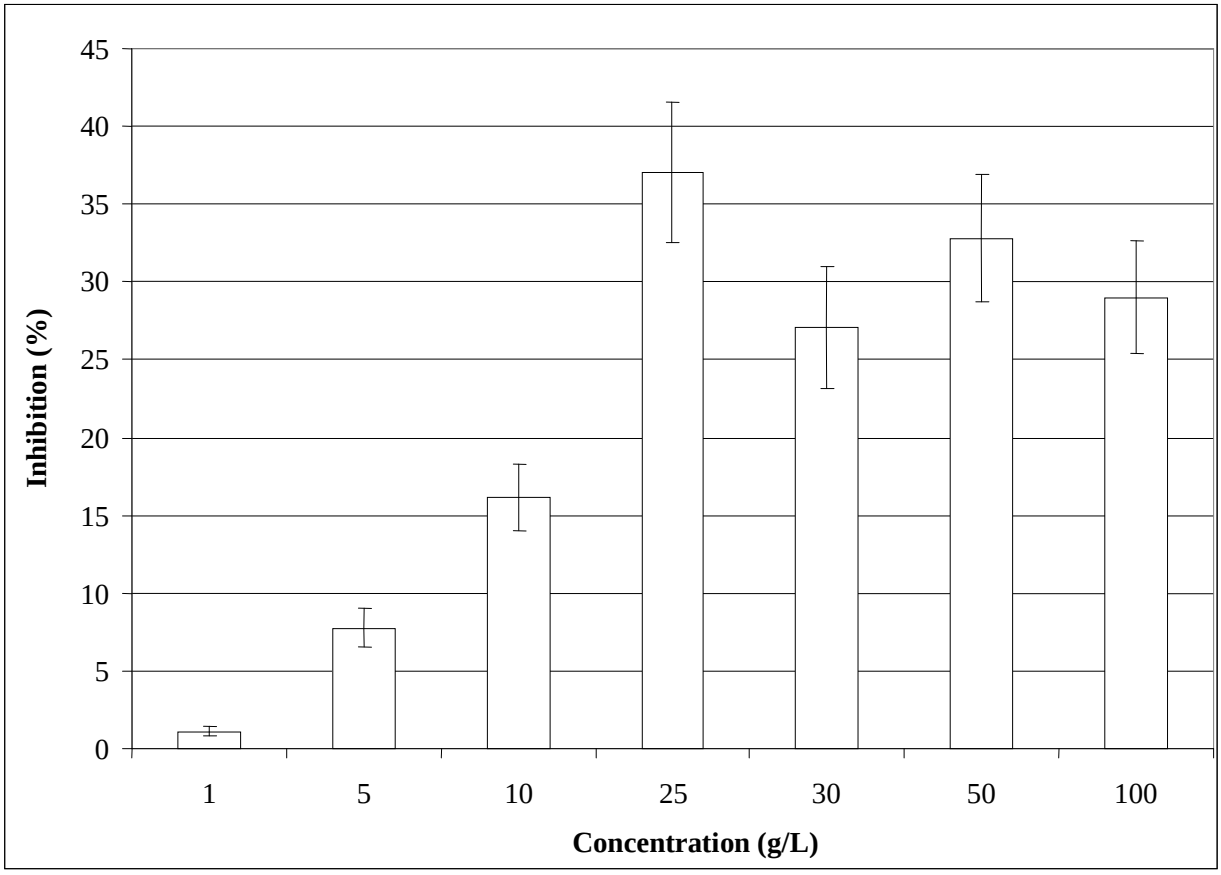

Figure 5. Antioxidant activity of *P. nigra* ssp. *dalmatica* essential oil tested by TBARS method.

highest antioxidant index of the essential oil was achieved at the concentration of 0.3 g/L in reaction system (37%), but relatively low compared to commercial antioxidants BHT and BHA (Politeo et al., 2009).

Low antioxidant activity of *P. nigra* ssp. *dalmatica* essential oil can be explained by dominant presence of monoterpene hydrocarbons which do not contain phenol groups known to be responsible for high radical scavenging effect.

Essential oil of endemic species *P. nigra* ssp. *dalmatica* has a complex chemical composition with monoterpene hydrocarbons (α- and β-pinene) and sesquiterpene hydrocarbons (germarcene D and ß-caryophyllene) as dominant components. Oil exhibits a relatively high acetylcholinesterase inhibition in comparison with other tested essential oils as well as commercial AChE inhibitor eserin. Relatively high AChE inhibitory activity of this essential oil could be explained by good AChE inhibitory activity of its main components, α- and β-pinene. Contrarily, *P. nigra* ssp. *dalmatica* essential oil does not contain compounds exhibiting significant antioxidant activity.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia, Project 011-2160547-1330. The authors thank PhD Mirko Ruscic, Faculty of Philosophy, University of Split, Croatia, for the plant material identification.

REFERENCES

Adams RP (1995). Identification of Essential Oil Components by Gas Chromatography / Mass Spectrometry. Allured Publishing Coorporation, IL.

Barbosa FJM, Medeiros KCP, Diniz MFFM, Batista LM, Athayde-Filho PF, Silva MS, da-Cunha EVL, Silva AJRG, Quintans-Júnior LJ (2006). Natural products inhibitors of the enzyme acetylcholinesterase. Rev. Bras. Farmacogn., 16: 258-285

Bartus RT, Dean RL, Beer B, Lippa AS (1982). The cholinergic hypothesis of geriatric memory dysfunction. Science, 217: 408-414.

