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Chemical and pharmacological analysis of *Ambrosia artemisiifolia*

Ph.D. Thesis

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LIST OF PUBLICATIONS RELATED TO THE THESIS

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II. **Kovács B**, Szemerédi N, Csikós O, Kiss T, Veres K, Spengler G, Csupor-Löffler B & Csupor D.
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TABLE OF CONTENTS

ABBREVIATIONS AND SYMBOLS

1D	one-dimensional
2D	two-dimensional
CH_3CN	acetonitrile
ATCC	American Type Culture Collection
CCP	carbonyl cyanide *m*-chlorophenylhydrazine
CI	combination index
COSY	correlated spectroscopy
DMSO	dimethyl sulfoxide
EB	ethidium bromide
ED_{50}	median effective dose
EDTA	ethylenediaminetetraacetic acid
EMEM	Eagle's Minimal Essential Medium
EtOAc	ethyl acetate
EO	essential oil
FAR	fluorescence activity ratio
FPP	2*E*,6*E*-farnesyl pyrophosphate
HMBC	heteronuclear multiple-bond correlation spectroscopy
HQC	heteronuclear single-quantum coherence spectroscopy
HPLC	high-performance liquid chromatography
IFNγ	interferon gamma
IL	interleukin
JMOD	J-modulated spin-echo experiment
LB	Luria-Bertani
MeOH	methanol
MEP	2-C-methyl-D-erythritol-4-phosphate
MIC	minimum inhibition concentration
Mill.	Miller
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVA	mevalonic acid
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	nuclear magnetic resonance

NOESY	nuclear Overhauser enhancement spectroscopy
OD	optical density
PBS	phosphate buffered saline
PTFE	polytetrafluoroethylene
RF	relative fluorescence
R_f	retention factor
RP	reverse phase
RSD	relative standard deviation
SDS	sodium dodecyl sulphate
subsp.	Subspecies
STAT3	signal transducer and activator of transcription 3
TSB	tryptic soy broth
TLC	thin-layer chromatography
TNFα	tumor necrosis factor alpha
UV	ultraviolet
var.	varietas
VIS	visible light spectroscopy

1. INTRODUCTION

In addition to isolating and characterizing naturally occurring components chemically and pharmacologically, modern pharmacognosy studies also seek to identify and screen for potentially hazardous and poisonous compounds. Research addressing the aforementioned issues has become increasingly relevant, particularly in light of the fact that some plants or plant parts have been consumed without adequate scientific background or empirical knowledge regarding their safe use.

Ambrosia artemisiifolia L., ragweed, or common ragweed belongs to the daisy family (Asteraceae), originates from the Sonoran Desert (USA) and in the past 150 years it is widespread in Europe mostly in agricultural and in disturbed territories. Although some ethnobotanical sources describe the use of *A. artemisiifolia* by Native Americans for certain medicinal purposes, these only deal with ethnographic aspects, and do not specify the pharmacological background. Ragweed had never been part of the folk medicine in Europe, and its widespread use as a medicinal herb has started recently and spreading fast.

Sesquiterpene lactones are characteristic secondary metabolites of the Asteraceae family. These naturally occurring plant metabolites, beside that they are structurally one of the most diverse terpenoid group, possess many biological activities, like cytotoxic, antiproliferative, antifungal, antibacterial, and antiprotozoal. *A. artemisiifolia* produces large amount of highly allergenic pollens, causing seasonal allergic rhinitis for millions of people. Besides its well-known allergic potential, dermal exposure to the plant can cause contact dermatitis, which is related to the sesquiterpene lactone content of the plant and which has previously been described also for other species belonging to Asteraceae. Our research group previously also conducted an experiment with the aerial parts of ragweed in a repeated-dose toxicity investigation on animals. Subchronic ragweed puree administration for rats reduced liver enzyme activities, significantly reduced liver weight and elevated brain weight relative to body weight.

The first aim of our present work was to investigate the chemical composition the aerial parts of *A. artemisiifolia*, with special focus on the sesquiterpene lactone content, including to reveal the bioactivities, which might be related to the possible cytotoxicity of this type of compounds. The second goal was to qualify and quantify the essential oil components in the aerial parts of ragweed by using different extraction methods, and to investigate the extracted essential oil bioactivities, including its possible cytotoxic and antibacterial activity. To study the seasonal variation of sesquiterpene lactones in

ragweed, we aimed to develop a high-performance liquid chromatography method, which allow us to identify and quantify the target sesquiterpene lactones from the aerial parts of ragweed, collected from different geographical origin, in its different vegetation period. Although the current perception of ragweed is generally not very good due to its well-known allergenicity, the aim of our work was to explore, by scientific means, its chemical and pharmacological characteristics that could facilitate the potential use of the plant or some of its extracts and compounds.

2. THE AIM OF THIS STUDY

The aim of this study was to:

- review the literature of *Ambrosia artemisiifolia*, from aspects of its botany, phytochemical characteristics and pharmacological properties of the plant;
- isolate the characteristic secondary metabolites focusing on the sesquiterpene lactones, representative metabolites from the Asteraceae family using different chromatographic techniques;
- extract the essential oil from the aerial parts of ragweed using two different extraction techniques (hydrodistillation and microwave-assisted extraction) and to compare the composition of the oils using GC and GC-MS methods;
- investigate the biological activities of the isolated sesquiterpene lactones and the extracted essential oil focusing on their possible cytotoxic, antiproliferative and antimicrobial activity;
- develop an HPLC analytical method for the identification and quantification of sesquiterpene lactones from the aerial parts of *A. artemisiifolia*, and to
- analyze the change of sesquiterpene lactone levels in plant samples harvested from different geographical origin and in different vegetation periods.

3. LITERATURE OVERVIEW

3.1 Botany of *Ambrosia artemisiifolia*

Common ragweed *(Ambrosia artemisiifolia* L.) is an annual in Ambrosinae subtribe and Heliantheae tribe in the Daisy family (Asteraceae). *A. artemisiifolia* is a branchy plant that grows 20–200 cm tall and has densely hairy stems. The blades are lanceolate or elliptic, pinnated with 2-3 oblong-lanceolate, toothed, or lobed segments on each side. The morphology of alternating leaves varies greatly. The flowers are arranged in capitula. The genus *Ambrosia* is mostly present in the southwest of the United States and near North Mexico, with an appearing center of origin and variety in the Sonoran Desert [1]. A few species may also be found in Central and South America. Later, the genus expanded outwards to the regions of North America and Mexico, which are currently considered to be the species native location [2]. The first ragweed seeds arrived in Europe around 1860 most likely with clover seed grains [3]. Nowadays it is widespread across the European continent (Hungary, the former Yugoslavian republics, Switzerland, Germany, France and Russia), but also Japan, South Korea, Australia, New Zealand, Central and South America, and the Middle East [4]. Though *A. artemisiifolia* is currently rare in the northern section of the continent (e.g., Scotland, Ireland, Norway, and Sweden), the climate change and the plant's high genetic diversity my boost infection in these areas in the near future [5]. The rapid spread of common ragweed can be attributed to its broad ecological niche and the genetic variability of the plant, which is compatible with the environmental conditions of the aforementioned territories [6]. Because of this, *A. artemisiifolia* is among of the world's most invasive plants species.

3.2 Ragweed as an invasive species

As an annual pioneer weed it produces a large amount of highly allergenic pollen, which can cause allergic symptoms such as rhinitis, conjunctivitis, and asthma. Although *Frederick W. Heyl* observed in 1917 that it caused hay fever, the pathophysiological background of this immune reaction was clarified only half a century later [7]. Amb a 1 and Amb a 2 endopeptidases are the main pollen allergens with immunoglobulin-E binding capacity, and responsible for the occurring rhinitis, conjunctivitis, and other hay fever symptoms [8]. The lipid content of the plant pollen influences the immune response mechanisms [9]. Dermal interactions with the plant can cause contact dermatitis, which

has previously been mentioned for other Asteraceae plants species. This reaction is induced by sesquiterpene lactones, the marker compounds of the Asteraceae family [10].

3.3 Ethnopharmacology of ragweed

The widespread use of common ragweed in the European folk medicine has not been documented, though ethnobotanical sources report some particular use of ragweed for medicinal purposes by Native Americans (e.g., to treat infected toes, minor skin eruptions, insect bites, hives; tea used for fever and nausea). The indigenous people used the plant leaves and herbs for different medicinal purposes, but there is no detailed information on the developmental stages of the plants used [11, 12, 13]. There are no data on ethnobotanical use of ragweed in Hungary, the medicinal application of the herb (mainly collected before the blooming period) has only begun in the past few years and is spreading rapidly. To the best of our knowledge, neither the long-term impacts of administration nor the expression of allergens (Amb a 1 and Amb a 2) have been investigated, therefore the risks of consuming the herb are unclear.

3.4 Chemical composition of *A. artemisiifolia*

The phytochemical investigations of the plant are mainly surveyed on the pollen. The plant and its pollen contain sesquiterpene lactones, polyphenolic compounds, flavonoids and phenolcarboxylic acids as well [14]. Some chemical analysis focused on the composition of the essential oil (EO) content [15, 16, 17].

3.4.1. Essential oil

A. artemisiifolia essential oil contains mostly sesquiterpenes (sesquiterpene hydrocarbons and oxygenated sesquiterpenes) and monoterpenes (monoterpene hydrocarbons and oxygenated monoterpenes). The most prominent components of the essential oil are germacrane D, β-pinene, limonene, β-caryophyllene [16]. Alcohols are also present, but at relatively smaller quantities: spathulenol, neointermedeol, borneol, cubebol, τ-muurolol, cedrenol. Other components (bornyl acetate, humulene epoxide II) are also present, but in amounts less than 1% [15].

3.4.2 Sesquiterpene lactones

Sesquiterpene lactones are the major terpenoids with a 15-carbon atom skeleton of the genus *Ambrosia*. To date, 60 sesquiterpene lactones have been isolated from ragweed (Fig 1). Among of the *Ambrosia* species, *A. artemisiifolia* is the most widely investigated plant

and the majority of papers have reported isolation of sesquiterpene lactones with guaiane, pseudoguaiane, seco-pseudoguaiane, germacrane, eudesmane and daucane sesquiterpene skeleton in samples from different geographical origins. The biological activities of most sesquiterpene lactones have been related to a γ-lactone ring (the suffix "olide" refers to the lactone ring) comprising an exocyclic methylene group coupled with a carbonyl group. The exomethylene moiety on the lactone ring can be replaced with a methyl group or a functionalized methylene group. Numerous secondary structural alterations affecting the methyl moieties are frequently generated by functional groups of alcohols, carboxylic acids, which can be reduced and oxidized (hydroxyl moieties, epoxides). Furthermore, the hydroxyl groups are usually esterified. Sesquiterpene lactones are distinguished by the presence of a β-oxygenated functional group at the C-6 terminal, which may exist as a free hydroxyl group, but in most cases it can be involved in the development of C-6 or C-12 γ-lactones.

Most of the *Ambrosia* sesquiterpenes in the Asteraceae family possess an oxygen functional group at the C-8 position that is mainly α-oriented (**37**), however in compounds isolated from *A. artemisiifolia* is an infrequent feature. Moreover, if present, the ester functional groups are generally connected to other positions. Only three guaiane-type sesquiterpenes (**1**-**3**) were isolated from *A. artemisiifolia* (Fig 1), they had a 1,5-trans junction with the exception of 4β-hydroxy-1α,5α,7α,9αH-guaia-10(14),11(13)-dien-12-acid-9-*O*-β-D-glucoside (**3**) [18]. The isolated guaiane compounds were *trans*-6,12 lactones, among them cumambrin B (**1**) has a C-8 oxygen functional group (a β-alcohol) [19]. The two glycosides (**2**, **3**) bear a β-D-glucopyranose moiety at the C-9 position [18]. Instead of a lactone ring, the 4β-hydroxy-1α,5α,7α,9αH-guaia-10(14),11(13)-dien-12-acid 9-*O*-β-D-glucoside (**3**) features an open-ring system with a carboxyl group at the C12 terminal. The occurrence of a C-3 or C-4 (**1**) and C-4 or C-5 (**2**) double bonds on the cyclopentane ring is a common feature, while compound **3** differs in this structural moiety.

