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Acetylcholinesterase inhibitory, antioxidant, and antimicrobial activities of *Salvia tomentosa* Mill. essential oil

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ABSTRACT

Chemical composition and bioactivity of essential oil from *Salvia tomentosa* Mill. natively grown in Bulgaria were investigated. GC-MS analysis identified 60 compounds which represented 98% of the oil constituents. The prevalent constituents were monoterpenes with eight dominant compounds being identified: borneol (10.3%), β-pinene (9%), camphor (7.9%), α-pinene (6%), camphene (4%), 1.8-cineole (3.8%), α-limonene (3.5%) and β-caryophyllene (3%). The essential oil showed considerable acetylcholinesterase inhibitory activity (IC_{50}=0.28±0.06 μg/mL), comparable with that of galanthamine. Study of antioxidant activity strongly suggested that the hydrogen atom transfer reaction was preferable over the electron transfer (ORAC=175.0±0.40 μM Trolox equivalents/g oil and FRAP=1.45±0.21 mM Trolox equivalents/g oil). The essential oil showed moderate antifungal and antibacterial activities against *Candida albicans* and Gram-positive bacteria, whereas it was almost inactive against the investigated Gram-negative strains. The results suggested that the essential oil of Bulgarian *S. tomentosa* could be considered as a prospective active ingredient for prevention of oxidative stress-related and neurodegenerative disorders in aromatherapy. Because of the high antioxidant capacity, the oil could be considered as natural supplement or antioxidant in cosmetics and food products.

Key words: Balsamic sage, antibacterial, aromatherapy, anti-acetylcholinesterase

Abbreviations

AD - Alzheimer's disease
ABTS - 2,2'azinobis (3)-ethylbenzthiazoline-6-sulfonic acid;
CUPRAC - cupric reducing antioxidant capacity;
DPPH - 1,1-diphenyl-2-picrylhydrazyl radical;
FRAP - ferric reducing antioxidant power;
GAL – galanthamine;
HAT - hydrogen atom transfer;
ORAC - oxygen radical antioxidant capacity;
SET – single electron transfer;
MIC - minimum inhibitory concentration;
MFC - minimum fungicidal concentration;
MBC - minimum bactericidal concentration;
IC_{50} - half maximal inhibitory concentration;
IR - inhibition rate.

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Introduction

Essential oils are complex mixtures of volatile chemicals, obtained by distillation of aromatic plants. As carriers of aroma, the essential oils are core ingredients in perfumery, cosmetics and food flavors. In addition, their constituents usually possess remarkable antimicrobial and antioxidant activities, which convert them into attractive therapeutic substances for application in alternative medicine techniques such as aromatherapy and phytotherapy (Dobetsberger & Buchbauer, 2011). The pharmaceutical value of essential oils has been additionally supported by the recent discovery of their potential to inhibit acetylcholinesterase enzyme. Currently, they are considered as prospective candidate drugs in prevention of brain associated neurodegenerative disorders, such as Alzheimer's disease (AD) (Ferreira et al., 2006; Dohi et al., 2009; Dobetsberger & Buchbauer, 2011; Chaiyana & Okonogi, 2012). Nowadays, the pharmacotherapy of AD is based on treatment with cholinergic drugs, mostly galanthamine (Georgiev et al., 2012; Ivanov et al., 2012). AD is closely associated with inflammatory processes associated with generation of reactive oxygen species (ROS) and consecutive damage of neuron cells (Şenol et al., 2010). Since antioxidants can scavenge ROS, their administration could be useful in prevention of AD symptoms (Eskici & Axelsen, 2012; Heo et al., 2013).

Plants from *Salvia* genus have a long history in traditional medicine of many cultures (Marchev et al., 2012; Georgiev et al., 2013). Infusions of various *Salvia* species have been widely used for treatment of simple brain disorders and enhancing memory functions (Şenol et al., 2010). Recent research outlined the possible benefits of essential oils and extracts from *Salvia* in prevention of neurodegenerative disorders, because of their potential acetylcholinesterase inhibitory and antioxidant activities (Perry et al., 1996; Perry et al., 2000; Savelev et al., 2004; Kivrak et al., 2009; Şenol et al., 2010).

S. tomentosa Mill. (Lamiaceae), popular as "balsamic sage", is traditionally used for preparation of high quality sage tea. In folk medicine, the plant is popular for treatment of stomachache, asthma, cold and inflamed wounds (Aşkun et al., 2010; Georgiev et al., 2011b). According to recent study, the essential oil and non-polar fractions of *S. tomentosa* plants, collected from different regions of Turkey, showed strong antimicrobial activities, whereas the polar extracts exhibited better antioxidant action (Haznedaroglu et al., 2001; Tepe et al., 2005). Şenol et al. reported that only dichloromethane extract from *S. tomentosa* leaves had AChE inhibitory activity, whereas the methanol and ethyl acetate extracts showed better antioxidant activities (Şenol et al., 2010). However there is no available data for complex evaluation of antimicrobial, antioxidant and AChE inhibitory activities of essential oil from *S. tomentosa.* Moreover, the possible effects of synergism and/or antagonism on AChE inhibitory activity of cholinergic drugs in combination with essential oil have not been investigated yet. In this study we reported the essential oil composition of *S. tomentosa* grown in Bulgaria, its AChE inhibitory, antioxidant and antimicrobial activities. The effect of essential oil on *in vitro* AChE inhibitory activity of galanthamin was investigated and existence of cumulative interaction was demonstrated and discussed for the first time.

