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Article

Comparative Study of Chemical Composition and Cholinesterase Inhibition Potential of Essential Oils Isolated from Artemisia Plants from Croatia

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Abstract: The essential oil (EO) of Artemisia plants contains a large number of bioactive compounds that are widely used. The aim of this study was to analyse the chemical composition of EOs of six Artemisia plants collected in Croatia and to test their cholinesterase inhibitory potential. GC-MS analysis of EO of *A. absinthium* showed that the dominant compounds are *cis*-sabinyl acetate and *cis*-epoxy-ocimene; in EO of *A. abrotanum* it is borneol; in EO of *A. annua* it is artemisia ketone, camphor, and 1,8-cineole; in EO of *A. arborescens* it is camphor and chamazulene; in EO of *A. verlotiorum* it is *cis*-thujone, 1,8-cineole, and *trans*-thujone; in EO of *A. vulgaris*, it is *trans*-thujone and *trans*-epoxy-ocimene. EO of the five studied Artemisia species from Croatia is rich in monoterpenoid compounds (1,8-cineole, artemisia ketone, *cis*-thujone, *trans*-thujone, *cis*-epoxy-ocimene, camphor, borneol, and *cis*-sabinyl acetate). EO of *A. arborescens* is also rich in chamazulene. The results also showed that the tested EOs have moderate cholinesterase inhibition potential, especially the EOs of *A. annua*, *A. vulgaris*, and *A. abrotanum*. This is the first analysis of the chemical composition of the EOs of four *Artemisia* plants and the first analysis of cholinesterase potential for plants collected in Croatia.

Keywords: artemisia; essential oil; GC-MS; AChE; BChE

1. Introduction

The genus Artemisia (family Asteraceae) includes a large number of species distributed in Europe, Asia, Africa and North America. Plants of the genus Artemisia are aromatic and are widely used in traditional medicine for their medicinal properties [1,2]. The genus Artemisia is of particular interest because in 2015 the Nobel Prize was awarded for the detection of the sesquiterpene lacone artemisinin in it and its antimalarial activity was demonstrated. The main constituents of Artemisia plants are mainly specific sesquiterpene lactones, essential oil, flavonoids, coumarins and phenolic acids [3]. The essential oil of these plants contains a large number of bioactive chemical compounds, which are widely used in the chemical industry as well as in medicine, cosmetics and food industry. The components of these oils show antifungal, antibacterial, and antiparasitic effects [4]. They also stimulate appetite, improve digestion by stimulating bile secretion, stimulate the liver, eliminate indigestion and flatulence. The species of this genus are used in modern medicine for their apoptosis-inducing, antitumor, and antiplasmodial effects, as well as for the treatment of viral infections [5]. It is used in the form of tea, extracts, and spirits, and the flower buds are dried and ground into powder. are used as a spice.

Essential oils (EOs) are secondary plant metabolites, characteristic ingredients of medicinal and aromatic plants. They are used in various industries and fields, from pharmaceuticals and cosmetics to food and aromatherapy [6]. Recently, the scientific community is paying more and more attention to the use of substances isolated from nature and their use in the therapy of pathological conditions.

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Alzheimer's disease (AD) is a progressive senile dementia that primarily affects the elderly. The decline in cognitive abilities is due to a deficiency of acetylcholine in the patient's brain tissue. This leads to an impairment of the patient's quality of life. There are two main forms of cholinesterase in the mammalian brain: Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE), both of which have the ability to degrade acetylcholine and butyrylcholine, respectively. AChE is found in the synaptic cleft (soluble form) and in synaptic membranes (membrane-bound form), whereas BChE is mainly associated with glial cells [7]. AChE is the major enzyme that hydrolyzes acetylcholine into choline and acetate. For this reason, inhibition of AChE is the mainstay of treatment for AD. Since existing inhibitors of these enzymes are associated with undesirable side effects, there is a constant need to research and invent new cholinesterase inhibitors isolated from nature [8].

The aim of this work was the isolation and identification of the chemical composition of EOs from six samples of plant species of the genus Artemisia, originating from the territory of Croatia: *A. absinthum*; *A. abrotanum*; *A. annua*; *A. arborescens*; *A. verlotiorum*; *A. vulgaris*. The isolated EOS were also evaluated for their ability to inhibit cholinesterase, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), to draw conclusions about the potential of the essential oils of these plants on these two enzymes, which are important in the treatment of AD. To our knowledge, this is the first report of the chemical composition of four of the six plants studied (*A. abrotanum*; *A. annua*; *A. arborescens*; *A. verlotiorum*) collected in Croatia, and the first test of the anticholinesterase potential of the EOs of *Artemisia* plants from Croatia.