The isolated pseudoguaianes (**4**–**23**) have the highest number (20 compounds) of derivatives. Their key difference from guaianes is that they have a methyl group at the C-5 position rather than the C-4 position. This type sesquiterpenes are usually C-6 or C-12-olides with a C-11 or C-13 dihydro moiety or exocyclic C-11 and C-13 double bond. In case of compounds **7**, **10**, **21**, **22** the lactone ring is absent, but unlike compounds **8**, **9**, **13**, **14**, **15**, **18**, **23** contained the C-8/C-12 olide ring. The following chemical properties of pseudoguaianes have been observed: C-15 nor (**21**) [20]; diol in **8**, **18**, **20**; C-4 ketone

in **4**–**7**, **9**–**12**, **16**, **17**, **19**, **21**, **22**; C-3/C-4 esters in **13**,**14**, **23**. These molecules resemble the pseudoguaiane group in that they are entirely C-6 or C-12 olides, and show a methyl moiety at the C-5 instead of the C-4 terminal.

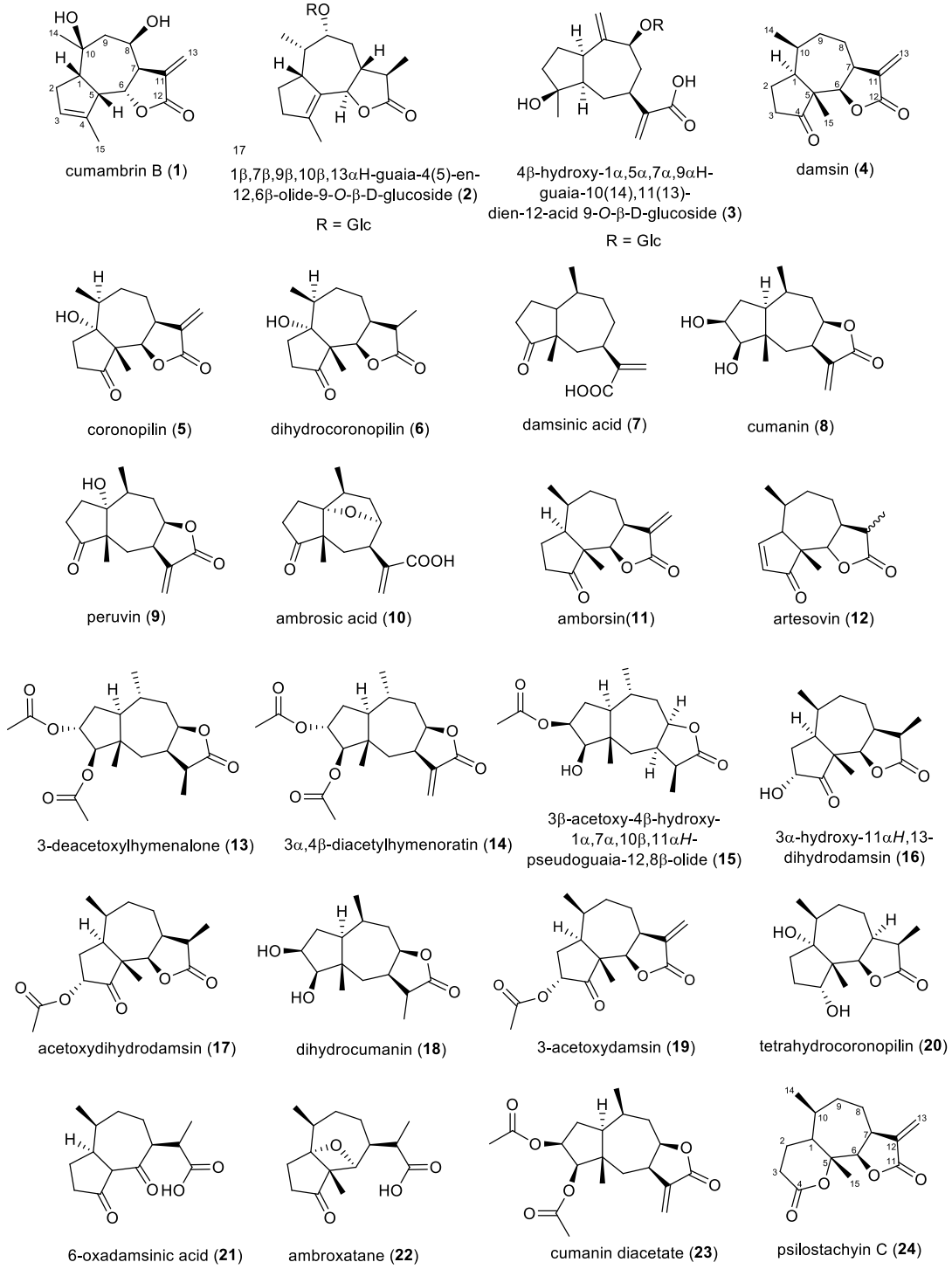


Figure 1. Sesquiterpene lactones isolated from *Ambrosia artemisiifolia*

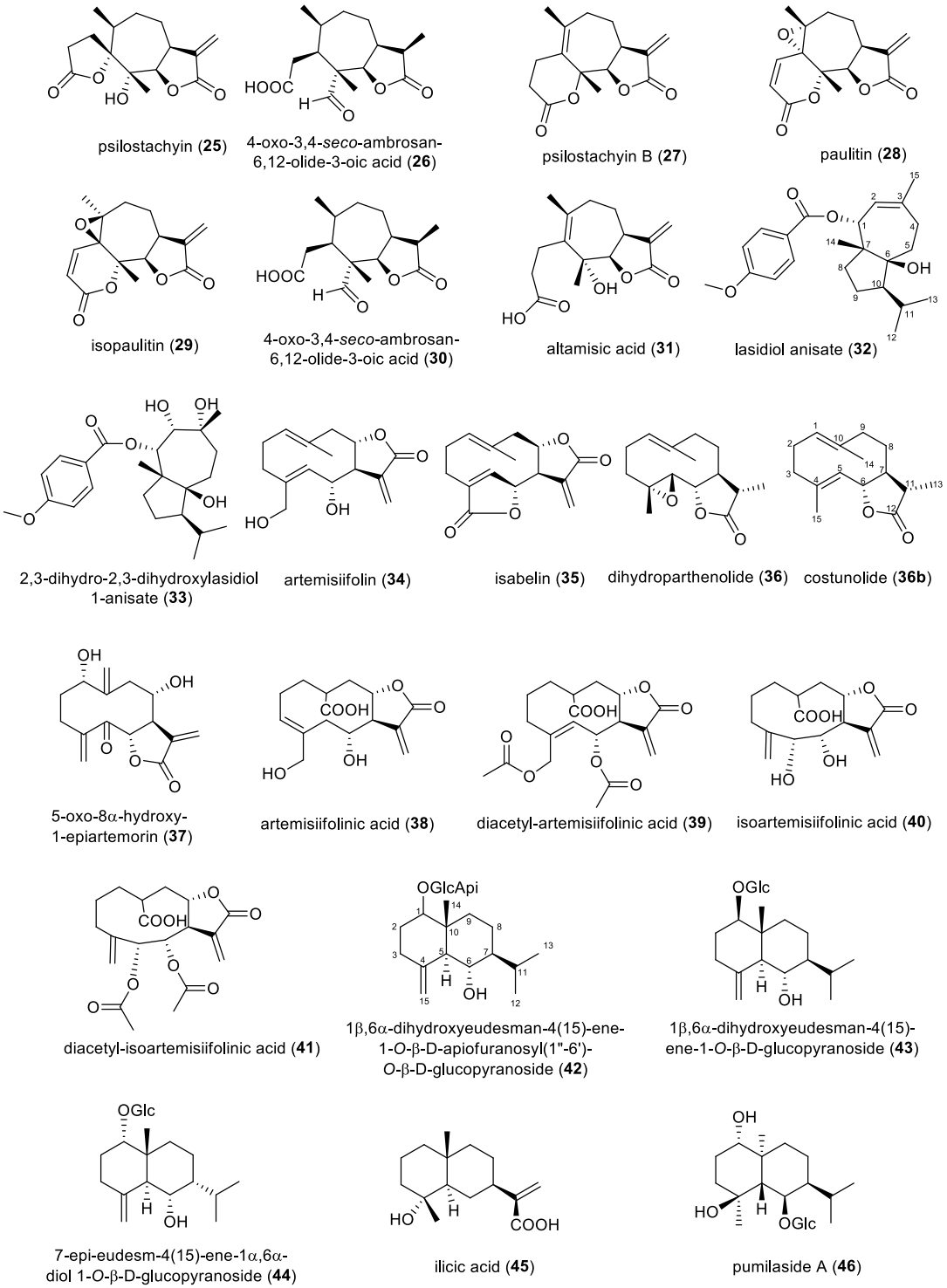


Figure 1. (continued)

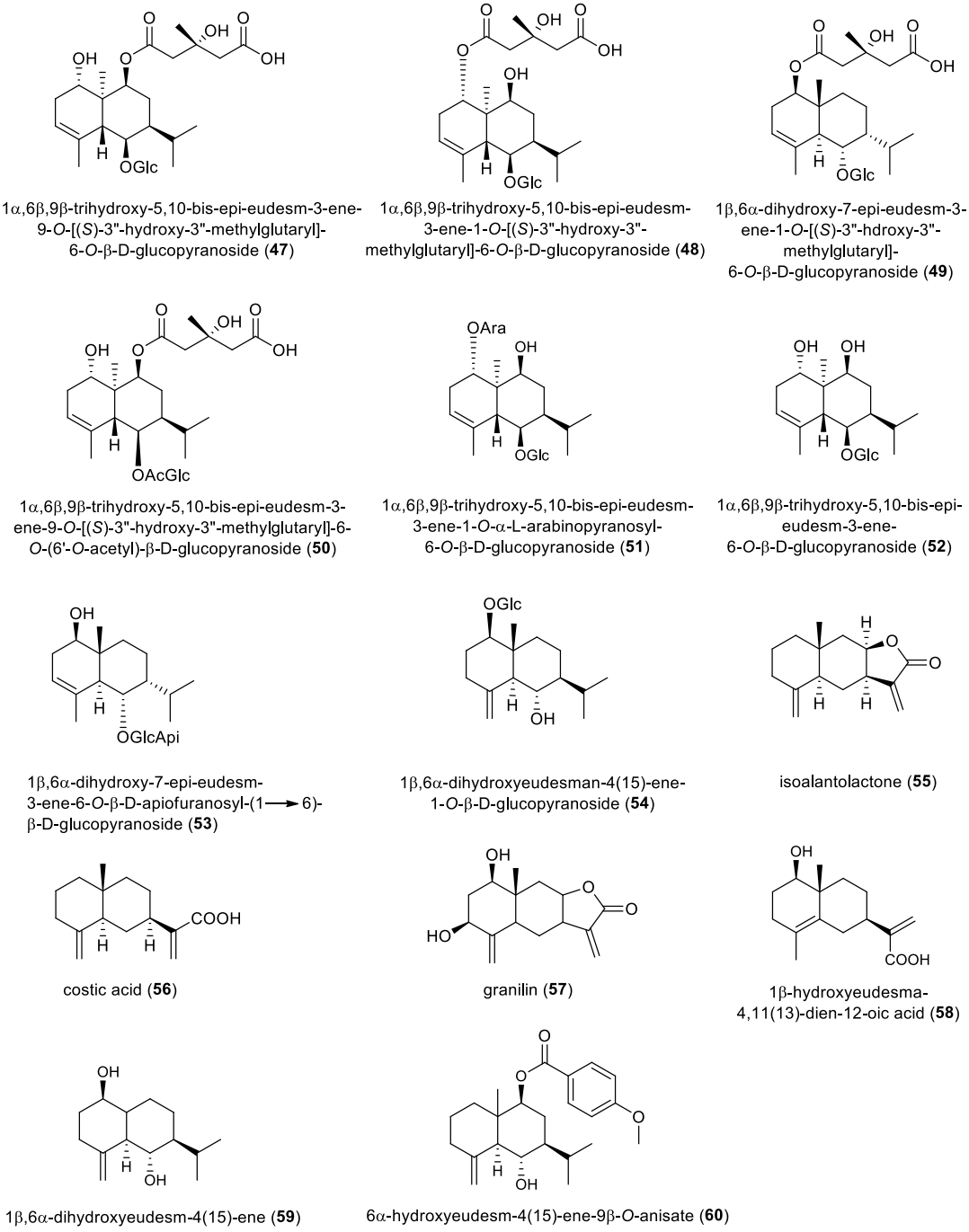


Figure 1 (continued)