Materials and Methods

Essential oil distillation

Fresh leaves of *S. tomentosa* were collected from experimental field-growing collection of Institute for Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences in June 2010. The plants were identified and grown by Dr. Milena Nikolova, Institute for Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences. One hundred grams of leaves were cut in pieces (1-2 cm long) and the essential oil was obtained by water distillation in Clevenger type laboratory glass apparatus according to British Pharmacopoeia, modified as described elsewhere (Balinova & Diakov, 1974). The moisture of leaves was analyzed according to Russian Pharmacopoeia. Distillation was performed for 2.3 h, and the oil was stored at 4°C prior the analyses.

GC/MS Analyses

One microliter of the essential oil was injected into Agilent 7890A gas chromatograph, coupled with Agilent 5975C mass spectrometer. Split ratio of 10:1 was used. The injector temperature was 250°C and the flow rate of carrier gas (helium) of 1.0 mL/min was used. Separation of compounds was on HP-5MS column (30 m / 250 μM / 0.25 μM) and the temperature program used was as follow: 60°C for 2 min, increase from 60°C to 260°C with the step of 5°C/min and then hold on 260°C for 8 min. EI/MS were recorded at 70 eV. MS source was 230°C and MS quad was 150°C. The RIs of compounds were recorded with a standard

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n-hydrocarbon calibration mixture (C9-C36, Restek, Teknokroma, Spain) using AMDIS 3.6 software. Identification of compounds was done by RI and mass spectra, compared to published data in NIST 08 database (NIST Mass Spectral Database, Version 5.0 - 2008). The relative percentages of separated compounds were determined from the total ion current by computerized integrator.

Acetylcholinesterase inhibitory activity assay

Sample of the essential oil was dissolved in 99.9% methanol (Merck) to final concentrations of 3, 5, 8 and 11 µg/mL. Higher concentrations were not tested because of formation of colloid system. Acetylcholinesterase inhibitory assays were performed by using colorimetric method as described previously (Georgiev et al., 2011a). For the assay 0.86 U AChE (type VI-S, Sigma) were dissolved in 1 mL 50 mM phosphate buffer (pH 8), supplied with 0.15 M NaCl (Sigma) and 0.05% (v/v) Tween 80 (Duchefa, The Netherlands). 20 µL of prepared enzyme solution were added into 2 mL 50 mM phosphate buffer (pH 8) mixed with 20 µL of methanol solution of essential oil with corresponding concentrations. The samples were incubated at 4ºC for 20 min in darkness, then the reaction was started by adding 20 µL 6 mM (in 50 mM phosphate buffer pH 7) acetylthiocholine iodide (Sigma) and 20 µL 5 mM (in 50 mM phosphate buffer pH 7) 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma). Samples were vigorously mixed on vortex and incubated at 37ºC for 20 min in darkness, then cooled down in ice. 20 µL of 1.8 mM (in 50 mM phosphate buffer pH 7) eserine salicylate (Sigma) was added to inactivate the enzyme. A blank sample (developed with pure methanol instead of essential oil solutions) was prepared as well. A positive controls (100% inhibition) were developed, following the same procedure, but the enzyme was fully inhibited by adding 20 µL eserine salicylate before substrate (acetylthiocholine iodide) addition. Changes in absorption at 405 nm of samples against their positive controls were measured (Shimadzu UV/Vis mini 1240, Japan) and used to calculate corresponding inhibition rates (IR, %) as described previously (Georgiev et al., 2011b). Because of inability to obtain miscible system with higher concentrations of essential oil, the half maximal inhibitory concentration (IC_{50}) was not determined by classical graphic approach, but it was calculated for each concentration by using following equation:

$IC_{50} = (G_{oil}x0.02x50)/ IR_{sample}$, µg/mL, whereas:

G_{oil} is the concentration of essential oil in current sample, µg/mL; 0.02 is the volume of sample in the reaction mixture, mL; 50 is the half inhibition rate, %; IR_{sample} is the observed inhibition rate of current sample concentration, %. The final IC_{50} value was expressed as mean value of calculated IC_{50} values in all investigated oil concentrations.

Effect of S. tomentosa essential oil on acetylcholinesterase inhibitory activity of galanthamine

For the analyses stock solution of 1.0 mg/mL galanthamin in 99.9% methanol (Merck) was prepared. The working samples were prepared by mixing a constant amount of 5 µL galanthamine solution with 3, 5, 8, 11 µg *S. tomentosa* essential oil solution and 99.9% methanol (Merck) up to final volume of 1 mL, respectively. The samples were analyzed for their abilities to inhibit AChE as described before and the altered IC_{50} values of galanthamine were calculated for corresponding mixtures. Cumulative action was expressed as percentage of improvement of galanthamine activity indicated by the decrease the amount of alkaloid, necessary to inhibit in half the activity of AChE enzyme.