2. Materials and Methods

2.1. Chemicals

Acetylcholinesterase (AChE, from Electrophorus electricus – electric eel, type V-S), Acetylthiocholine iodide (ATChI), Butyrylcholinesterase (BChE, from equine serum), Butyrylthiocholine iodide (BTChI), and 5,5-dithiobis (2-nitrobenzoicacid) (DTNB, Ellman's reagent), were purchased from Sigma-Aldrich GmbH (Steinheim, Germany); Ethanol was purchased from Kemika, Zagreb, Croatia;

2.2. Plant material

Plant parts of six different species of the genus *Artemisia* were collected immediately after full flowering at different locations in Croatia, Table 1. Collection and identification of plant material was performed by botanist Prof. Mirko Ruscic. The voucher specimens of the plant material were deposited in the Herbarium of the Department of Biology, Faculty of Natural Sciences, University of Split (AABS_2020, AABR_2021, AANN_2020, AABR_2020, AVER _2020, AVUL_2020).

Table 1. Location, coordinates and year of collection *Artemisia* plants.

Species	Species code	Locality/Year	Coordinates
			Geogr. latitude (N)
			Geogr. longitude (E)
Artemisia absinthium L.	AABS	Sinj, Croatia / 2020	43°43'27.29"
			16°40'28.29"
Artemisia abrotanum L.	AABR	Vrgorac, Croatia / 2021	43°12'36.63"
			17°24'4.83"
Artemisia annua L.	AANN	Split, Croatia /2020	43°31'34.24"
			16°28'2.95"
Artemisia arborescens (Vaill.) L.	AARB	Split, Croatia / 2020	43°30'30.5"

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Artemisia verlotiorum Lamotte	AVER	Zagreb, Croatia /2020	16°25'17.76" 45°48'59.08" 15°55'55.59"
Artemisia vulgaris L.	AVUL	Sinj, Croatia / 2020	43°43'27.29" 16°40'28.29"

2.3. Isolation of The Essential Oil

The EOSs of six different *Artemisia* plants were isolated from previously dried plant material by hydrodistillation in a Clevenger apparatus using according to the method previously described by Bektasevic et al. [9]. The isolated essential oils were filled into vials, dried over anhydrous Na_2SO_4 and stored at 4 °C until analysis.

2.4. Identification and Quantification of the Chemical Constituents of the Essential Oil by GC-MS

Separation and analysis of the essential oils from Artemisia plants was performed by GC-MS using a gas chromatograph (gas chromatograph model 8890 equipped with an automatic liquid injector model 7693A), and a tandem mass spectrometer (MS) model 7000D GC/TQ (Agilent Inc., Santa Clara, CA, USA). Chromatographic separation was performed on the nonpolar HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent Inc.). Helium was used as the carrier gas at a flow rate of 1.0 mL min, the sample injection volume was 1 μL, and the split ratio was 1:50. Analyses were performed using MS full scan (33-350 m/z). The ion source temperature was set at 230 °C, the interface temperature was set at 250 °C, and the ionization energy was 70 eV. The column temperature programme was set at 70 °C for the first 2 min and then heated to 200 °C at 3 °C/min and kept isothermal for 18 min. The analysis was performed twice, and the results are presented as the mean of the obtained results.

The compounds of the essential oils were identified by comparing their retention indices with the series of *n*-hydrocarbons (C_8-C_{40}) analyzed under the same conditions as the essential oil. Individual components were identified by comparing their mass spectra to library entries from two commercial databases, Wiley 7 MS library (Wiley, NY, USA) and NIST02 (Gaithersburg, MD, USA), and by comparing their mass spectra and retention indices to published data [10]. The relative proportions of oil components (%) were calculated based on the peak areas on the chromatography column. Retention indices (RI) were calculated based on the retention times of series of alkanes and using the equation of van den Dool and Kratz [11].

2.5. Cholinesterase Inhibitory Assay

Cholinesterase inhibitory activity was determined against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) at a concentration of 1 mg/mL using an ELISA microplate reader according to the Ellman method [12]. The method was based on the reaction of Ellman's reagent (DTNB) and thiocholine, yielding a yellow colored product. Enzyme activity was measured according to the method previously described by Bektasevic et al. [9] and lasted 6 min with three replicates each time. The percentage of AChE / BChE enzyme inhibition by essential oils or extracts was calculated according to the following formula:

$$\% \text{ inhibition of AChE / BChE} = \{[(Ae - Abe) - (Au - Abu)]/(Ae - Abe)\} \times 100;$$

Ae—absorbance of enzyme without an inhibitor, Abe—absorbance of a blank for enzyme without a substrate, Au—absorbance of enzyme with an inhibitor, Abu—absorbance of blank for enzyme without an inhibitor

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3. Results and Discussion

In this work, the chemical composition and cholinesterase inhibition potential of the essential oils (EOs) of six Artemisia plants (*A. absinthum; A. abrotanum; A. annua; A. arborescens; A. verlotiorum; A. vulgaris*) collected in Croatia were studied.

3.1. Phytochemical Profile

EOs of six species of the genus *Artemisia* collected immediately after full flowering in Croatia were isolated from dried plant material by hydrodistillation and analysed by coupled gas chromatography-mass spectrometry system (GC-MS).