Apart from the C-5 methyl group terminal, seco-pseudoguaianes **25**, **31** bear an α-hydroxyl group. The lactone ring between C-4 and C-1 or C-4 and C-5 positions was lacking in three seco-pseudoguaianes (**26**, **30**, **31**). In the case of 4-oxo-3,4-*seco*-ambrosan-6,12-olide-3-oic acid (**26**) the A ring opening occurred at the C-3 or C4 position, leading in a unique structure [21]. The remaining seco derivatives are dilactones with a higher oxygen content than the other skeletal forms. This type of sesquiterpenes

isolated from *A. artemisiifolia* have a total absence of oxygen functional or hydroxy groups at the C-8 position.

Two daucanes (carotanes **32** and **33**) were isolated from ragweed, which are substituted with aromatic acid 1α-anisoyloxy derivatives in the C-1 position [20]. At C-6 position in both daucanes, an alternate oxygen functional group, a β-alcohol is a common feature. Lasidiol anisate (**33**) contains a double bond at the C-2 or C-3 position, while in 2,3-dihydro-2,3-dihydroxylasidiol 1-anisate (**32**) is bearing two α-oriented hydroxyl groups.

Eight germacranes were isolated from *A. artemisiifolia* so far. The olefinic group is always *trans* at the C-1 or C-10, C-3, and C-4 or C-5 positions. The olefinic group in isoartemisiifolinic acid (**40**) and diacetyl isoartemisiifolinic acid (**41**) was positioned at the C-4 or C-5, whereas in the case of 5-oxo-8α-hydroxy-1-epiartemorin (**37**) the exomethylene moieties were found at the C-10 or C-14 and C-4 or C-15 locations [20]. Except for dihydroparthenolide (**36**), wich has a C-11 or C-13 dihydro moiety, germacranes of *A. artemisiifolia* are nearly completely C-8 or C-12 olides (**34**, **35**, **38**–**41**), except compounds **36** and **37** which are C-6 or C-12 olides with a characteristic exocyclic C-11 or C-13 double bound [22]. The artemisiifolin derivatives **34**, **35**, **38**-**41** bear an olide ring and an oxygen functional group at the C-6 position. A primary alcohol functional group was found in compounds **34** and **38,** but an acetoxy at the C-15 location was only detected in diacetyl-artemisiifolinic acid (**39**) [20]. Only one germacranolide has a C-15 methyl group (**36**), an α-oxygen functional group at the C-8 position (**37**), whereas isabelin (**35**) is a dilactone derivative [23].

Eudesmanes, totally 19 isolated from *A. artemisiifolia* are characterized by the presence of a *trans* fused decalin (decahydronaphthalene) moiety. This is the most diversified category of sesquiterpene lactones in terms of their substituents and they are mainly non-lactonized compounds, only two compounds **55** and **57** bears this moiety, which are C-8 or C-12 olides.

3.4.3. Other non-volatile small molecules

Flavonoids, polyphenolic chemicals, and spermidine derivatives were also determined from the aerial parts of *A. artemisiifolia.* The plant pollen frequently contains flavonoids and hydroxycinnamic acid derivatives, notably their glycosides. Investigations on the occurrence and distribution of the flavonoid aglycons have revealed, that free aglycons are likely to be accumulated externally on the surfaces of twigs, leaves and florets as

components of resinous excretions or thin epicuticular layers [24]. Flavones and flavonols are extensively found often with additional substituent in the 6-and/or 8-positions [25].

A. artemisiifolia contains some C(6)-oxygenated polar flavonoid aglycones (e.g. axillarin, tomentin, and patuletin) [25]. The presence of phenolcarboxylic acids (chlorogenic, caffeic, isoferulic, ferulic acids and caffeic acid glycosides) and coumarins (skimmin, umbelliferone, esculin, esculetin, scopoline, scopoletin) have also been reported [26].

The Asteraceae family is well known that contains a variety of structural forms of polyacetylenes including various sulfur derivatives [27]. Among these, the sulfur-containing polyacetylenes, a relatively small group of 1,2-dithiea-3,5-cyclohexadiene derivatives have received a lot of interest in recent years due to their distinct chemical characteristics and potent biological activities [28]. From *Ambrosia artemisiifolia*, C-13-monothiophenes have been reported so far [27]. These secondary metabolites are derived from fatty acids and sometimes referred to as thiarubrines because of their deep red color [29]. From the 1,2-dithiins, various species of *Ambrosia* including *A. artemisiifolia* contains thiarubrin A and B [30].

3.5 Biological activities of *Ambrosia* sesquiterpenes

The primary bioactivities of the naturally occurring sesquiterpenes from the genus *Ambrosia* are cytotoxic and antiproliferative effects. It has been established that almost all known cytotoxic sesquiterpenes from the *Ambrosia* genus contain an exocyclic methylene linkage and a lactone function, which is α,β-unsaturated. The cytotoxicity of the isolated sesquiterpenes depends on the presence of a C-11 or C-13 exocyclic double bound conjugated to the γ-lactone. Several investigations have revealed that the structures and reactivities of *Ambrosia* sesquiterpenes may be related to the observation that these metabolites can alkylate the nucleophilic groups of enzymes controlling cell division [32, 33, 34, 35]. Functional groups next to the α-CH_2 γ-lactone, such as the epoxide, hydroxyl, unsaturated ketone, or *O*-acyl, might increase the reactivity of these compounds toward biological nucleophiles.

Pseudoguaianolide sesquiterpenes damsin (**4**) and coronopilin (**5**) showed anticancer activity against several cancer cell lines (human breast cancer cell line, human epithelial cancer cell line). These type of sesquiterpenes were shown to inhibit tumor necrosis factor-α-induced translocation of NF-κB from the cytoplasm to the nucleus. Coronopilin (**5**) exerts cell population growth-inhibitory activity by triggering caspase-

dependent apoptosis and robust arrest in G_2/M [35]. It is known that due to its nucleophilic sites, DNA is a potential target for the α,β-unsaturated carbonyl functional group of SLs. Both, damsin (**4)** and coronopilin (**5**) exert antiproliferative activity trough inhibiting DNA biosynthesis and the formation of cytoplasmic DNA histone complexes [36]. Also, these types of compounds have an impact on different molecular mechanisms: inhibition of IFNγ-induced expression of STAT3 and TNFα-induced expression of NFκB. Seco-pseudoguaianolides psilostachyin C (**24**) and psilostachyin (**25**) isolated from common ragweed are checkpoint inhibitors: arresting cell mitosis, causing the formation of aberrant microtubule spindles. The α,β-unsaturated compounds covalently bind to target proteins through the Michael-type addition. Another pseudoguaianolide, cumanin (**8**) bearing two hydroxyl groups which allows the preparation of its acetylated, silylated and triazole derivatives, thus the modified derivatives had improved activities (higher cytotoxic activity and selectivity) on different cancer cell lines (A549, HBL100, HeLa, SW1573, T47-D and WiDr) [37].

Some authors hypothesized that these type of secondary plant metabolites growth-inhibitory activity could be due to the alkylation of thiol groups of cysteine residues of the UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) associated with the biosynthesis of bacterial cell wall [38].

3.6. Bioactivities of the essential oil

The investigations with the essential oil focused mainly on the chemical composition of the extracted oil, and just few studies investigated the possible biological activity of EO. Some studies revealed the antibacterial and antimycotic activity of the essential oil extracted from a Serbian ragweed collection [39]. Other *Ambrosia* species (*A. trifida* collected in Northeast China) essential oils demonstrated bactericidal activity against Gram-positive and Gram-negative bacteria and fungicidal activity [40].

The phytotoxic evaluation of essential oil from an Chinese collection revealed that the plant EO can inhibit seed germination, seedling development, also it can cause root cell viability decline, while foliar spray results visible injury in leaves and decreased chlorophyll content in case of monocot (*Poa annua*, *Setaria viridis*) and dicot (*Amaranthus retroflexus*, *Medicago sative*) species [41]. Other allelopathic activity investigations with different *Ambrosia* species resulted similar results, where *A. psilostachya* DC. essential oil presented a great potential for reducing seed germination and seedling vigor in lettuce seeds, this may be considered with the high monoterpene content of the plant EO [42]. *A. trifida* EO showed similar allelopathic results on seed

germination and seedling growth of lettuce, watermelon, cucumber and tomato: an increase in essential oil leads to decrease in seed germination, shoot and radical length of lettuce, watermelon, cucumber and tomato [43]. Other investigations revealed, that the EO from *A. psilostachya* caused inhibition on the fibrinogenolysis and clotting induced by *Bothrops moojeni* and *Lachesis muta* venoms [44]. It can be hypothesized, that the plant EOs show promise as adjuvants for the treatment of snakebites injuries. *A. peruviana* Willd (Peruvian ragweed) is frequently used in indigenous traditional medicine to treat rheumatic disorders, menstruation disorders, neurological problems and as vermifuge and insecticide [45]. The EO obtained from the Ecuadorian plant sample showed acaricidal efficacy (high larval mortality, inhibited oviposition, egg hatching) against the common cattle tick *Rhipicephalus microplus*, which is the most importance due to its significant, detrimental effect on farm's economy [46]. In the search of potential sources of antiparasitic compounds, some authors reported, that *A. tenuifolia* and *A. scabra* EO showed *in vitro* trypanocidal activity against chloroquine resistant F32 and chloroquine sensitive (W2) *Plasmodium falciparum*, and also *Trypanosoma cruzi* epimastigotes [47].

4. MATERIALS AND METHODS

4.1 General experimental procedure

Analytical grade solvents were purchased from Chem-Lab NV (Zedelgem, Belgium). Solvents for extraction were obtained from Molar Chemicals Kft. (Halásztelek, Hungary). HPLC grade solvents were acquired from VWR Chemicals International S. A. S. (Fontenay-sous-Bois, France). HPLC grade water was purified using a Millipore Direct-Q® 3 UV pump (Millipore S. A. S., Molsheim, France). For the GC-MS analysis the MS grade *n*-hexane was purchased Sigma-Aldrich (St. Louis, MO, USA). Silica gel 60 RP-18 F_{254}s and silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany) were used for TLC analysis. MN-Polyamide SC-6 (polycaprolactam, 0.05-0.16 mm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) was applied for open column chromatography.

Vacuum liquid chromatography was carried out with a Büchi Rotavapor R-300 vacuum pump V-300 (BÜCHI Labortechnik AG, Flawil, Switzerland) using silica gel 60 GF_{254} (mean particle size 15 µm, Merck).

The ***preparative rotation planar chromatography*** was performed on a Harrison Chromatotron (Harrison Research, Palo Alto, CA, USA), equipped with a Büchi pump Manager C-615 and with two Büchi Pump Module C-605 using silica gel (0.040-0.063 mm, Merck).