Antioxidant activity determination

DPPH assay. The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma) scavenging activity assay was performed according to the procedure described by Thaipong et al. (2006) with some modifications. A solution of 0.1 mM DPPH in methanol was prepared and 2.85 mL of the solution were mixed with 0.15 mL sample. The reaction mixture was incubated at 37°C in darkness and the decrease in the absorbance was measured after 15 min at 517 nm against methanol. The antioxidant activity was expressed as mM Trolox equivalents (TE) per gram essential oil, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4 and 0.5 mM).

ABTS assay. The procedure was previously described by Re at al. (1999) and some modifications were applied. ABTS radical was generated after 16 h at ambient temperature in darkness by mixing in equal quantities of two stock solutions: 7 mM 2,2'azinobis (3)-ethylbenzthiazoline-6-sulfonic acid (Sigma) in dd H_2O and 2.45 mM $K_2S_2O_8$ (Sigma) in dd H_2O. Prior analysis, 2 mL of $ABTS^{\cdot+}$ solution were diluted to 60 mL with methanol to final absorbance of 1.1±0.02 units at 734 nm. 0.15 mL of the samples were allowed to react with 2.85 mL freshly prepared $ABTS^{\cdot+}$ solution and after 15 min at 37°C the decrease in coloration of $ABTS^{\cdot+}$ was recorded at 734 nm against methanol.

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Results were expressed as Trolox equivalent antioxidant capacity (mM Trolox equivalents per gram essential oil), using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4 and 0.5 mM).

Ferric reducing antioxidant power (FRAP). FRAP assay was conducted by following the modified method of Benzie & Strain (1996). Fresh FRAP reagent was prepared by mixing the following stock solutions: 10 parts of 300 mM sodium acetate buffer with pH 3.6; 1 part of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma) solution in 40 mM HCl and 1 part of 20 mM $FeCl_3x6H_2O$ (Sigma) solution in dd H_2O. The working sample was prepared by mixing 3 mL of FRAP reagent with 0.1 mL of investigated sample [0.025 mL essential oil dissolved in 0.475 mL 7% (w/v) solution of randomly methylated β-cyclodextrin (RMCD) in 1:1 acetone:water mixture]. Blank sample was prepared by the same way, but the sample was replaced with pure 7% (w/v) solution of RMCD in 1:1 acetone:water mixture. After 4 min at 37°C the absorption was read against the blank sample at 593 nm. The results were expressed as mM Trolox equivalents per gram essential oil, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4 and 0.5 mM).

Cupric reducing antioxidant capacity (CUPRAC) assay. The method was adapted according to Apak et al. (2004). Stock solutions of 10 mM $CuCl_2x2H_2O$ (Sigma) in dd H_2O; 1.0 M ammonium acetate buffer (pH 7.0); 7.5 mM neocuproine (Sigma) in 96% ethanol were prepared. The reaction was performed in the following order: 1.0 mL 10 mM $CuCl_2x2H_2O$ + 1.0 mL 7.5 mM neocuproine + 1 mL 1 M ammonium acetate buffer + 0.1 mL of investigated sample [0.025 mL essential oil dissolved in 0.475 mL 7% (w/v) solution of RMCD in 1:1 acetone:water mixture] + 1.0 mL methanol. A blank sample was prepared in the same order, but the essential oil was replaced with pure 7% (w/v) solution of RMCD in 1:1 acetone:water. The reaction mixture was heated to 50°C for 20 min and the absorbance was measured at 450 nm against the blank sample. Results were expressed as mM Trolox equivalents per gram essential oil, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4 and 0.5 mM).

Oxygen radical antioxidant capacity (ORAC) assay. The lipophilic Oxygen Radical Absorbance Capacity (L-ORAC) assay measures the antioxidant scavenging function of lipophilic antioxidants against peroxyl radical induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37°C. Fluorescein is used as a fluorescent probe. The loss of fluorescein fluorescence is an indication of the extent of damage from its reaction with the peroxyl radical. The detailed procedure of the assay was described by Huang et. al. (2002). Briefly, working solution of fluorescein (63 nM) was prepared by dissolving fluorescein disodium salt in phosphate buffer (75 mM, pH=7.4). The total reaction mixture volume was 200 µL and all solutions were prepared in a phosphate buffer (75 mM, pH=7.4). Seven percent solution (w/v) of randomly methylated β-cyclodextrins (RMCD) in a 50% acetone−H_2O mixture was used as solubility enhancer of lipophilic samples. 170 µL of fluorescein solution (53.6 nM final concentration) and 10 µL of the sample were placed in the well of the 96 microplate and incubated at 37°C directly in the FLUOstar plate reader for 20 min. After the incubation 20 µL of AAPH (51.5 mM final concentration) were added rapidly using a multichannel pipette to start the reaction. The fluorescence was recorded every minute and the microplate was automatically shaken prior to each reading. A blank using 7% RMCDs solution, instead of the antioxidant and calibration solutions of Trolox in 7% RMCDs solution (6.25, 12.5, 25 and 50 µM) as antioxidant were also carried out in each assay. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) as compared to that of blank in which no antioxidant was added. The final L-ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the curve (AUC). The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. ORAC values were expressed as µM Trolox equivalents per gram oil. All measurements were performed on FLUOstar OPTIMA fluorimeter (BMG LABTECH, Offenburg, Germany) and excitation wavelength of 485 nm, and emission wavelength of 520 nm were used.