The chemical composition of the essential oils is given in Table 2, while the GC-MS total ion chromatograms are shown in Figure 1. The compounds in the Table 2 are grouped by compound class and by ascending retention index (RI).

Table 2. The chemical composition of *Artemisia* essential oils from Croatia.

		AABS	**AABR**	**AANN**	**AARB**	**AVER**	**AVUL**
% EO (w/w)		0.5	1.6	0.6	1.1	0.3	0.2
Compounds	**RI**						
ß-thujene	928	1.9	-	0.9	-	-	-
α-pinene	937	0.4	1.7	1.2	1.6	0.5	1.2
camphene	952	-	7.0	2.9	2.4	-	0.5
ß-pinene	976	-	-	1.3	-	1.4	2.3
sabinene	979	-	5.2	0.9	-	0.2	1.8
2-pentylfuran	989	-	0.9	-	-	-	-
ß-myrcene	993	-	-	-	2.1	-	-
α-phellandrene	1006	-	-	-	-	-	0.7
α-terpinene	1018	-	-	0.4	0.8	-	0.2
p-cymene	1027	0.8	1.9	0.4	0.5	1.2	2.9
limonene	1031	-	-	-	-	0.2	0.4
γ-terpinene	1061	-	-	0.6	1.4	0.3	0.4
Monoterpenes		**3.1**	**16.7**	**8.6**	**8.8**	**3.8**	**10.4**
yomogi alcohol	1000	-	-	1.2	-	-	-
1,8-cineole	1034	1.0	3.0	16.2	-	10.9	3.2
artemisia ketone	1065	-	-	22.3	-	-	-
cis-sabinene hydrate	1069	-	-	0.3	1.5	0.3	0.3
artemisia alcohol	1086	-	-	3.2	-	-	-
linalool	1100	0.8	-	1.5	-	-	-
trans-sabinene hydrate	1102	-	-	-	-	0.3	-
trans-3-caren-2-ol	1103	-	-	-	-	0.5	-
cis-thujone	1107	3.1	-	-	-	46.3	5.6
trans-thujone	1118	0.9	-	-	-	9.0	40.3
cis-p-menth-2-en-1-ol	1122	-	0.9	0.3	-	0.4	-
chrysanthenone	1127	-	4.7	0.3	-	-	-
cis-epoxy-ocimene	1136	28.8	-	-	-	-	15.5
ß-pinone	1139	-	0.8		-	-	-
trans-p-menth-2-en-1-ol	1141	-	0.8	0.6	-	0.5	-
trans-sabinol	1142	0.8	-	0.6	-	-	0.2
trans-epoxyocimene	1143	1.1	-	-	-	-	-
camphor	1146	-	9.5	22.0	39.5	0.7	2.7

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ß-pinene oxide	1160	-	-	1.2	-	0.5	-
pinocarvone	1165	-	0.9	0.6	-	0.4	-
borneol	1167	-	48.0	0.3	0.5	0.3	0.7
lavandulol	1169	-	-	0.4			
terpinen-4-ol	1178	0.8	-	1.2	3.2	0.7	0.5
trans-p-mentha-1(7),8-dien-2-ol	1188	0.5	-	-	-	-	-
α-terpineol	1191	-	-	0.5	0.4	-	-
myrtenol	1196	-	-	-	-	0.3	0.4
myrtenal	1202	-	0.7	0.8	-	-	-
trans-piperitol	1210	-	1.0	-	-	-	-
trans-carveol	1220	-	-	-	-	2.3	-
neral	1229	-	-	-	-	0.3	-
carvotanacetone	1245	-	-	-	-	0.7	-
cis-chrysanthenyl acetate	1264	1.8	-	-	-	-	-
perilla aldehyde	1275	-	-	-	-	0.5	-
isobornyl acetate	1287	-	3.6	-	-	-	-
thymol	1293	-	0.5	-	-	-	-
perilla alcohol	1297	-	-	0.2	-	0.3	-
cis-sabinyl acetate	1299	38.5	-	-	-	-	-
Monoterpenoids		**78.1**	**74.4**	**74.0**	**45.5**	**75.2**	**69.4**
α-copaene	1377	-	-	1.1	-	-	0.3
ß-bourbonene	1385	-	-	-	-	-	0.2
ß-caryophyllene	1419	1.3	-	1.3	0.5	5.8	1.3
α-humulene	1454	-	-	-	-	0.9	0.1
γ-muurolene	1477	-	-	0.5	-	-	0.3
γ-himachalene	1480	0.4	-	-	-	-	-
α-amorphene	1484	0.5	-	-	-	-	-
germacrene D	1485	-	-	0.4	1.1	1.1	-
ß-selinene	1486	1.5	-	2.3	-	0.3	1.2
α-selinene	1496	-	-	-	-	0.3	-
δ-cadinene	1524	-	-	-	-	0.4	-
Sesquiterpenes		**3.7**	**0.0**	**5.6**	**1.6**	**8.8**	**4.4**
spathulenol	1577	-	-	0.2	-	1.5	-
caryophyllene oxide	1582	1.3	-	5.3	-	6.0	2.5
davanone	1589	-	-	-	-	-	3.2
humulene epoxide	1608	-	-	0.3	-	0.4	1.1
α-copaen-4-ol	1611	-	-	0.3	-	-	0.3
10-epi-*γ*-eudesmol	1623	-	-	0.7	-	-	-
longifolenaldehyde	1629	-	-	0.6	1.8	-	0.8
torreyol	1655	-	-	-	-	0.5	3.7
cubenol	1656	-	-	0.2	-	-	-
ß-bisabolol	1671	-	-	0.3	-	-	-
eudesma-4,15(7)-dien-1ß-ol	1685	-	-	0.3		0.4	0.2
chamazulene	1728	0.8	-	-	33.9	-	0.2
Sesquiterpenoids		**2.1**	**0.0**	**8.2**	**35.7**	**8.8**	**12.3**
hexanal	800	-	-	-	-	-	0.3
trans-2-hexen-1-ol	853	-	-	-	-	0.9	0.8
1-octen-3-ol	980	-	-	-	-	0.8	
phenylacetaldehyde	1046	-	0.8	-	-	-	0.5