Preparative HPLC was performed on a Shimadzu LC-2010 C_{HT} system (Shimadzu Corp., Japan), equipped with an UV-VIS detector with two channels, on-line degasser and autosampler using a Kinetex XB C_{18} (5 µm, 100 Å, 250 × 10.0 mm) column (Phenomenex, Torrance, CA, USA).

Analytical HPLC experiments were performed on a Shimadzu LC-20 system, equipped with SPD-M20A Diode Array Detector (DAD), on-line degasser unit, automatic injector, auto sampler and column oven and operating by a CBM-20A Communication Bus Module (Shimadzu Crop., Japan) using a Kinetex C_{18} (5 µm, 100Å, 150 × 4.6 mm) column.

Microwave-assisted essential oil extraction was carried out with an ETHOS XL system (Milestone Ltd., Sorisole, Italy).

GC analysis was performed on a HP 5890 Series II gas chromatography equipped with a flame ionization detector (FID), using a 30 m × 0.35 mm × 0.25 µm HP-5 fused silica capillary column and N_2 as the carrier gas. The temperature program was from 60 °C to 210 °C at 3 °C min^{-1}, and from 210 to 250 °C (2 min hold) at 5 °C min^{-1}. The detector and injector temperature were 250 °C and the carrier gas was N_2, with split

sample introduction. The quantity of the individual components of EO was expressed as the percent of peak area relative to the total peak area from the GC/FID analysis.

GC-MS analysis was performed on a Shimadzu GC-MS-QP2010SE system with a ZB-5MS (30 m × 0.25 mm × 0.25 μm) column. The carrier gas was He at a linear velocity of 31.9 cm s^{-1} and the capillary column was ZB-5MS (30 m × 0.25 mm × 0.25 μm). The positive ion electron ionization mode was used, with ionization energy of 70 eV. Ion source temperature of 200 °C, interface temperature of 250 °C was applied, scan range was 40–550 m/z. Identification of the compounds was based on comparisons with published MS data [31] and a computer library search (FFNSC) and by comparison of their retention indices with literature values [32]. Retention indices were calculated [33] against C8 to C32 *n*-alkanes on a ZB-5MS column. A mixture of aliphatic hydrocarbons was injected in hexane (Sigma–Aldrich, St. Louis, MO, USA) by using the same temperature program that was used for analyzing the EO.

NMR spectra were recorded in $CDCl_3$ and CD_3OD on a Bruker Avance DRX 500 spectrometer at 500 MHz (^1H) and 125 MHz (^{13}C JMOD). The residual peaks of the deuterated solvents were taken as reference points. Two-dimensional (2D) experiments were performed with a standard Bruker software. In the ^1H-^1H COSY, HSQC, and HMBC experiments, gradient-enhanced version was applied. The data were acquired and processed with MestReNova v11.0.4-18998 software.

4.2 Plant material

For the preparative phytochemical experiments, the raw plant material of *A. artemisiifolia* was harvested around Mórahalom, Hungary in July 2017 during the blooming stage. Before processing, the above ground parts were dried and kept at room temperature. A voucher of specimen of the plant (No. 894) was deposited in the Herbarium of the Department of Pharmacognosy, University of Szeged (Southeast Hungary, N 46.28, E 20.14).

For the essential oil extraction experiments, the blooming aerial parts of *A. artemisiifolia* were gathered in August 2022 in the suburban region of Szeged (Southeast Hungary, N 46.28, E 20.14). The harvested plant material was air dried at room temperature in a shady, ventilated area. A voucher specimen of the samples (No. 898) was deposited in the Herbarium of the Institute of Pharmacognosy, University of Szeged.

For the analysis of the seasonal variation of sesquiterpene lactone content, aerial parts of *A. artemisiifolia* were collected in June to October 2021 in the suburban region

of Szeged (Southeast Hungary, N 46.28, E 21.44, height above mean sea level: 70 m); and Nyíri (Northeast Hungary, N 48.50, E 21.14, height above mean sea level: 240 m) Voucher specimens of the collected herbal samples (Szeged samples: 2021/VI-20 to 2021/VI-39; Nyíri samples: 2021/VI-40 to 2021/VI-60) were deposited in the herbarium of the Institute of Pharmacognosy, University of Szeged.

4.3 Extraction and isolation of sesquiterpenes from the above-ground parts

The dried plant material (5 kg) was crushed in a Retsch® SM 100 cutting mill and extracted with 51 L of MeOH at room temperature using ultrasonication. To evaporate the solvent a Büchi Rotavapor R-220 SE was using under reduced pressure. The extract was redissolved in 0.375 L H_2O and 0.625 L MeOH and was subjected to liquid-liquid extraction with chloroform (11×1 L). The crude chloroform fraction weighted 128.57 g. Open column chromatography using polyamide and a gradient system of MeOH-H_2O with increasing MeOH concentration (from 20% to 100%) (17.5 L 20% MeOH, 30 L 40% MeOH, 42.5 L 60% MeOH, 55 L 80% MeOH) was applied to separate the $CHCl_3$-partition, and gaining 15 fractions (AA20/1, AA20/2, AA40/1, AA40/2, AA60/1-AA60/4, AA80/1-AA80/6, AA100). Fraction AA20/1 (66 g) was subjected to vacuum chromatography on silica gel column with a gradient system of *n*-hexane-$CHCl_3$-MeOH (50:30:00, 50:30:01, 50:30:02, 50:30:05, 50:30:10, 50:30:20, 50:30:30, 50:30:40, 50:30:50, 50:50:60, 50:60:70, 00:50:50, 100% MeOH), gaining 10 fractions (AA/C1/C1-AA/C1/C10). Fraction AA/C1/C4 (3.83 g) eluted by *n*-hexane-$CHCl_3$-MeOH (50:30:40) were further separated by rotation planar chromatography (RPC) on silica gel and eluted with cyclohexane-EtOAc (16:4, 14:6, 12:8, 10:10, 8:12, 100% MeOH) with a flow rate 7 mL/min gaining 7 fractions (AA/C1/C4/R1*-AA/C1/C4/R7*). Fraction AA/C1/C4/R2* (433 mg) was purified by rotation planar chromatography on silica gel (gradient elution with cyclohexane-dichloromethane-MeOH 16:4:0.5, 14:5:0.5, 14:5:1, 12:7:1, 100% MeOH in the final step, flow rate 7 mL/min), 4 fractions were collected (AA/C1/C4/R2*/R1-AA/C1/C4/R2*/R4). Finally compound 5 (17 mg) was obtained from fraction AA/C1/C4/R2*/R2 (184.9 mg) by using semipreparative HPLC gradient solvent system, consisting of methanol and water (0 min: MeOH-H_2O 6.5:3.5, 6 min: MeOH:H_2O 6.5:3.5, 9 min: MeOH:H_2O 8:2, 10 min: MeOH:H_2O 1:0, 11 min: MeOH:H_2O 6.5:3.5, flow rate 2.8 mL/min)). Fraction AA/C1/C4/R6* (400 mg) was subjected to rotation planar chromatography using silica gel with gradient elution (cyclohexane-dichloromethane-MeOH 16:4:0.5, 14:5:0.5, 14:5:1, 12:7:1, 100% MeOH in the final step,

flow rate 7 mL/min). 2 fractions were collected AA/C1/C4/R6*/R1 (213.3 mg) and AA/C1/C4/R6*/R2 (138.4 mg). Fraction AA/C1/C4/R6*/R1 (213.3 mg) was dissolved in MeOH-dichloromethane 9:1, stored in the refrigerator for 24 h. Compound 2 (35 mg) was obtained by crystallization from this solvent mixture. Fraction AA/C1/C4/R5* (385 mg) was separated by rotation planar chromatography on silica gel with the same method gaining two fractions, AA/C1/C4/R5*/R1 (177.5 mg) and AA/C1/C4/R5*/R2 (161.1 mg). Fraction AA/C1/C4/R5*/R2 (161.1 mg) was further separated by RPC on silica gel eluting with toluene-acetone (18:2, 17:3, 16:4, 15:5, 100% MeOH in the final step), affording 6 fractions (AA/C1/C4/R5*/R2/R1-AA/C1/C4/R5*/R2/R6). Fraction AA/C1/C4/R5*/R2/R1 (10.5 mg) was purified by semipreparative HPLC (gradient elution, MeOH-H_2O system, 0–1 min: MeOH-H_2O 40:60, 1–12 min: MeOH-H_2O 40 →100, 12–13 min: MeOH-H_2O 100:0, 13–14 min: MeOH-H_2O 100 → 40, flow rate 3.0 mL/min) to obtain compound 3 (6 mg). Fraction AA/C1/C4/R4* (380 mg) was subjected on silica gel and separated by RPC (gradient elution, cyclohexane-dichloromethane-MeOH 16:4:0.5, 14:5:0.5, 14:5:1, 12:7:1, 100% MeOH in the final step, flow rate 7 mL/min). 3 fractions were collected (AA/C1/C4/R4*/R1-AA/C1/C4/R4*/R3). Fraction AA/C1/C4/R4*/R1 (104 mg) was dissolved in MeOH-dichloromethane 9:1, stored in the refrigerator for 24 h. Compound 6 (27 mg) was obtained by crystallization form this solvent system. Fraction AA/C1/C4/R4*/R2 (71.2 mg) was subjected to semipreparative HPLC (gradient solvent system, 0–1 min: MeOH-H_2O 40:60, 1–10 min: MeOH 40 → 60, 10–12 min: MeOH-H_2O 60:40, 12–15 min: MeOH 60 → 100, 15–16 min MeOH-H_2O 100:0, 16–17 min: MeOH 100 → 40, flow rate 2.8 mL/min) to obtain compound 4 (34 mg). Fraction AA/C1/C4/R3* (415.2 mg) was purified by rotation planar chromatography using silica gel (gradient elution, cyclohexane-dichloromethane-MeOH 16:4:0.5, 14:5:0.5, 14:5:1, 12:7:1, 100% MeOH in the final step) and gaining 4 fractions (AA/C1/C4/R3*/R1-AA/C1/C4/R3*/R4). Fraction AA/C1/C4/R3*/R2 (149.9 mg) was further separated by RPC on silica gel (gradient elution, toluene-acetone 18:2, 17:3, 16:4, 15:5, 100% MeOH in the final step, flow rate 7 mL/min). 4 fractions were collected (AA/C1/C4/R3*/R2/R1-AA/C1/C4/R3*/R2/R4). Subfraction AA/C1/C4/R3*/R2/R2 (30 mg) was purified by semipreparative HPLC (gradient solvent system, 0–1 min: MeOH-H_2O 40:60, 1–10 min MeOH 40 → 60, 10–12 min: MeOH-H_2O 64:40, 12–15 min: MeOH 60 → 100, 15–16 min: MeOH-H_2O 100:0, 16–17 min: MeOH 100 → 40, flow rate 2.8 mL/min) to obtain compound 7 (4 mg). Fraction AA/C1C6 (4.12 g) was subjected to RPC with gradient elution system (cyclohexane-dichloromethane-MeOH 16:4:0.5, 14:5:0.5,

14:5:1, 12:7:1, 100% MeOH in the final step, flow rate 7 mL/min). 6 fractions were collected (AA/C1/C6/R1*-AA/C1/C4/R6*). Fraction AA/C1/C6/R3* (126 mg) was further separated by rotation planar chromatography in the same way to gain five fractions (AA/C1/C6/R3*/R1-AA/C1/C6/R3*/R5). Subfraction AA/C1/C6/R3*/R1 (69 mg) was purified by HPLC (Shimadzu LC-10AS system, isocratic elution, MeOH-H_2O 60:40, flow rate 0.8 mL/min) to obtain compound 1 (2 mg). Fraction AA/60/3 (3.4229 g) was chromatographed by open column chromatography on silica gel with a gradient system of *n*-hexane-EtOAc (9:1, 8:2, 4:1, 1:1, 1:4, 1:9, 0:1) and EtOAc-MeOH (9:1, 4:1, 1:1, 1:4, 1:9, 0:1) gaining 6 fractions (AA60/3/C1-AA60/3/C6). Subfraction AA60/3/C5 (322 mg) was further separated by size-exclusion chromatography (Sephadex LH-20) with MeOH gaining seven subfraction (AA60/3/C5/S1-AA60/3/C5/S7). Compound 8 (34 mg) was obtained in subfraction AA60/3/C5/S7.