Behl C, Lezoualc'h F, Trapp T, Widmann M, Skutella T, Holsboer F (1997). Glucocorticoids enhance oxidative stress induced cell death in hippocampal neurons *in vitro*. Endocrinology, 138: 101-106.

Benzie IFF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem., 239: 70-76.

Brand-Williams W, Cuvelier ME, Berset C (1995). Use of free radical method to evaluate antioxidant activity. Lebensm. Wiss. Technol., 28: 25-30.
Cao W, Carney JM, Duchon A, Floyd RA, Chevion M (1988). Oxygen free radical involvement in ischemia and reperfusion injury to brain. Neurosci. Lett., 88: 233-238.
Chalchat JC, Gorunovic MS (1995). Chemotaxonomy of pines native to the Balkans (III): Variations in the composition of essential oils of the *Pinus nigra* Arnold ssp. *dalmatica* according to age of specimens. Pharmazie, 50: 575-576.
Floyd RA (1999). Antioxidants, oxidative stress, and degenerative neurological disorders. Proc. Soc. Exp. Biol. Med., 222: 236-245.
Hartman D (1995). Free radical theory of aging: Alzheimer's disease pathogenesis. Age, 18: 97-119.
Halliwell B, Gutteridge JMC (1989). Free radicals in biology and medicine. University Press, Oxford, UK.
Halliwell B (2001). Role of free radicals in the neurodegenerative diseases: Therapeutic implications for antioxidant treatment. Drugs Aging, 18: 685-716.
Howes MJR, Perry NSL, Houghton PJ (2003). Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. Phytother. Res., 17: 1-18.
Jukic M, Politeo O, Maksimovic M, Milos M, Milos M (2007). *In Vitro* acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. Phytother. Res., 21: 259-261.
Kulisic T, Radonic A, Katalinic V, Milos M (2004). Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem., 85: 633-640.
Kulisic-Bilusic T, Katalinic V, Dragovic-Uzelac V, Ljubenkov I, Krisko A, Dejanovic B, Jukic M, Politeo O, Pitaf G, Milos M (2008). Antioxidant and acetylcholinesterase inhibiting activity of several aqueous tea infusions *in vitro*. Food Technol. Biotechnol., 46: 68-375.
Macchioni F, Cioni PL, Flamini G, Morelli I, Maccioni S, Ansaldi M (2003). Chemical composition of essential oils from needles, branches and cones of *Pinus pinea, P. halepensis, P. pinaster and P. nigra* from central Italy. Flavour Fragr. J., 18: 139-143.
Maciag A, Milakovic D, Christensen HH, Antolovic V, Kalemba D (2007). Essential oil composition and plant-insect relations in Scots pine (*Pinus Sylvestris* L.). Food Chem. Biotechnol., 71: 71-95
Markesbery WR (1997). Oxidative stress hypothesis in Alzheimer's disease. Free Rad. Biol. Med., 23: 134-147.
Mastelic J, Politeo O, Jerkovic I (2008). Contribution to the analysis of the essential oil of *Helichrysum italicum* (Roth) G. Don.-Determination of ester bonded acids and phenols. Molecules, 13: 795-803.
Mukherjee PK, Kumar V, Mal M, Houghton PJ (2007). Acetylcholinesterase inhibitors from plants. Phytomedicine, 14: 289-300.
Nostrandt AC, Duncan JA, Padilla A (1993). A modified spectrophotometric method appropriate for measuring cholinesterase activity in tissue from carbaryl-treated animals. Fundam. Appl. Toxicol., 21: 196-203.
Ochocka JR, Asztemborska M, Sybilska D, Langa W (2002). Determination of Enantiomers of Terpenic Hydrocarbons in Essential Oils Obtained from Species of Pinus and Abies. Pharm. Biol., 40: 395-399.
Pakaski M, Kalman J (2008). Interactions between the amyloid and cholinergic mechanisms in Alzheimer's disease. Neurochem. Int., 53: 103-111.
Persson M (2003). Chemodiversity and functions of monterpene hydrocarbons in conifers. Doctoral Thesis. Royal Institute of Technology Stockholm, Sweden.
Politeo O, Jukic M, Milos M (2010). Comparison of chemical composition and antioxidant activity of glycosidically bound and free volatiles from clove (*Eugenia caryophyllata* Thunb.). J. Food Biochem., 34(1): 129-141.
Politeo O (2009). Antioxidant capacity of glycosidically bound and free volatiles from selected spice plants in two lipid model systems. Chem. Nat. Compd., 45(2): 227-230.
Ruberto G, Baratta MT, Deans SG, Dorman HJD (2000). Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritime* essential oils. Planta Med., 66: 687.
Ruberto G, Baratta MT (2000). Antioxidant activity of selected essential oil components in two lipid model systems. Food Chem., 69: 167-174.
Thompson S, Lanctot KL, Herrmann N (2004). The benefits and risks associated with cholinesterase inhibitor therapy in Alzheimer's disease. Expert Opin. Drug Saf., 3: 425-440.
Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982). Alzhimer's disease and senile dementia: loss of neurons in basal forebrain. Science, 215: 1237-1239.