Antimicrobial activity assay

Antimicrobial activity of *S. tomentosa* essential oil was tested against reference strains *Staphylococcus aureus* ATCC 6538, *E. coli* ATCC 8739, *Salmonella abony* ATCC 6017, *Pseudomonas aeruginosa* ATCC 9627 and *Candida albicans* ATCC 10231. Clinical isolates of *Staphylococcus epidermidis*, *Salmonella abony* and *Candida albicans*, and food isolates of *E. coli* and *Pseudomonas aeruginosa* were also used.

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The strains are deposited in microbial culture collection of Department "Biochemistry and microbiology", Biological faculty, Plovdiv University. Serial broth dilution method was used to evaluate the antimicrobial activity of *S. tomentosa* essential oil according to CLSI (Clinical Laboratory Standard Institute) recommendations (CLSI, 2006, 2008). The inoculum was prepared by transferring 5 to 6 24h old single colonies of each strain into sterile saline solution to final optical density (OD) of 0.5 McFarland units, corresponding to 1.10^6-5.10^6 cfu/mL. The resulted stock solutions were diluted (1:100 v/v) to obtain working suspensions. The analyses were carried out in Nunc™ 96-well microtitration plates. Prior analyses, the essential oil was dissolved in dimethyl sulfoxide (DMSO, Sigma) to obtain varying final concentrations in microtitration wells between 0.125 mg/mL and 16 mg/mL. Each well was filled with 90 µL RPMI 1640 (Sigma) nutrient medium, buffered to pH 6.9-7.1 with 3-N-morpho-linepropanesulfonic acid (MOPS) and 90 µL of working microbial suspensions. After 15 min incubation, 10 µL of diluted essential oil in each investigated concentrations were added to corresponding wells. The plates were cultivated at 37°C for 18-20 h in a case of bacterial cultures and for 34-48 h in the case of investigated yeast strains. Control wells contained only culture medium, inoculum and equal amounts of DMSO without essential oil. Minimum inhibitory concentration (MIC) was defined as the concentration at which no microbial growth was detected. To determine minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of *S. tomentosa* essential oil, 100 µL of all wells without observed growth were transferred on solid medium in Petri dishes with Nutritional Agar (HiMedia Laboratories Ltd.) for bacteria and Sabouraud Agar for yeasts and incubated at 37°C. The lowest concentration at which no growth occurred was defined as minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC), correspondingly.

Statistical analyses

Results are presented as mean ± standard deviations (SD) of tree independent analyzes (n=3), measured in triplicate for obtaining mean record values. Data analysis was done by using Sigma Plot 2007 software. The significance of the differences between means (presented as small letters in superscript) were determined by one-way ANOVA with Duncan posttest (P=0.01) by using SPSS Statistic 17.0.

Results

Chemical composition of essential oil from Bulgarian S. tomentosa

Water distillation of fresh *S. tomentosa* leaves (67% moisture) yields in 0.96% v/w (dry weight) essential oil. Chemical composition of produced oil was analyzed by GC/MS. Sixty compounds were recognized, representing 98.0% of the total oil constituents (Table 1). The major compounds (above 3%) were borneol (10.3%), β-pinene (9.0%), camphor (7.9%), α-pinene (6.0%), camphene (4.0%), 1.8-cineole (3.8%), α-limonene (3.5%) and β-caryophyllene (3.0%) (Table 1).

Identified constituents belong to monoterpenes, sesquiterpenes, aromatics and other cycloaliphatics (non-terpenoids, structurally related to cyclic terpenes) classes.

The major monoterpenes include: hydrocarbons (47.5%), alcohols (23.0%), aldehydes and ketones (17.1%), ethers (8.3%), and esters (4.1%). Sesquiterpenes are presented by hydrocarbons (67.1%), alcohols (23.6%), ethers (6.4%), aldehydes and ketones (2.9%).

Acetylcholinesterase inhibitory activity of S. tomentosa essential oil

The ability of *S. tomentosa* essential oil to inhibit AChE has been studied and its half maximal inhibitory concentration (IC_{50}) was determined. The obtained IC_{50} value was compared to that of the well-known AChE inhibitor galanthamine (GAL) (Figure 1). The statistical analysis showed no significant differences (P=0.01) between obtained IC_{50} values of investigated essential oil (0.28±0.06 µg/mL) and galanthamine (0.28±0.01 µg/mL) (Figure 1).