4.4 Extraction of essential oil

4.4.1 Hydrodistillation

The aerial parts of the plant were crushed using a blender (Retsch Grindomix GM 200). From the dried, homogenized sample (16 × 50 g), the EO was extracted by hydrodistillation after mixing it with 500 mL of distilled water. The EO was hydrodistilled using a Clevenger's apparatus for 2 h until the total amount of the EO was enough for all the further trials. The oil was separated, dried on anhydrous sodium sulfate, and kept in a sealed brown glass vial in a refrigerator at –20 C° before any further analytical and bioassay studies. The following equation was used to determine the yield of *A. artemisiifolia* EO:

$$\text{Oil yiel}\left(\%, \frac{\text{V}}{\text{w}}\right) = \frac{\text{volume of EO in mL}}{\text{dried weight of plant material in g}} \cdot 100\%$$

4.4.2 Microwave-assisted extraction

For the microwave-assisted extraction of the aerial parts of the plant (2921 g) an ETHOX XL microwave instrument (Milestone Ltd., Sorisole, Italy) was used, operating at 2450 MHz. The ground plant material was loaded into cotton bags and then into the drum of the system. The system automatically loaded the water into the bottom of the cavity and started the heating process. A Clevenger-type apparatus was connected to the outside of the microwave oven. The extraction was performed using 3000 W power in the first 31 min and the temperature was adjusted to 110 °C, after 31 min the power was reduced to

2200 W. The oil layer was separated using *n*-hexane. The extracted sample of EO was stored in sealed air-tight glass vials at –20 °C until further analysis.

4.5 Sample preparation for HPLC analysis and quantification

Ragweed samples collected near Szeged contained less foliage, smaller leaves, and taller branches on average, compared to samples from Nyíri, which appears shrubbier with lower shoots and dense foliage. The botanical identification was carried out on the key anatomical features of the species *A. artemisiifolia* var. *elatior,* the only ragweed variety which occurs in Hungary [48]. The plant is green or dark green in the early stages, and its leaves are usually pinnate. *A. artemisiifolia* may be misidentified with *Artemisia vulgaris* at this stage; however, the lower leaf blade of juvenile *A. vulgaris* is less divided, or more lobed, and has a serrated border. The ragweed has tall, glabrous to rough, hairy stems when it is fully grown. The leaves are opposite below, alternate above, tri-pinnatifid. Occasionally, the leaves at the top differ from the ones at the bottom in that the top leaves have unlobed blades. As a monoecious plant, ragweed has either male or female flowers in its flower heads [49].

The collected plant material was air dried for a week at room temperature in a well-ventilated, shady area. A blender (Retsch Grindomix GM 200) was used to grind the 100 g aerial parts of the raw plant material. The powdered plant material (10 g) was extracted twice with 100 mL of acetone for ten minutes in an ultrasonic bath at room temperature, respectively. The extract was filtered through a paper filter (Watman 4 filter papers, 125 mm in diameter) and centrifuged at 5000 rpm for 5 minutes using a Rotanta 460 Hattich centrifuge (Kirchlengem, Germany). The supernatants obtained from two-step extraction were combined and transferred quantitatively to rounded bottom flask, and the solvent was evaporated under vacuum (Büchi Labortechnik AG, Flawil, Switzerland). After being redissolved in 20 mL HPLC-grade MeOH, the dry residue was put into a volumetric flask and diluted to 25 mL. Before performing HPLC analysis, every sample was filtered using a syringe filter equipped with a polytetra-fluoroethylene (PTFE) membrane (d=13 mm, porosity 0.45 μm) (Nantong FilterBio Membrane Co., Ltd., Nantong City, China) and immediately injected. The quantitative analysis was performed in triplicates.

4.6 HPLC analysis of the samples

The Shimadzu LC20 Liquid Chromatograph (Shimadzu Corp., Kyoto, Japan) was used for the HPLC analysis. It was operating by a CMB-20A Communication Bus Module and

equipped with an SPD-M20A Diode Array Detector (DAD), a degasser unit, an automatic injector, an auto sampler, and a column oven.

The separation was performed on a reverse phase Kinetex® C_{18} column (5 µm, 100Å, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA) with guard columns (4.6 × 10.0 mm) that was packed with the same stationary phase. The mobile phase A was water with 0.1% (v/v) TFA, whereas the mobile phase B was acetonitrile with 0.1% (v/v) TFA. The chromatographic elution of the samples was carried out with a gradient solvent system by the following: 20% CH_3CN (0–1 min), 20–70% CH_3CN (1–19 min), 70–100% CH_3CN (19–21 min), and kept at 100% CH_3CN for 2 min, 100 to 20% (23–24 min) and kept at 20% for 6 min at a flow rate of 1.4 mL/min, the temperature of the column was set at 30 °C; the injection volume was 10 µL. The UV-DAD detector was adjusted to record between 205 and 800 nm, and chromatograms were taken at 210 nm to detect the targeted chemicals. A set of reference standards including acetoxydihydrodamsin (**17**), peruvin (**9**), psilostachyin (**25**), costunolide (**36b**), and isoalantolactone (**55**) were used to make a methanolic dilution series to generate the calibration curves. The concentration levels (1, 0.1 and 0.01 mg/mL) were obtained by preparing a dilution series of reference compound, which starting at 1 mg/mL. The linear calibration plot at eight to ten different concentration levels covering the linear range shown in **Table 1** were obtained by injecting the appropriate volume of solutions. All calibration levels were measured four times. Linear regression was used to establish the calibration curve. The AUC was utilized to determine the results.

4.7 Meteorological data

We acquired rainfall, average temperature, and global radiation data form the Hungarian Meteorological Service's Database (http://odp.met.hu). For the sample set collected near Szeged, data from the meteorological station Szeged-külterület (No. 58116) [51, 52] were used to describe the conditions; for the sample set collected near Nyíri, dta from the nearest meteorological station Hidasnémeti (No. 61104) [53, 54] and the nearest Sátoraljaújhely (No. 61709) [55, 56] were analyzed for global solar radiation and average temperature and rainfall, respectively.

4.8 Statistical analysis

The Shapiro-Wilk test was used to check the normal distribution of the data. One-way ANOVA or T-test with Bonferroni post-hoc test was used to determine there are any statistically significant differences between the samples investigated by our group. The

relationship between meteorological data and levels of sesquiterpene lactone concentration was examined using the Pearson correlation coefficient. The correlations or the differences were considered significant when $p<0.05$ (*), and $p<0.01$ (**), and $p<0.001$ (***). R was used to perform the statistical analysis (version 4.0.3, The R Foundation Statistical Computing, Vienna, Austria, http://www.r-project.org).

4.9 Culture media and chemicals used in biological assays

The following culture mediums were utilized in the experiments: Mueller-Hinton broth (MHB; Sigma-Aldrich, St- Louis, MO, USA), Luria-Bertani broth (LB-B; Sigma, St. Louis, MO, USA) and Tryptic Soy broth (TSB; Scharlau Chemie S. A., Barcelona, Spain) were purchased. Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS; pH 7.4), ethidium bromide (EB), reserpine (RES), CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), promethazine (PMZ) and crystal violet (CV) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). DMSO (Sigma-Aldrich, St Louis, MO, USA), phosphate-buffered saline (PBS; pH 7.4), Mueller Hinton (MH) broth, tryptic soy broth (TSB), tryptic soy agar (TSA), Luria-Bertani broth (LBB), Luria-Bertani agar (LBA), reserpine, ethidium bromide (EB), thioridazine (TZ) were purchased from Sigma.

4.10 Biological assays evaluated with the isolated sesquiterpene lactones

4.10.1 Cell lines

For biological assays, human colonic adenocarcinoma cell lines (Colo 205 doxorubicin-sensitive and Colo 320/MDR-LRP multidrug resistant overexpressing ABCB1 (MDR1)-LRP), ATCC-CCL-220.1 (Colo 320) and CCL-222 (Colo 205)), and MRC-5 human embryonal lung fibroblast cell lines (ATCC CCL-171) were used.

4.10.2 Bacterial strains

Wild-type *Escherichia coli* AG100, expressing the AcrAB-TolC efflux pump (EP) at its basal level, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 49619, *K. pneumoniae* ATCC 49619 and *Pseudomonas aeruginosa* ATCC 33362 strains were used as Gram-negative strains. As a Gram-positive strains, *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 25923 (methicillin-susceptible refences), *S. aureus* MRSA 272123, were used.

4.10.3 Antibacterial assay

The minimum inhibitory concentration (MIC) of the substances was used to evaluate their antibacterial activity. The concentrations tested ranged from 100 µM to 0.195 µM.

4.10.4 Assay for antiproliferative and cytotoxic effects

MRC-5 non-cancerous human embryonic lung fibroblast, doxorubicin-sensitive Colo 205 and multidrug resistant Colo 320 colonic adenocarcinoma cells were used to determine the effects of increasing concentration of tested compounds on cell proliferation. The density of the cells was adjusted to $6x10^3$ cells (antiproliferative assay) or $1x10^4$ cells (cytotoxicity assay). The plates were incubated at 37 °C for 72 h (antiproliferative test) or 24 h (cytotoxicity test). At the end of the incubation period, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to each well and cell growth was determined by measuring the optical density (OD) at 540/630 nm with Multiscan EX ELISA reader. Cell growth inhibition was calculated using the following formula:

$$Inhibition\% = 100 - \left[\frac{OD\ sample - OD\ medium\ control}{OD\ cell\ control - OD\ medium\ control}\right] \cdot 100\%$$

4.10.5 Evaluation of rhodamine 123 (R123) retention by flow cytometry

Colonic adenocarcinoma cells (the doxorubicin-sensitive Colo 205 and the multidrug resistant Colo 320) were treated with different concentrations of the tested compounds, then rhodamine 123 a fluorescent dye was added to measure ABCB1 transporter activity and identify possible inhibiting activity of the isolated sesquiterpene lactones. Without overexpressing the P-glycoprotein, cells accumulate rhodamine 123, resulting in increased fluorescence intensity. The cell population fluorescence intensity was measured with a Partec CyFlow cytometer. Compounds with a fluorescence activity ratio (FAR) above 2 are considered effective ABCB1 inhibitors.

4.10.6 Checkerboard combination assay

In order to investigate the impact of drug interactions between the compounds and doxorubicin, a checkboard microplate method and the multidrug resistant Colo 320 colonic adenocarcinoma cells expressing the ABCB1 transporter were used in the experiment. The dilutions of doxorubicin were made in a horizontal direction, and the dilutions of the tested compounds vertically in the microtiter plate using resuspended cells in each well. The cell growth rate was determined after MTT staining. Optical density (OD) was measured at 540/630 nm with Multiscan EX ELISA reader. Combination index (CI) values at 50% of the growth inhibition dose (ED_{50}), were determined using CompuSyn software, where $CI < 1$, $CI = 1$, and $CI > 1$ represent synergism, additive effect (or no interaction), and antagonism, respectively.