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benzyl isovalerate	1388	-	-	1.6	-	-	-
eugenol	1359	-	-	0.3	-	0.3	
2-ethyl-4-methyl-1,3-pentadienyl benzene*#	1515	2.9	-	-	0.8	-	0.2
2-ethyl-4-methyl-1,3-pentadienyl benzene*#	1616	4.5	-	-	1.9	-	0.6
hexadecanoic acid	1960	-	-	-	-	-	0.3
Other Compounds		**7.4**	**0.8**	**1.9**	**2.7**	**2.0**	**2.7**
TOTAL		**94.4**	**91.9**	**98.3**	**94.3**	**98.6**	**99.2**

AABS-*A. absinthum*; AABR_*A. abrotanum*; AANN_*A. Annua*; AARB-*A. arborescens*; AVER-*A. verlotiorum*; AVUL-*A. vulgaris*; * correct isomer is not identified; # identification performed only on the basis of MS and confirmed on the basis of the identification previously performed by Juteau et al. [14]; "-"- not identified. The presented results are given as the mean of two ranalyses.

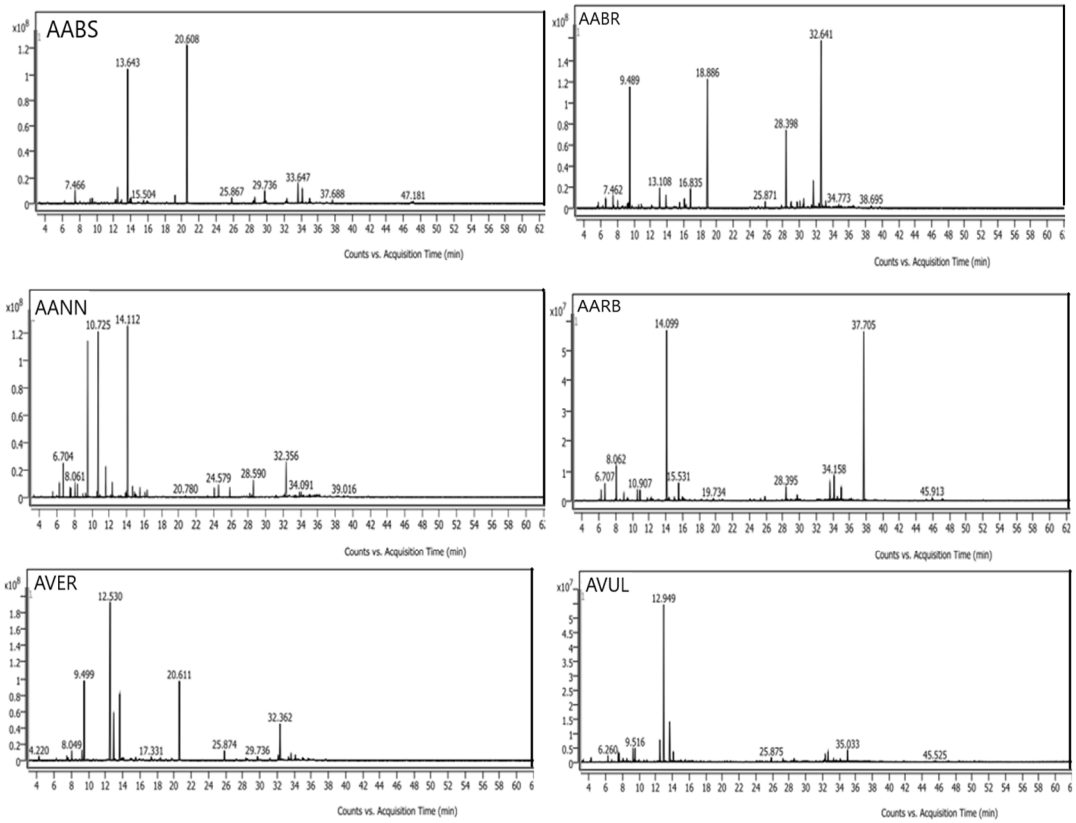

Figure 1. GC-MS total ion chromatograms of Artemisia essential oils (AABS-*A. absinthum*; AABR-*A. abrotanum*; AANN-*A. Annua*; AARB-*A. arborescens*; AVER-*A. verlotiorum*; AVUL-*A. vulgaris*).