Effect of S. tomentosa essential oil on acetylcholinesterase inhibitory activity of galanthamine

To study the effect of *S. tomentosa* essential oil on AChE inhibitory activity of galanthamine, various concentrations of essential oil were added to fixed amount (5 µg/mL) of galanthamine. The results are presented in Figure 2.

The data showed a significant decrease in IC_{50} values of galanthamine (from 0.19 µg/mL to 0.11 µg/mL, correlated with the increase of essential oil concentration from 3 µg/mL to 11 µg/mL (Figure 2). It should be pointed that, the addition of 8.0 µg *S. tomentosa* essential oil contributed to 51.1% improvement in galanthamine effectiveness against AChE (Figure 2).

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Table 1. *Relative percentages of the compounds identified in the essential oil from Salvia tomentosa Mill. leaves.*

№	Compound	RI*	RI**	%	№	Compound	RI	RI**	%
1	Tricyclene	922	924 **a**	0.2	**31**	Bornyl acetate	1277	1286 **a**	1.5
2	Dehydrosabinene	927	956 **a**	0.6	**32**	α-Cubebene	1356	1351 **a**	0.6
3	α-Pinene	936	936 **a**	6.0	**33**	Neryl acetate	1366	1368 **i**	0.2
4	Camphene	943	951 **a**	4.0	**34**	α-Ylangene	1392	1374 **a**	0.4
5	(+)-Sabinene	964	974 **a**	0.3	**35**	α-Copaene	1397	1376 **a**	0.8
6	β-Pinene	970	978 **a**	9.0	**36**	Geranyl acetate	1402	1392 **j**	0.9
7	β-Myrcene	979	990 **a**	1.8	**37**	β-Bourbonene	1408	1406 **k**	1.1
8	α-Phellandrene	997	1005 **a**	0.4	**38**	cis-Jasmone	1414	1430 **l**	0.2
9	α-Terpinene	1012	1016 **a**	0.2	**39**	Dihydro- γ-ionone	1419	1417 **m**	0.5
10	p-Cymene	1018	1025 **a**	2.7	**40**	β-Caryophyllene	1424	1420 **a**	3.0
11	α-Limonene	1028	1028 **b**	3.5	**41**	β-Cobaene	1444	1430 **n**	0.9
12	1.8-cineole	1030	1030 **b**	3.8	**42**	α-Caryophyllene(α-Humulene)	1456	1452 **j**	2.6
13	β-trans-Ocimene	1034	1037 **b**	2.0	**43**	γ-Muurolene	1490	1477 **a**	2.6
14	β-cis-Ocimene	1042	1048 **b**	1.8	**44**	Germacrene D	1495	1480 **a**	2.8
15	γ-Terpinene	1047	1059 **b**	0.5	**45**	β-Eudesmene (β-Selinene)	1508	1494 **o**	0.3
16	Terpinolene	1052	1052 **c**	0.2	**46**	α-Muurolene	1519	1517 **k**	0.6
17	cis-Linalyl Oxide	1069	1071 **d**	1.5	**47**	γ-Cadinene	1535	1526 **o**	1.9
18	β-Linalool	1081	1097 **b**	1.4	**48**	δ-Cadinene	1544	1541 **k**	2.7
19	(-)-Thujone	1096	1091 **e**	0.3	**49**	α-Calacorene	1567	1560 **p**	0.3
20	trans-2-Menthen-1-ol	1112	1114 **f**	tr***	**50**	Cubenol	1577	1574 **c**	0.4
21	(-)-trans-Pinocarveol	1131	1131 **g**	0.9	**51**	(-)-Cedreanol	1595	1599 **c**	0.5
22	Camphor	1141	1139 **b**	7.9	**52**	Germacrene D-4-ol	1601	1595 **q**	0.9
23	Pinocamphone	1151	1159 **a**	0.3	**53**	(-)-Spathulenol	1607	1619 **r**	0.8
24	Pinocarvone	1158	1161 **a**	0.5	**54**	Caryophyllene oxide	1622	1632 **l**	2.0
25	Borneol	1170	1165 **a**	10.3	**55**	β-Guaiene	1635	1663 **s**	0.4
26	Isopinocamphone	1177	1173 **h**	0.7	**56**	Ledol	1643	1620 **t**	1.1
27	(-)-Terpinen-4-ol	1187	1178 **a**	0.7	**57**	tau-Cadinol	1658	1639 **j**	1.6
28	α-Terpineol	1199	1191 **a**	0.8	**58**	δ-Cadinol	1666	1651 **s**	0.5
29	(-)-Myrtenol	1212	1194 **a**	0.4	**59**	α-Cadinol	1672	1650 **j**	1.7
30	(-)-Verbenone	1221	1206 **a**	0.7	**60**	(+)-Valeranone	1690	1679 **u**	0.9

a- (Salido et al., 2002); **b**- (Siani et al., 1999); **c**- (Tzakou et al., 2007); **d**- (Sibanda et al., 2004); **e**- (Weyerstahl et al., 1997); **f**- (Raina et al., 2003); **g**- (Gkinis et al., 2003); **h**- (Adams et al., 1998); **i**- (Oliveira et al., 2006); **j**-(Hazzit et al., 2006); **k**-(Jalali-Heravi et al., 2006); **l**- (Sarikurkcu et al., 2008); **m**- (Todua, 2011); **n**- (Kukić et al., 2006); **o**- (Yang et al., 2009); **p**- (Gauvin et al., 2004); **q**- (Karlsson et al., 2009); **r**- (Özel et al., 2008); **s**- (Babushok et al., 2011); **t**- (Ramalho et al., 1999); **u**- (Kundakovic et al., 2007).
* - Retention Index (n-alkane series Kovats indices); ** - Referent Retention Index; *** - traces.