4.10.7 Real-time ethidium bromide accumulation assay

The EB accumulation assay provides insights into the EP function on *E. coli* AG100 and *S. aureus* ATCC 25923 strains. EP inhibitors increase the fluorescence of the intercalating substance EB, which is used as fluorescence dye due its accumulation within bacteria. The automated EB technique was used to analyze the effects of substances on EB accumulation using a CLARIOstar Plus plate reader (BMG Labtech, UK). The fluorescence was tracked every minute in real-time for an hour at 525 and 615 nm for excitation and emission to determine the relative fluorescence index (RFI) of the EB accumulation experiment final time point (minute 60).

4.11 Biological assays evaluated with the extracted essential oil

4.11.1 Bacterial strains

A wild-type *E. coli* K-12 AG100 expressing the AcrAB-TolC efflux pump (RP) at its basal level, *K. pneumoniae* ATCC 700603 were used as Gram-negative bacterial strains. *S. aureus* ATCC 25923 as methicillin-susceptible and *S. aureus* MRSA ATCC 43300 strains were investigated as Gram-positive bacterial strains.

4.11.2 Cell lines

For the antiproliferative and cytotoxic assays, human colonic adenocarcinoma cell lines (Colo 205 doxorubicin-sensitive and Colo 320/MDR-LRP multidrug resistant overexpressing ABCB1 (MDR1)-LRP), ATCC-CCL-220.1 (Colo 320) and CCL-222 (Colo 205)), hormone-responsive MCF-7 breast cancer and A549 lung carcinoma cells were used. While MRC-5 human embryonal lung fibroblast cell line (ATCC CCL-171) was used as normal cell lines.

4.11.3 Antibacterial assay

The antibacterial activity of the extracted essential oil was assessed through the minimum inhibitory concentration (MIC). The tested concentrations ranged from 1% to 0.0019%. The essential oil was dissolved in DMSO at subinhibitory concentrations (1% v/v). Four parallel measurements were performed on all tested concentration of the essential oil.

4.11.4 Real-time ethidium bromide accumulation assay

In the EB accumulation assay which gives insight into the bacterial EP function the essential oil was applied at MIC/2 concentrations: it was 0.0075% in *S. aureus* ATCC 25923 and 0.125% in MRSA. If the essential oil had no antibacterial effect, it was added at 0.5% or 1% in the case of *E. coli* and *K. pneumoniae* to the samples containing a non-toxic concentration of EB (2 μg/mL). Reserpine (RES) was applied at 25 μM as a positive

control on *S. aureus* strains, while carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was applied at 50 µM as positive control on *E. coli* and *K. pneumoniae*. The fluorescence was tracked in every minute using a CLARIOStar plate reader in real-time for an hour at 525 and 615 nm for excitation and emission to determine the relative fluorescence index (RFI) of the EB accumulation experiment final time point (minute 60).

4.11.5 Inhibition of biofilm formation

The Gram-negative *E. coli* K-12 AG100, *K. pneumoniae* ATCC 700603 and the Gram-positive *S. aureus* ATCC 25923, *S. aureus* MRSA ATCC 43300 and *S. aureus* MRSA 272123 strains were used in this experiment, where the dye crystal violet was applied to detect the development of biofilms. The biofilm formation was determined by measuring the OD_{600} using a Multiscan EX ELISA plate reader and the anti-biofilm effect of the compounds was expressed in the percentage of decrease of biofilm formation.

4.11.6 Assay for antiproliferative and cytotoxic effects

Two colonic adenocarcinoma cell lines (doxorubicin sensitive Colo 205 and multidrug-resistant Colo 320), MCF-7 and A549 cancer cells and the non-cancerous MRC-5 cell were used to test the effects of the increasing concentrations of the tested essential oil on cell growth and proliferation, in 96-well flat-bottom plates.

The MRC-5 embryonal lung fibroblast cells were cultured in EMEM supplemented with 10% heat-inactivated fetal bovine using 96-well flat bottom plates. The density of the cells was adjusted 1×10^3 cells (antiproliferative assay) or 1×10^4 cells (cytotoxicity assay) in 100 µL per well. After 24 h of seeding at 37 ºC the medium with the cells was removed.

Two-fold serial dilutions of the EO were prepared for the colonic adenocarcinoma cells in 100 µL of RPMI 1640, horizontally. Trypsin-Versene (EDTA) solution was used to treat the semi-adherent colonic adenocarcinoma cells. The cell density was adjusted to 6×10^3 cells for antiproliferative assay, and 6×10^4 for cytotoxicity assay. Doxorubicin and cisplatin were used as positive controls. The plates were incubated at 37 ºC for 72 h in case of antiproliferative test or 24 h for the cytotoxicity test. At the end of the incubation period, 20 µL of MTT (3-(4,5-dmiethylthiazol-2-yl)-2,5-diphenytetrazolium bromide) solution were added to each well. After incubation for 4 h at 37 ºC, 100 µL of sodium dodecyl sulfate solution (10% in 0.01 M HCl) was added to the wells and incubation was continued at 37 ºC overnight. Cell growth was determined based on optical density (OD)

measurements at 540/630 nm with Multiscan EX ELISA reader (Thermo Labsystems, USA). Using the following formula, the cell growth inhibition was calculated:

$$\text{Inhibition } \% = 100 - \left[\frac{OD\,sample - OD\,medium\,control}{OD\,cell\,control - OD\,medium\,control} \right] \times 100$$

5. Results

5.1 Isolation and identification of *Ambrosia* sesquiterpene lactones

During the phytochemical investigation the chromatographic purification steps resulted 8 pure compounds (compound **1**–**8**) from the MeOH extract of ragweed aerial parts (**Figure 2**). Based on comparison of NMR and HR-MS spectra with literature data, the isolate compounds were identified. Compound **1** was identified as psilostachyin C (**24**) [56], compound **2** as acetoxydihydrodamsin (**17**) [57], compound **3** as peruvin (**9**) [58], compound **4** as psilostachyin (**25**) [59], compound **5** as 1′-noraltamisin (**61**) [60], compound **6** as psilostachyin B (**27**) [58], and compound **8** as axillarin (**62**) [61].

Compound **7** was identified as a rare *seco*-psilostachyinolide based on NMR and MS experiments [61, 60], and named as 1,10-dihydro-1′-noraltamisin (**63**). Compound **7** was successfully obtained from natural sources and its ^{1}H and ^{13}C NMR spectral was completely revealed for the first time (**Table S1**).

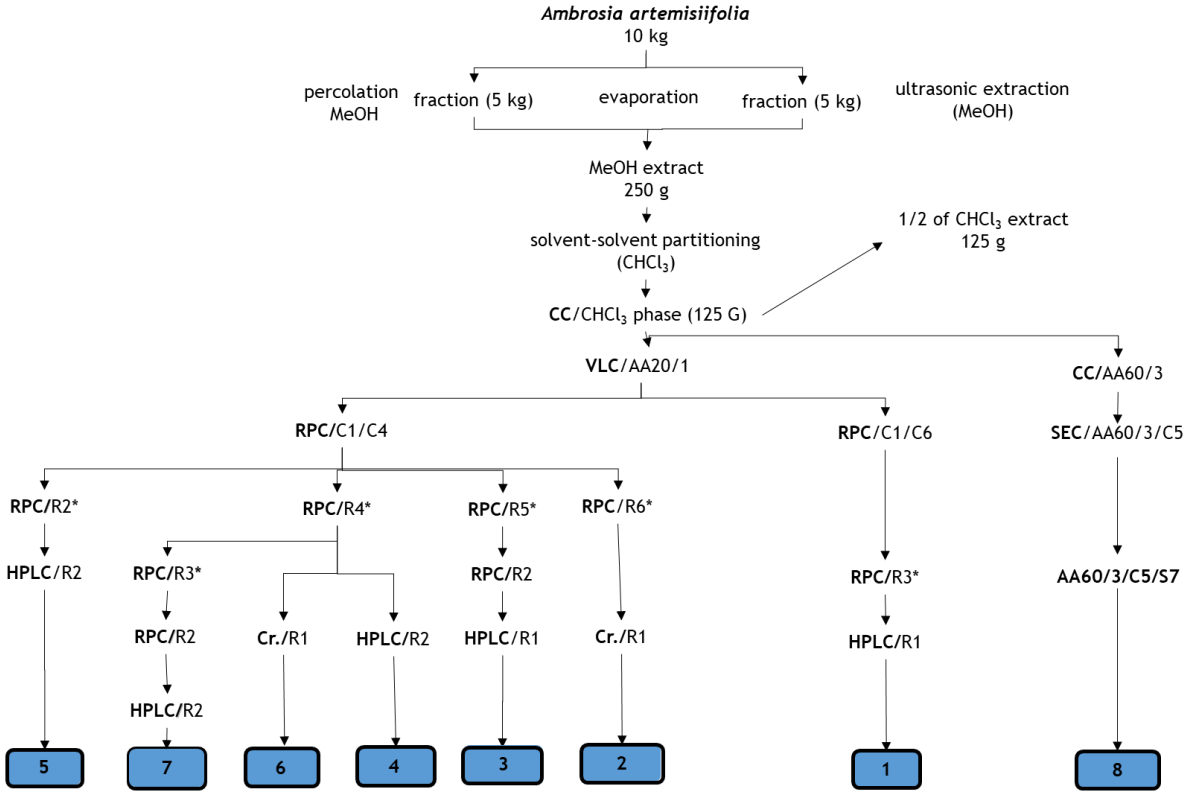


Figure 2. Isolation of sesquiterpene lactones from *Ambrosia artemisiifolia*

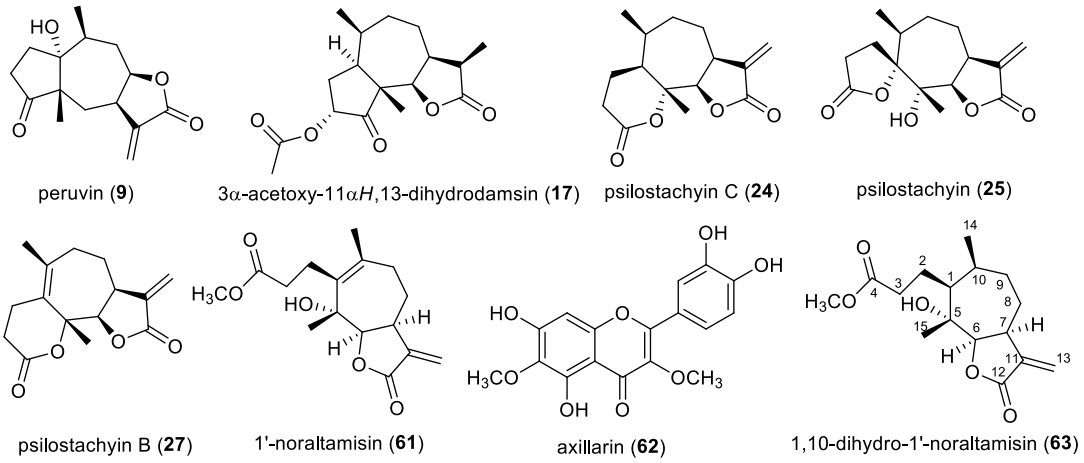


Figure 3. The structure of the isolated compounds from *Ambrosia artemisiifolia*

5.2 Extraction and characterization of *Ambrosia* essential oil

The conventional hydrodistillation technique yielded 0.13% essential oil from the aerial parts of *A. artemisiifolia* (v/w, estimated on dried plant material). The extracted yellowish oil had a strongly unpleasant odor. The essential oil components of *A. artemisiifolia* has been examined using GC/FID and GC-MS (**Table S2).** The GC-MS analysis revealed 73 components in *A. artemisiifolia* accounting for 91.19% of the total compounds. The essential oil was mostly composed of terpenes and their derivatives, with 74.40% being sesquiterpenes (including 31.91% oxygenated sesquiterpenes and 39.49% sesquiterpene hydrocarbons) and 19.78% monoterpenes (consisting of 8.72% monoterpene hydrocarbons and 11.06% oxygenated monoterpenes). The main components were germacrane D (18.81%), spathulenol (6.98%), caryophyllene oxide (6.45%), myrtenal (4.3%) and *trans*-β-ocimene (2.89%). These five chemicals made up 39.43% of the total oil mass, while monoterpene hydrocarbons (8.78%) and oxygenated monoterpenes (11.06%) were found in lesser levels.