The EOs of the studied *Artemisia* species in the dry plant material from which they were isolated ranged from 0.2% (*A. vulgaris*) to 1.6% (*A. absinthium*). The essential oil of *A. absinthium* was reddish-brown, the oil of *A. arborescens* was dark blue, while all other studied oils were yellow. The most abundant compounds in the EO of *A. absinthium* are the monoterpenoids *cis*-sabinyl acetate (38.5%) and *cis*-epoxy-ocimene (28.8%). All other components of this EO have a proportion of less than 5%. The monoterpenoids are present in this EO in a high proportion of 78.1% (w/w). This is followed by other compounds (7.4%), sesquiterpenes (3.7%), monoterpenes (3.1%), and sesquiterpenoids (2.1%).

According to Orav et al. [13], four chemotypes characteristic of *A. absinthium* growing in Europe were found: sabinene- and myrcene-rich oil, α- and ß-thujone-rich oil, epoxy-ocimene-rich oil, and (*E*)-sabinyl acetate-rich oil. Some mixed chemotypes were also found. According to this classification, the oil isolated from the plant collected in Croatia belongs to the mixed chemotype (epoxy-ocimene-rich oil and (*E*)-sabinyl acetate-rich oil).

The EO of this plant species collected in **Croatia** (it is not specified where, full flowering, dried and powdered) was previously analyzed by Juteau et al. [14]. Analysis of this oil revealed *ß*-thujone (26.0%), (*Z*)-6,7-epoxyocymene (9.0%), linalool (5.9%), and sabinene (5.5%) as the main constituents. All other constituents of this oil were present in amounts less than 4.5%.

Analysis of EO of this plant collected in the southern part of neighboring **Serbia** (Bela Palanka and Nis, above ground and previously dried) showed that the main components are *ß*-thujone (19.8 and 63.4%), *cis*-*ß*-epoxy-ocimene (10.7 and 0.0%), *trans*-sabinyl acetate (8.8 and 0.0%), sabinene (8.1 and 10.8%), and linalyl-3-methylbutanoate (7.5 and 4.5%) [15]. The composition of the essential oil of *A. absinthium* collected in the northwestern Italian Alps, Piedmont (full bloom, air-dried) revealed *cis*-epoxyocimene (24.8%), *trans*-chrysanthenyl acetate (21.6%), and camphor (17.1%) as the main constituents [16].

The main constituent of the EO of *A. abrotanum* is the monoterpene alcohol borneol (48.0%). Camphor (9.5%), camphene (7.0%), sabinene (5.2%), and chrysanthenone (4.7%) are also present in significant proportions. Other identified constituents of this EO account for less than 4%. The predominant compound class in this oil is monoterpenoids (74.4%). This is followed by monoterpenes (16.7%) and other compounds (0.8%).

There are many different chemotypes of *A. abrotanum* from different geographical locations ((+)-piperitone chemotype, *trans*-sabinyl acetate/*α*-terpineol chemotype, 1,8-cineole/*α*-thujene/*α*-pinene chemotype, eucalyptol chemotype, davanol/davanone/hydroxydavanon chemotype) [17]. The EO of the *A. abrotanum* from Croatia is particularly rich in borneol, and we could conclude that it is a borneol chemotype. This is not the case with any other oil of this plant.

To date, not one analysis of EO of this plant species collected in Croatia has been performed. Two analyzes of EO of this plant has been performed in neighboring countries, Austria and Italy. The results of the Austrian EO analysis (plant from the Botanical Garden of the University of Veterinary Medicine Vienna, Austria, in full bloom) showed that the most abundant components of this EO are the derivative davanone (22.5%) and 4-methyl-pent-2-enolide (15.7%) [18]. The EO composition of *A. abrotanum* from the northwestern Italian Alps, Piedmont (full bloom, air-dried) revealed 1,8-cineole (34.7%), bisabolol oxide (18.4%) and ascaridol (16.0%) as the predominant components [16].

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The monoterpenoids artemisia ketone (22.3%), camphor (22.0%), and 1,8-cineole (16.2%) were identified as the dominant constituents of EO from *A. annua*. Caryophyllene oxide (5.3%) and artemisia alcohol (3.2%) were also identified with lower proportions. All other constituents of this EO were present in minor proportions. The predominant compound class in this EO was monoterpenes (74.0%). This was followed by monoterpenes (8.6%), sesquiterpenoids (8.2%), sesquiterpenes (5.6%) and other compounds (1.9%).