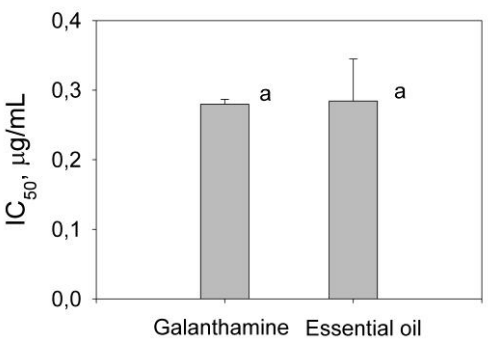


Figure 1. *Acetylcholinesterase inhibitory activities of essential oil from Bulgarian S. tomentosa Mill. and galanthamine (IC_{50} values). Presented values are mans with standard deviations of three independent analyses (n=3). Small letters in superscript represent the absence of significant differences between means according to ANOVA with Duncan post-test (P=0.01)*

Antioxidant activity of S. tomentosa essential oil

Antioxidant properties of essential oils from *Salvia* plants are well documented (Tepe et al., 2005; Kivrak et al., 2009; Şenol et al., 2010). In this paper, five experimental methods based on different reaction mechanisms were applied to achieve a comprehensive evaluation of antioxidant activity of *S. tomentosa* essential oil. The oil showed high capacities in scavenging free radicals (1.76±0.36 and 22.09±4.19 mM Trolox equivalents/g oil, by DPPH and ABTS methods, respectively), reducing ferric (1.45±0.21 mMl Trolox equivalents/g oil) and cupric (26.08±0.21 mM Trolox equivalents/g oil) ions, and absorbing peroxyl radical (ORAC value of 175.0±0.40 µM Trolox equivalents/g oil) (Table 2).

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Table 2. *Antioxidant activity of essential oil from Salvia tomentosa Mill. leaves and some referent standards. Presented values are means with standard deviations (n=3).*

Sample	DPPH (mM TE/g)	ABTS (mM TE/g)	FRAP (mM TE/g)	CUPRAC (mM TE/g)	ORAC (μM TE/g)
Essential oil	1.76±0.36	22.09±4.19	1.45±0.21	26.08±0.21	175.50±0.40
α-pinene	4.76±0.21	8.06±0.11	6.35±0.05	82.94±0.37	24.8±1.50
β-pinene	4.23±0.32	6.31±0.06	4.48±0.10	35.47±0.55	35.5±2.10
Camphor	4.64±0.26	7.40±0.11	1.12±0.25	44.06±0.55	3.4±0.20
Caryophyllene	18.52±0.21	184.18±1.72	59.05±0.35	87.75±0.55	551.8±25.40

Table 3. *Antimicrobial activity (Minimum Bactericidal Concentration and Minimum Fungicidal Concentration) of essential oil from Bulgarian Salvia tomentosa Mill. Presented values are means from three independent experiments (n=3).*

Referent strains	Origin	MBC (MFC)*	MBC (MFC)** /positive controls/	
			Ciprofloxacin	Fluconazole
Staphylococcus epidermidis	Clinical isolate	4.0	0.125	-
Staphylococcus aureus	ATCC 6538	4.0	0.125	-
Escherichia coli	Food isolate	8.0	0.25	-
Escherichia coli	ATCC 8739	8.0	0.25	-
Salmonella abony	Clinical isolate	8.0	0.50	-
Salmonella abony	ATCC 6017	8.0	0.50	-
Pseudomonas aeruginosa	Food isolate	Not active***	1.0	-
Pseudomonas aeruginosa	ATCC 9627	Not active ***	1.0	-
Candida albicans	Clinical isolate	4.0	-	0.25
Candida albicans	ATCC 10231	4.0	-	0.25

* the concentrations are in mg/mL; ** The concentrations are in μg/mL; *** no antimicrobial activity in investigated concentrations between 0.125 and 16 mg/mL.