The microwave-assisted isolation yielded different essential oil compositions: along with sesquiterpene hydrocarbons (39.91%), oxygenated monoterpenes were the second most prevalent, accounting for 30.60% of the total oil content. Borneol (7.17%) and bornyl acetate (8.76%) are the most oxygenated monoterpenes, with germacrene D (25.22%) having the largest amount. **Table S2.** shows the components of the cohobated water after hydrodistillation following liquid-liquid separation with *n*-hexane. Oxygenated monoterpenes made up 81.97% of this phase, with borneol (36.67%) and

trans-verbenol (18.07%) being the most prevalent. Besides the terpenoids, the following components are found in smaller quantities: eugenol (1.91%) as a phenylpropanoid.

5.3 HPLC/DAD method for targeted screening and quantification of sesquiterpene lactones in *A. artemisiifolia*

We analyzed qualitatively and quantitatively five marker compounds, psilostachyin (**25**), peruvin (**9**), acetoxydihydrodamsin (**17**), costunolide (**36b**) and isoalantolactone (**55**) from the aerial parts of *A. artemisiifolia* collected in two different regions of Hungary from June to October using an HPLC/DAD method developed by our group.

The new HPLC method enabled accurate characterization of these chemicals in ragweed samples (**Figure 4**).

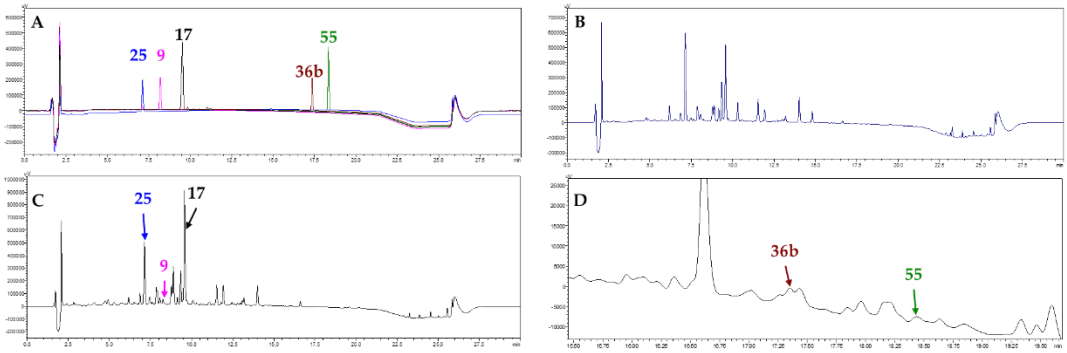


Figure 4. HPLC chromatograms of the reference compounds (**A**) and the crude extract (**B**, **C,** and **D**) at 210 nm. (**25**: psilostachyin, **9**: peruvin, **17**: acetoxydihadryodamsin, **36b**: costunolide, **55**: isoalantolactone).

The calibration curves rely on 8-10 calibration points. The correlation coefficient of the calibration curves was at least 0.997. To evaluate the analytical system's suitableness, a combination of reference standards was injected four times. The low RSD% values of AUCs and retention times, along with the tailing factors listed below, demonstrate that the system is appropriate for the measurement of these substances (**Table 2**).

Table 2. Characteristics of the calibration curve and limit of detection and quantification values, and results of the system suitability test. (LOD: limit of detection, LOQ: limit of quantification, RSD: relative standard deviation, RT: retention time).

Standard	LOD (µg/inj)	LOQ (µg/inj)	Calibration points	Range covered (µg/inj)	Regressions equations	AUC (RSD%)	Tailing factor	RT (RSD%)
psilostachyin (**25**)	0.04155	0.12590	9	0.02–20.00	y = 621593.7970x – 150728.6813 R^2 = 0.9970	1.00	0.876–1.224	0.16
peruvin (**9**)	0.06877	0.20841	9	0.05–4	y = 4929518558x – 46339884 R^2 = 0.9999	0.78	0.947–1.216	0.11
acetoxydihydrodamsin (**17**)	0.02688	0.08121	10	0.02–5	y = 1178552.7162x + 52571.8051 R^2 = 0.9992	0.54	1.081-1.110	0.13
costunolide (**36b**)	0.17653	0.53496	8	0.02–2.5	y = 417313.4111x – 32627130 R^2 = 0.9999	0.78	1.108–1.789	0.12
isoalantolactone (**55**)	0.07902	0.23947	8	0.02–2.5	y = 851443.9139x + 6909.0185 R^2 = 0.9999	0.29	1.108–1.239	0.11

5.4 Quantification of sesquiterpene lactones

The levels of the marker compounds in aerial parts of ragweed samples collected near Szeged and Nyíri are presented in **Table 3** and **Table 4**. Fluctuations of the detected compounds were graphically presented in **Figures 5–6** throughout time. The aerial parts of the ragweed collected from June to October near Szeged contained mainly psylostachyin (**25**) and peruvin (**9**), and in lesser quantities of acetoxydihydrodamsin (**17**), costunolide (**36b**), and isoalantolactone (**55**) in the concentration range of 0.02-0.10, 0.210–3.729, 0.499–10.917, 0.0561–1.128, and 0.019–0.090 mg/g, respectively. The compounds acetoxydihydrodamsin (**17**) and costunolide (**36b**) were found on minor concentration ranges in the set of samples collected near Nyíri, while psilostachyin (**25**) and peruvin (**9**) were observed in the highest amount (26.66 mg/g and 4.80 mg/g, respectively), while isoalantolactone (**55**) could not be identified. Sesquiterpene lactone levels were lower in plant samples obtained near Szeged compared to Nyíri. Significant differences ($p<0.0001$) were found for psilostachyin (**25**), peruvin (**9**), and acetoxydihydrodamsin (**17**), but costunolide (**36b**) and isoalantolactone (**55**) did not change significantly.

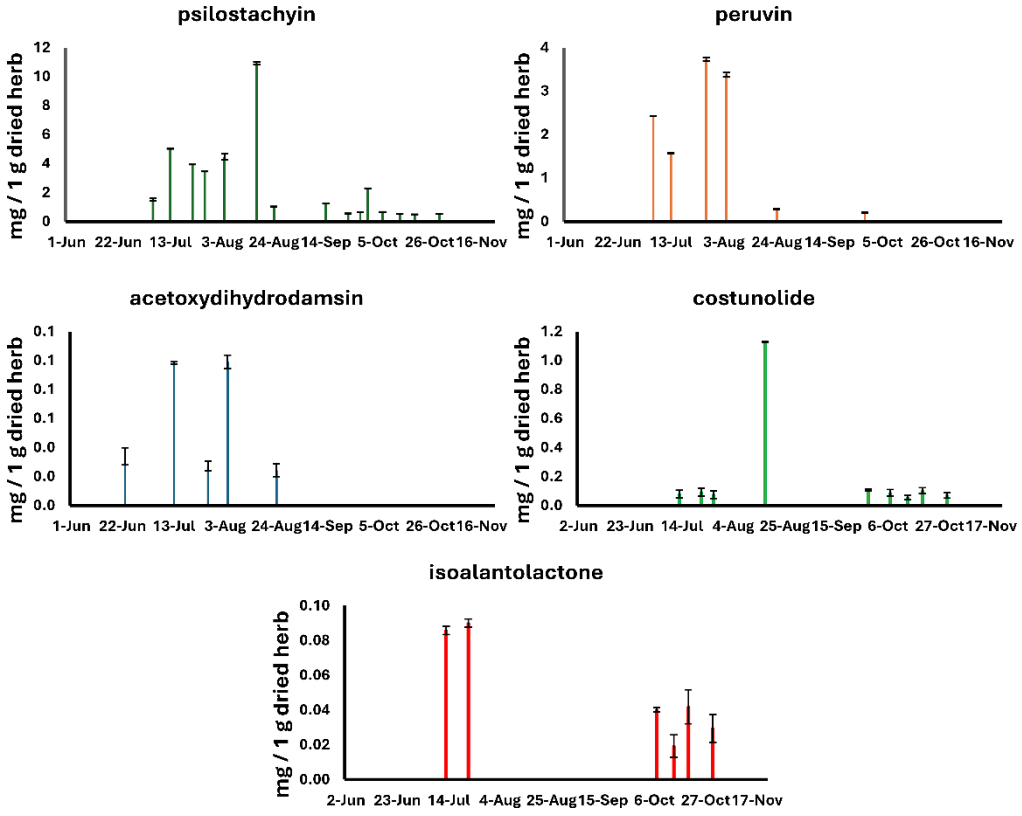


Figure 5. Concentration levels of sesquiterpene lactones in the aerial parts of *A. artemisiifolia* collected near Szeged from June to October.

Characteristic trends were found in the fluctuation of sesquiterpene lactone concentration during the examined time period. In general, the level of concentration peaked between the end of July and the middle of August. Differences in sesquiterpene variation trends might be found in sample sets of different geographical origins. Ragweed collected in Szeged contained higher amounts in the second half of July and the first half of August for the major components, psilostachyin (**25**) and peruvin (**9**). Interestingly, psilostachyin (**25**) and a minor chemical costunolide (**36b**) showed identical variation trends. Ragweed samples near Nyíri had higher sesquiterpene concentrations earlier in the vegetational season, but decreased significantly in early August for the major components (psilostachyin (**25**), peruvin (**9**), and isoalantolacton (**55**)).

Psilostachyin (**25**), a main component of ragweed aerial parts, peaks in early August in samples taken near Szeged and in mid-August in those collected near Nyíri. Samples collected near Szeged showed constant elevation until the middle of August, followed by a substantial decline at the end of August. A considerable increase in the level of psilostachyin (**25**) was followed by maxima at the beginning and second half of August, respectively, and then the level of this compound steadily decreased until the end of October. The Nyíri sample had the highest concentration of psilostachyin (**25**), measuring 26.66 mg/g. In contrast, samples collected during the same summer near Szeged contained significantly less psilostachyin (**25**) (10.92 mg/g). Isoalantolactone (**55**) was found to be a minor compound with concentrations below the limit of detection (LOQ) in samples collected near Szeged, but not near Nyíri. In contrast, costunolide (**36b**) was present in both samples in trace amounts (0.06-1.13 mg/g). The highest concentrations of the compounds detected in ragweed samples occurred during the bloom period, which lasted from the end of June (June 23, 2021) to the last weeks of September (September 21, 2021) at both collection sites (**Figure 5-6**).

Acetoxydihydrodamsin (**17**) levels varied significantly from 0.39 to 9.23 mg/g in samples collected near Nyíri from June to October. The highest concentration of the aforementioned sesquiterpene in the aerial parts was detected in samples collected in the middle of July, while samples from the last weeks of October contained the lowest concentration (0.39 mg/g) (**Figure 4**). The concentration of peruvin (**9**), the third major compound, varied from 0.01 mg/g (July 4) to 4.80 mg/g (July 13) in samples collected near Nyíri. In contrast, the samples collected in Szeged contained the largest quantities at the end of July (3.73 mg/g) and in the early weeks of August (3.38 mg/g) (**Figure 5**).