Depending on the variety, the dominant compounds of EO, isolated from *A. annua*, were artemisia ketone and camphor, camphor and 1,8-cineole, *α*-pinene and pinocarvone, artemisia ketone and 1,8-cineole, and a chemotype with phenolic compounds [19]. According to the chemical composition, the EO isolated from *A. annua* collected in Croatia, belongs to the artemisia ketone/camphor/1,8-cineole chemotype.

To date, not one analysis of EO of *A. annua* collected in Croatia has been performed. A few analyzes have been performed in neighboring countries. The EO of the cultivated plant collected in spring in Bosnia and Herzegovina (Kiseljak, near Sarajevo) and previously dried contains a high percentage of artemisia ketone (30.7%) and artemisia alcohol (6.5%) [20]. The analysis of this plant species cultivated near Sarajevo, Bosnia and Herzegovina (air-dried and hydrodistillated), contains

artemisia ketone (28.3%) and camphor (16.9%) as the main components [19], while the analysis of *A. annua* harvested after flowering period from the natural habitat, air-dried and hydrodestilated after one year of storage revealed selina-3,11-dien-6α-ol (9.6%), *cis*-thujopsenoic acid (7.0%), caryophyllene oxide (7.0%) and alloaromadendrene epoxide (4.7%) as the main constituents [21]. The most abundant volatile compounds of *A. annua* EO from Serbia were artemisia ketone (25.4 %) and *trans*-caryophyllene (10.2 %), followed by 1,8-cineole, camphor, germacrene D and β-selinene [22] . Ickovski et al. [23] identified artemisia ketone (55.8%) and α-pinene (12.7%) as main components components of *A. annua* collected near Nis, Serbia (fresh aerial parts). Radulovic et al. [24] were also performed an analysis of *A. annua* EO from Serbia (Nis) (air-dried) and identified artemisia ketone (35.7%), α-pinene (16.5%) and 1,8-cineole (5.5%) as the most abundant components while The analysis of this EO collected in Belgrade, Serbia (aerial and air-dried), contains pinocarvone (29.40%), artemisia ketone (19.19%), caryophyllene oxide (5.93%), and 1,8-cineole (4.72%) as the most abundant constituents [25]. The flowering aerial parts of *A. annua* collected from the banks of the Arno River in Pisa (Italy) in late September 2015 and previously air-dried contained artemisia ketone (22.1%), 1,8-cineole (18.8%), and camphor (16.9%) as main constituents [26]. The essential oil of plants collected in Sesto Fiorentino, Italy, at the full flowering stage (fresh plant material) contained numerous constituents, of which the most important were germacrene D (21.2%), camphor (17.6%), (*E*)-β-farnesene (10.2%), (*E*)-β-caryophyllene (9%), and bicyclogermacrene (4.2%) [27]. The composition of EO of *A. nnua* collected in the northwestern Italian Alps, Piedmont (full flower, air-dried), revealed 1,8-cineole (34.7%), α-pinene (19.6%), bisabolol oxide (18.4%), ascaridole (16.0%), and camphor (15.5%) as the main constituents [16]. The chemical composition of the EO of 85 individuals of *A. annua* cultivated in Budaörs, near Budapest, Hungary, (fresh plant material) showed that the main constituents were artemisia ketone (33–75%) and artemisia alcohol (15–56%) [28].

The monoterpenoid camphor (39.5%) and the bicyclic unsaturated hydrocarbon, the sesquiterpene camazulene (33.9%), were identified as the major constituents of EO isolated from *A. arborescens*. Terpinen-4-ol (3.2%), camphene (2.4%), and ß-myrcene (2.1%) occur in lower proportions, while the other constituents of this oil occur in proportions of less than 2%. The dominant class of compounds in this oil are monoterpenoids (45.5%) and sesquiterpenoids (35.7%). They are followed by monoterpenes (8.8%), other compounds (2.7%) and sesquiterpenes (1.6%).

Different chemotypes have been identified for the essential oils of *A. arborescens*: a ß-thujone/camphor chemotype (Sardinia, Italy, around Usellus) and Morocco; a chamazulene/camphor chemotype (northwestern United States and in southern parts of Italy, Calabria, Sicily, and the Aeolian Islands); and a ß-thujone/chamazulene chemotype (Liguria (Sacco), Sicily, Sardinia, and Algeria) [29]. According to this classification, the EO isolated from the plant collected in Croatia belongs to the chamazulene/camphor chemotype.