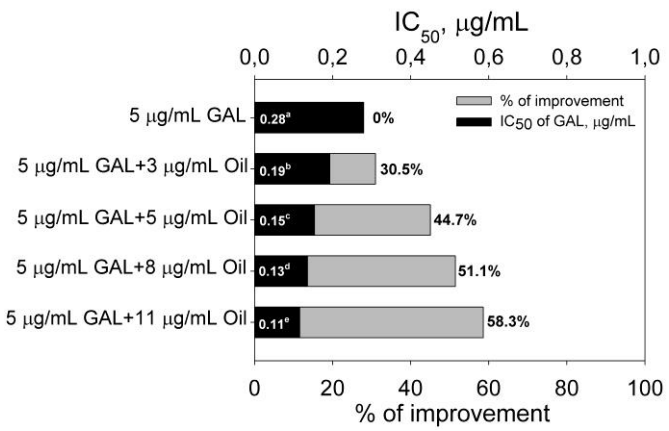


Figure 2. *Effect of S. tomentosa Mill. essential oil on acetylcholinesterase inhibitory activitiy of galanthamine (GAL) (IC_{50} values and % of improvements). Small letters in superscript represent significant differences between means according to ANOVA with Duncan post-test (P=0.01).*

Antimicrobial activity of S. tomentosa essential oil

Antimicrobial activity of *S. tomentosa* essential oil was tested against six Gram-negative bacteria strains (*Escherichia coli*, *Salmonella abony*, *Pseudomonas aeruginosa*), two Gram-positive bacteria strain (*Staphylococcus aureus*, *Staphylococcus epidermidis*), and two fungi strains (*Candida albicans*) (Table 3). The essential oil was investigated in concentrations between 0.125 mg/mL and 16 mg/mL and the minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC) were determined for susceptible strains (Table 3). The essential oil of *S. tomentosa* had a moderate antibacterial and antifungal activity against *Staphylococcus* and *Candida* respectively, but was inactive against *Pseudomonas* (Table 3).

The most sensitive strains were the Gram-positive *S. epidermidis* and *S. aureus* with MICs = 4 mg/mL, while the MIC values against the Gram-negative *E. coli* and *S. abony* were two fold higher (8 mg/mL) independently of that registered strains or clinical isolates were tested.

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Discussion

Chemical composition of essential oil from Bulgarian S. tomentosa

Chemical composition of the essential oil of *S. tomentosa* grown in Bulgaria reported in this study differs significantly from the previous reports for essential oils from *S. tomentosa* grown in other geographic locations, such as Turkey and its provinces (Tepe et al., 2005; Aşkun et al., 2010; Ulukanli et al., 2013), Hungary (Máthé & Csedő, 2007), Greece and Croatia (Putievsky et al., 1990). Moreover, the comparative analysis of available data shows the existence of high degree of variability in both oil yields and chemical composition between the essential oils from *S. tomentosa* plants, collected at different geographic locations.

Schulz et al. reported that the dominant volatile compounds in Turkish *S. tomentosa* were α-pinene and 1.8-cineole (Schulz et al., 2005). However, our results reported here are rather in agreement with the previous conclusions of Tepe et al. that the predominant constituents in *Salvia* essential oils are borneol and 1.8-cineole (Tepe et al., 2005). In addition, our research is in agreement with the earlier report and confirms the sustainable distribution of major constituents in essential oil of Bulgarian *S. tomentosa* reported by Tsankova et al. (1994). Moreover, in our study we provide an update to the number of identified minor compounds from 25 (Tsankova et al., 1994) to 52 reported here (Table 1).

Acetylcholinesterase inhibitory activity of S. tomentosa essential oil

The observed high AChE inhibitory effect is probably due to the specific chemical composition of the oil (Table 1). Study on essential oil of Spanish sage (*S. lavandulaefolia* Vahl.) and its main constituents showed that α-pinene, 1.8-cineole and camphor are the active AChE inhibitory substances (Perry et al., 2000). Acethylcholinesterase inhibitory activity of α-pinene was also confirmed by Kivrak et al. during investigation of essential oil from *S. potentillifolia* (Boiss. et. Heldr.) ex. Bentham (Kivrak et al., 2009). On the other hand, the α-pinene isomere, *β*-pinene, has been reported as effective butyrylcholinesterase inhibitor (Savelev et al., 2004; Kivrak et al., 2009). Recently, Savelev et al. demonstrated that the AChE inhibitory activity of essential oil from *S. lavandulaefolia* Vahl. have not been determined by the activity of single compound, but rater was in correlation with the complex interaction between all constituents (Savelev et al., 2004). The authors suggested that the existence of both synergistic and antagonistic interactions among volatile components were essential for observed AChE inhibitory activity of *Salvia* oils (Savelev et al., 2004).

Effect of S. tomentosa essential oil on acetylcholinesterase inhibitory activity of galanthamine

The results clearly demonstrated the existence of cumulative effect of investigated oil on AChE inhibitory activity of galanthamine. Because of the volatile nature of essential oils, they can be easily inhaled and absorbed in respiratory tract (Kohlert et al., 2000). It was demonstrated that during inhalation with rosemary essential oil, the blood concentration of 1.8-cineole was increased up to the levels of that in the air used for inhalation (Kovar et al., 1987). 1.8-cineole (one of the major compounds in our study) was found to be one of the strongest AChE inhibitor in *S. lavandulaefolia* Vahl. essential oil (Perry et al., 2000). Recently, Lima et al. demonstrated that α- and β-pinene are involved in potentiating acetylcholine-induced contractions in rat trachea (Lima et al., 2010). Inhalation with essential oils is the base of aromatherapy (Lee et al., 2012), but applied in treatment of dementia gave ambiguous results (Overshott & Burns, 2005; Nguyen & Paton, 2008). However, currently there are rather few clinical studies on therapeutic effects of aromatherapy (Lee et al., 2012). To our knowledge, this is the first report for possible interactions between volatiles in essential oils and parasympathomimetic medicins. Our results strongly suggested that the effects of essential oils used in aromatherapy should be taken into account when the patient is under medical treatment with cholinergic drugs.