To date, not a single analysis of the essential oil of this plant species collected in Croatia has been performed. Several analyzes of the oil of this plant have been carried out in neighboring countries. The analysis of EO of this plant (above-ground biomass of plant, blossom stage) collected from two sites in Italy (Capo Zafferano and Termini Imerese) revealed that the most abundant constituents of the EO are chamazulene (43.12 and 36.83%), ß-thujone (19.57 and 19.89%), and camphor (8.78 and 8.68%). The results of GC-MS analysis of this plant collected in Italy in three locations (Sicily, Calabria and the Aeolian Islands, Lipari) (fresh plant material, leaves; at vegetative phase; EO isolated by microwave assisted hydrodistillation) showed that the most abundant components of this EO are camphor (21.4, 39.5 and 20.1%), camazulene (37.6 27.1 and 34.6%) [31]. The EO of *A. arborescens* from Sardinia, Italy, isolated from plant material collected at three developmental stages of the plant (from vegetative state to postflowering), belongs to the ß-thujone / chamazulene chemotype. The most abundant constituents of this EO were chamazulene (51.5; 34.2 and 25.6%), ß-thujone (38.8; 33.8 and 53.2%) and germacrene D (3.2; 5.4 and 4.3%) [29]. The EOs of the aerial parts of several *A. arborescens* populations (flowering stage) collected from different sites in Sicily (Petru, Diga, Felice) were analyzed by GC–FID and GC–MS systems. β-Thujone (20.5–55.9%), chamazulene (15.2–49.4%), camphor (1.3–8.4%) and germacrene D (2.8–3.4%) were identified as the most abundant compounds of these oils [32]. The analysis of EO, isolated from the fresh plant material of this plant collected in the

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vegetative stage (January) in the northwestern part of Sicily, Italy, showed that the most abundant constituents of this oil (steam distillation) are ß-thujone (45.04%), chamazulene (22.71%), and camphor (6.78%) [33]. GC- MS analysis of this EO oil collected in Montenegro (Budva and Stari Ulcinj island) (aerial parts, air-dried) showed that the most abundant constituents were α thujone (0.0 and 28.59%), camphor (6.44 and 39.46%) and camphene (7.08 and 2.35%) [25].

The monoterpenoids *cis*-thujone (46.3%), 1,8-cineole (10.9%), and *trans*-thujone (9.0%) were identified as the predominant constituents of the EO of *A. verlotiorum*. Caryophyllene oxide (6.0%) and *ß*-caryophyllene (5.8%) were presented in slightly lower proportions. Other compounds of this oil were identified in amounts of less than 2.5%. The dominant class of compounds in this EO was monoterpenoids (75.2%). This was followed by sesquiterpenes (8.8%) and sesquiterpenoids (8.8%), as well as monoterpenes (3.8%) and other compounds (2.0%). As for the chemical composition, the analyzed essential oil from Croatia belongs to the thujone/1,8-cineole chemotype.

So far, not one analysis of EO from this plant has been performed on a plant collected in Croatia, but several EO analyzes have been performed on plant material collected in neighboring countries. Seasonal variations in the chemical composition of the oil isolated from this plant collected during the year in Pisa Province, **Italy** (aerial parts, air-dried, showed that the most abundant constituents of this oil were 1,8-cineole (12.8–32.2%), germacrene D (3.8–18.1%), *α*-thujone (2.3–8.0%), *ß*-thujone (8.3–14.7%), *ß*-caryophyllene (1.8–10.6%), borneol (3.3–9.9%), camphor (3.6–8.3%), and myrcene (0.4–11.2%) [34]. The composition of the EO of *A. verlotiorum* from the northwestern Italian Alps, Piedmont (full bloom, air-dried) revealed caryophyllene oxide (21.4%), borneol (17.6%), camphor (11.2%), 1,8-cineole (10.6%), and spathulenol (9.2%) as the main components [16].

The monoterpenoids trans-thujone (40.3%) and *cis*-epoxy-ocimene (15.5%) were identified as dominant constituents of *A. vulgaris* EO. The EO also contains *cis*-thujone (5.6%), toreiol (3.7%), davanone (3.2%), 1,8-cineole (3.2%), and other compounds in lesser amounts. The predominant compound class in this EO was monoterpenoids (69.4%). Followed by sesquiterpenoids (12.1%), monoterpenes (10.4%), sesquiterpenes (4.4%) and other compounds (2.7%). Four different chemotypes of EO from A. vulgaris were found: One with the coexistence of ar-curcumene and *α*-zingiberene; two characterized by the presence or absence of thujone and santolinatriene; and a fourth characterized by the presence of crysanthenyl acetate (40%) [35]. Accordingly, the Croatian EO of A. vulgaris belongs to the thujone chemotype.

GC-MS analysis of the EO of this plant collected in Dalmatia, Croatia, (aerial plant material, air-dried) revealed that the most abundant constituents of this oil at the of full flowering (August) were ß-thujone (20.8%), *α*-pinene (15.1%), 1,8-cineole (11.7%), camphor (8.7%), and *α*-thujone (8.5%) *trans*-chrysanthenyl acetate (18.5%), 1,8-cineole (15.2%), and *α*-phellandrene (12.9%) [36]. Chemical analysis of the essential oil of this plant, collected in the area of Niš, Serbia, at the time of full flowering, showed that the dominant compounds in the oil of the aerial part of the plant (isolated directly after drying and after one year of storage) are 1,8-cineole (28.9%), sabinene (13.7%) and *ß*-thujone (13.5%) [15]. The composition of the EO of *A. vulgaris* collected from north-west Italian Alps, Piedmont (full bloom, air-dried) revealed camphor (47.7%) as dominant compounds. In this EO, camphene (9.1%), verbenone (8.6%) and *trans*-verbenol (7.0%) were also identified as components contained in larger propositions [16].

The chemical composition of EOs of the studied plant species of the genus Artemisia (*A. absinthium, A. abrotanum, A. annua, A. verlotiorum, A. vulgaris*) revealed that the studied EOs are dominated by monoterpenoid components: 1,8-cineole, artemisia ketone, *cis*-thujone, *trans*-thujone, *cis*-epoxyocimene, camphor, borneol, *cis*-sabinyl acetate. In one plant species (*A. arborescens*), the azulene derivative chamazulene occurs as a major compound. It is a blue-violet azulene derivative biosynthesized from the sesquiterpene matricin

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3.2. Cholinesterase inhibition potential of Artemisia essential oils from Croatia

The ability of EOs from *Artemisia* plants collected in Croatia to inhibit the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was tested by the Ellman method [12]. The concentration of tested EOs in the solutions was 1 mg/mL, while the concentration of EOs in the reaction systems was 45.45 μg/mL. The results are shown in Table 3.

Table 3. Cholinesterase inhibition potential of *Artemisia* essential oils.

Inhibition %	AABS*	AABR*	AANN*	AARB*	AVER*	AVUL*	huperzineA[&]	galantamine[#]
AChE	29.7	49.6	55.2	41.1	34.3	54.4	90.7	78.60
BChE	33.8	47.0	35.8	33.5	31.4	23.0	58.8	40.9

Tested concentrations *1 mg/mL; [&]0,1 mg/mL; [#]5 μg/mL.

The essential oils isolated from *Artemisia* from Croatia show moderate ability to inhibit the enzyme AChE (29.7 – 55.2%). Among these oils, the oil of *A. annua* shows the best inhibitory effect, while the EO of *A. absinthium* shows the weakest effect on this enzyme at the tested stock solution concentration of 1 mg/mL. As expected, the inhibition of BChE by these EOs shows a slightly weaker activity compared to the inhibition of AChE, with the exception of EO from *A. absinthium*. The results obtained were compared with those of the known good inhibitors of these enzymes, huperzine A and galantamine, Table 3.

To the best of our knowledge, we report here the first results on cholinesterase inhibitory activity of selected *Artemisia* plants collected in Croatia. Only one study was conducted on the antiAChE potential of the EOs of the tested *Artemisia* species collected in the areas of neighboring countries. The flowering aerial parts of *A. annua* collected in late September in Pisa (Italy) along the Arno riverbank showed an AChE inhibition potential IC_{50}=472.4 mg/L [25].

Numerous researchers have evaluated the pure compounds included in the essential oil composition for their ability to inhibit AChE. Less pure compounds have been tested for their BuChE inhibition [37]. Despite major differences in methodology, the results of these tests showed that monoterpenoids are the most potent inhibitors of these enzymes. Among them, 1,8-cineole and camphor, which are present in greater proportions in the essential oils of *Artemisia* plants, are quite potent inhibitors, especially of AChE. It can be concluded that these are the components of the oil that can be attributed with the ability to inhibit AChE. 1,8-Cineole has also been shown to be a good BChE inhibitor. At the same time, synergistic or antagonistic effects must also be taken into account, so it is difficult to say with absolute certainty which constituents of a mixture of compounds are responsible for the biological effect [37].

Few more tests on the inhibitory potential of EOs from *Artemisia* plants on AChE/BChE were performed: *A. absinthium* collected in Pakistan [38] and Algeria [39] and *A. annua* (flowers) from China [40].

4. Conclusions

The chemical composition of EOs of the studied plant species of the genus Artemisia (*A. absinthium, A. abrotanum, A. annua, A. verlotiorum, A. vulgaris*) revealed that the studied EOs are dominated by monoterpenoid components: 1,8-cineole, artemisia ketone, *cis*-thujone, *trans*-thujone, *cis*-epoxyocimene, camphor, borneol, *cis*-sabinyl acetate. In one plant species (*A. arborescens*), the azulene derivative chamazulene occurs as a major compound.

Artemisia essential oils isolated from Croatia showed moderate ability to inhibit the enzyme AChE. Among these oils, the oil of *A. annua* showed the best inhibitory activity compared to the known ChE inhibitors galantamine and huperzine A. EO isolated from *A. vulgaris* and *A. abrotanum* also showed significant inhibitory activity, especially on AChE. Inhibition of BChE by these EOs shows a slightly weaker activity compared to the inhibition of AChE, with the exception of EO from *A. absinthium*.

This is the first analysis of the chemical composition of the EOs of four Artemisia plants studied and the first analysis of cholinesterase potential for Artemisia plants collected in Croatia.

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