Antioxidant activity of S. tomentosa essential oil

The highest values, obtained by ABTS (run in neutral pH, where the hydrogen atom transfer (HAT) mechanism is preferable) and ORAC (utilizing only HAT reaction mechanism) strongly suggested that the volatiles in *S. tomentosa* essential oil preferably utilize the hydrogen atom transfer as mechanism of their antioxidant action. The significantly lower values, obtained by DPPH method (utilizing mixed reaction types but generally running at electron transfer mechanism (SET)) and FRAP method (utilizing only SET mechanism), suggested that the electron transfer was less involved in antioxidant activity of *tomentosa* essential oil (Table 2). The unusually high value (26.08±0.21 mM Trolox eq./g oil) obtained by CUPRAC method (the system with the lowest redox potential among the tested) was probably due to the running process of redox

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cycling, which could be considered as indicator for presence of compounds with potential pro-oxidant activity. The ability of *S. tomentosa* essential oil to scavenge ROS in multiple reaction mechanisms outlines it as potential additive in prevention of oxidative stress-related conditions such as aging, chronic inflammation and neurodegenerative disorders. Our data showed that the antioxidant activities of major compounds (α- and β-pinenes) were significantly lower than the activity of minor compound caryophillene (Table 2). Thus, it could be suggested that the strong antioxidant activity of essential oil is result of positive interactions between both major and minor constituents. The unique combination of pleasant aroma with strong antioxidant properties outlines the essential oil of *S. tomentosa* as valuable antioxidant protector for application in cosmetics, pharmaceuticals and food products.

Antimicrobial activity of S. tomentosa essential oil

Our results are in agreement with previous reports, where the essential oil of *S. tomentosa* had stronger antimicrobial effect against Gram-positive than the Gram-negative bacteria (Haznedaroglu et al., 2001; Tepe et al., 2005). Other reports showed that the essential oil of *S. tomentosa* was completely inactive against *E. coli* and *P. aeruginosa* (Haznedaroglu et al., 2001; Sarac & Ugur, 2009). Recently Ulukanli et al. reported significant antibacterial activity of essential oil from *S. tomemtosa*, grown in Osmaniye, Turkey (Ulukanli et al., 2013). The authors reported MIC values ranging from 10 mg/mL (*S. aureus*, *B. subtilis* and *E. aerogenes*) to 20 mg/mL (*B. cereus*, *M. luteus*, *E. coli* and *Y. enterocolitica*) and proposed the application of *S. tomentosa* essential oil as bactericidal agent in food products (Ulukanli et al., 2013). Similar results were reported previously for essential oil from *S. tomentosa*, grown in the same location (Tepe et al., 2005). However, the reported MICs were significantly higher and the oils were less active than to the oil described in this paper. Tepe et al. also reported moderate activity of *S. tomentosa* essential oil against *C. albicans*, which is in good correlation with our results (Tepe et al., 2005) (Table 3). It is worth to be pointed that here we report a moderate antimicrobial activity of *S. tomentosa* essential oil against clinical and food isolated pathogenic strains. Food-born and multi-drug resistant microorganisms are responsible for number of diseases and are usually extremely difficult to treat.

Because of its good antimicrobial activity, *S. tomentosa* essential oil could be recommended as natural additive for enhancing the shelf life of pharmaceuticals, cosmetics and food products.

Conclusion

In this paper we reported the results of complex study on AChE inhibitory, antioxidant and antimicrobial activities of essential oil from *S. tomentosa* natively grown in Bulgaria. The oil showed a considerable AChE inhibitory activity, comparable with that of commercially available cholinergic drug galanthamine. Here for the first time we demonstrated the existence of cumulative effect between *S. tomentosa* essential oil and galanthamine, which should be taken into account if patients on cholinergic drugs are subjects to aromatherapy. However, more investigations on this direction are necessary to prove our statement. In addition, the essential oil showed high potential to scavenge ROS by utilizing various reaction mechanisms, but preferably those based on hydrogen atom transfer. The high antioxidant activity made the oil especially interesting for application as oxidative protector in food and cosmetic products or as phytopharmaceutical in prevention of oxidative stress-related conditions. The oil also showed moderate antifungal and antibacterial activities, especially against Gram-positive bacteria. The observed biological activities of *S. tomentosa* essential oil were probably due to the specific interactions and unique balanced between the major and minor presented volatiles including borneol, β-pinene, camphor, α-pinene, camphene, 1.8-cineole, α-limonene and β-caryophyllene. In addition, our study updated the available data for chemical composition of essential oil from *S. tomentosa* natively growing in Bulgaria by identifying 27 new minor compounds.

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