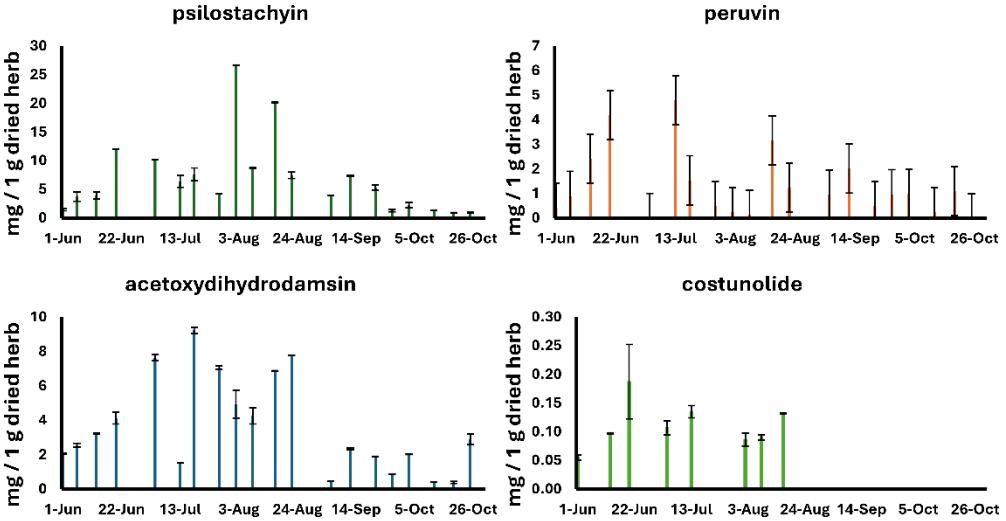


Figure 6. Concentration levels of sesquiterpene lactones in the aerial parts of *A. artemisiifolia* collected near Nyíri from June to October.

Table 3. Content of marker compounds in aerial parts of *A. artemisiifolia*; collected near Szeged, Hungary.

Date of harvest	psilostachyin	peruvin	acetoxydihydrodamsin	costunolide	isoalantolactone
June 2, 2021	ND	ND	ND	ND	ND
June 8, 2021	ND	ND	ND	ND	ND
June 15, 2021	ND	ND	ND	ND	ND
June 23, 2021	ND	ND	<LOQ	ND	ND
July 6, 2021	1.52±0.10	2.43±0.00	ND	ND	ND
July 13, 2021	5.03±0.02	1.57±0.02	0.10±0.00	<LOD	<LOQ
July 22, 2021	3.94±0.00	ND	ND	<LOD	<LOQ
July 27, 2021	3.46±0.01	3.73±0.04	0.03±0.00	<LOD	ND
August 4, 2021	4.47±0.22	3.38±0.05	0.10±0.01	ND	ND
August 17, 2021	10.92±0.10	ND	ND	1.13±0.00	ND
August 24, 2021	1.03±0.01	0.03±0.01	<LOQ	ND	ND
September 14, 2021	1.25±0.00	ND	ND	ND	ND
September 23, 2021	0.54±0.03	ND	ND	ND	ND
September 28, 2021	0.64±0.01	0.21±0.01	ND	<LOD	ND
September 31, 2021	2.26±0.03	ND	ND	ND	ND
October 7, 2021	0.66±0.02	ND	ND	<LOD	<LOD
October 14, 2021	0.53±0.02	ND	ND	<LOD	<LOD
October 20, 2021	0.50±0.00	ND	ND	<LOD	<LOD
October 30, 2021	0.51±0.01	ND	ND	<LOD	<LOD

The values are presented as mean (n=3) of marker compounds amount in mg/1 g of dried aerial part of the herb. (LOQ: limit of detection, LOD: limit of detection, ND: not detected.).

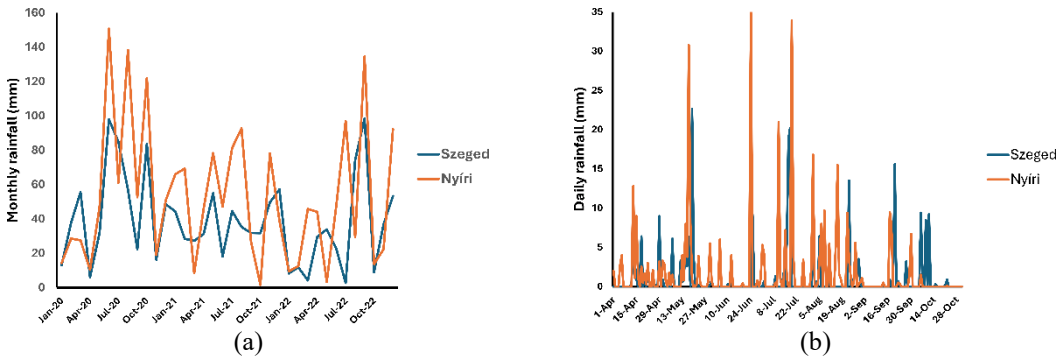
The results of the statistical analysis of sample sets gathered in Szeged and Nyíri showed that there were significant differences in the concentration level of psilostachyin (**25**) ($t(2)=6.32$, $p<0.001$), peruvin (**9**) ($t(2)=3.69$, $p<0.001$), acetoxydihydrodamsin (**17**) ($t(2)=11.93$, $p<0.001$), and costunolide (**36b**) ($t(2)=6.32$, $p<0.001$), respectively. The Nyíri sample did not contain any isoalantolactone (**55**).

Table 4. Contents of marker compounds on aerial parts of *A. artemisiifolia*; collected near Nyíri, Hungary.

Date of harvest	psilostachyin	peruvin	acetoxydihydrodamsin	costunolide	isoalantolactone
June 1,2021	1.46±0.19	0.42±0.02	2.06±0.02	<LOD	ND
June 6, 2021	3.73±0.79	0.90±0.01	2.60±0.10	ND	ND
June 13, 2021	3.91±0.60	2.42±0.03	3.23±0.02	<LOD	ND
June 20, 2021	12.01±0.03	4.19±0.04	4.13±0.34	<LOD	ND
July 4, 2021	10.23±0.00	<LOD	7.63±0.18	<LOD	ND
July 13, 2021	6.39±1.02	4.80±0.00	1.51±0.00	<LOD	ND
July 18, 2021	7.64±1.11	1.54±0.03	9.23±0.17	ND	ND
July 27, 2021	4.30±0.03	0.48±0.01	7.06±0.10	ND	ND
August 2, 2021	26.66±0.08	0.25±0.01	4.94±0.81	<LOD	ND
August 8, 2021	8.76±0.04	<LOQ	4.25±0.47	<LOD	ND
August 16, 2021	20.13±0.09	3.16±0.08	6.87±0.02	<LOD	ND
August 22, 2021	7.47±0.56	1.24±0.00	7.78±0.01	ND	ND
September 5, 2021	4.00±0.01	0.96±0.01	0.46±0.00	ND	ND
September 12, 2021	7.39±0.14	2.02±0.03	2.35±0.05	ND	ND
September 21, 2021	5.32±0.48	0.49±0.00	1.90±0.00	ND	ND
September 27, 2021	1.32±0.22	0.97±0.00	0.86±0.02	ND	ND
October 3, 2021	2.26±0.48	0.98±0.04	2.02±0.01	ND	ND
October 12, 2021	1.37±0.03	0.24±0.03	0.40±0.00	ND	ND
October 19, 2021	0.91±0.06	1.11±0.16	0.39±0.06	ND	ND
October 25, 2021	0.94±0.07	ND	2.90±0.31	ND	ND

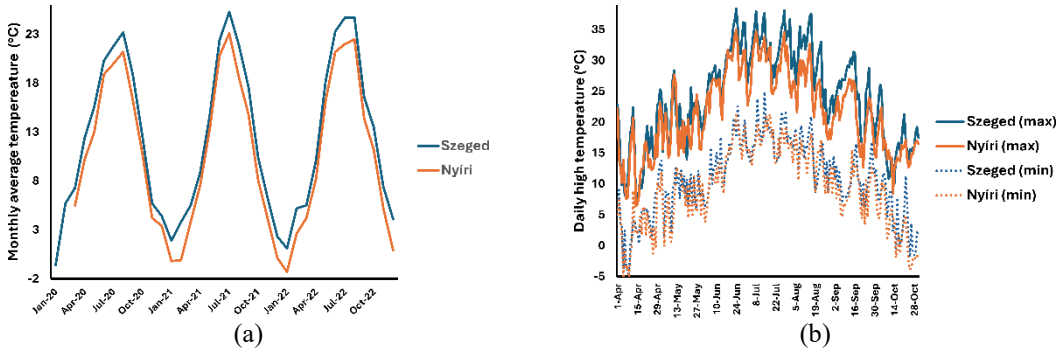
Values are presented as mean (n=3) of marker compounds in mg/1 g of dried part of the herb. (LOQ: limit of quantification, LOD: limit of detection, ND: not detected).

Climatic parameters, such as daily temperature (average, minimum, and maximum), global radiation, and daily precipitation, were sourced from the Meteorological Database of the Hungarian Service (http://odp.met.hu) [51, 52], [53, 54], [55,56]. These data are presented in **Figures 7–9**. Pearson correlation was used to examine the relationship between sesquiterpene lactone concentration levels and climate parameters. **Table 5** represents the correlation data. These findings show a weak to moderate positive association between temperature and sesquiterpene lactone concentration levels. Nyíri samples showed weak correlations. Surprisingly weak negative and non-significant correlation was found in the Szeged samples between sesquiterpene lactones and daily rainfall; however, this association needs to be interpreted in light of the fact that Szeged only had one day of rain throughout the studied period. There was no observable correlation with global radiation.



(a) (b)

Figure 7. Monthly average rainfall recorded from 2020 to 2022 (a) and daily rainfall recorded from April to October in 2021 (b). Data source: HungaroMet.



(a) (b)

Figure 8. Monthly average temperatures registered from 2020 to 2022 (a) and the daily lowest and highest temperatures registered from May to October in 2021 (b). Data source: HungaroMet.

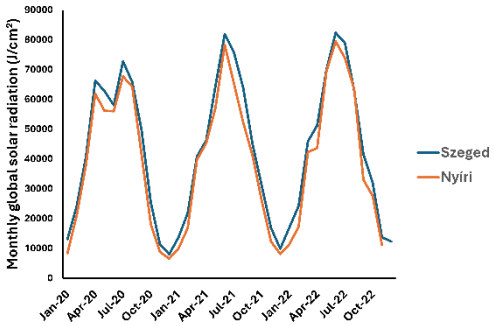


Figure 9. Monthly global solar radiation recorded at collection sites. Data source: HungaroMet.

Table 5. Correlation data between climatic factors and concentration levels of sesquiterpene lactones.

Climatic factor	Collection site	psilostachyin	peruvin	acetoxydihydrodamsin	costunolide	isoalantolactone
temperature (average)	SZEGED	0.34	0.59 (**)	0.55 (*)	0.00	0.01
	NYÍRI	0.46 (*)	0.46 (*)	0.58 (**)	0.61 (**)	ND
temperature (minimal)	SZEGED	0.46 (*)	0.52 (*)	0.50 (*)	0.18	-0.09
	NYÍRI	0.59 (**)	0.43	0.61 (**)	0.54 (*)	ND
temperature (maximal)	SZEGED	0.24	0.55 (*)	0.52 (*)	0.09	-0.26
	NYÍRI	0.43	0.45 (*)	0.55 (*)	0.62 (**)	ND
global radiation (daily)	SZEGED	0.04	0.33	0.30	–0.18	0.02
	NYÍRI	0.12	0.39	0.35	0.46	ND
precipitation (daily)	SZEGED	–0.12	–0.12	–0.11	–0.01	0.20
	NYÍRI	0.53 (*)	0.02	0.21	0.37	ND

Values are Pearson correlation coefficients for Szeged (df=17) and Nyíri (df=18) sample sets. The correlation is significant if $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***). (ND: not detected.)

5.5 Pharmacological activities of *Ambrosia* sesquiterpene lactones

5.5.1 Antibacterial activity of the isolated sesquiterpene lactones

None of the tested compounds had an antibacterial effect. The MIC values for Gram-negative and -positive bacteria were greater than 100 μM.

5.5.2 Antiproliferative and cytotoxic effects of sesquiterpene lactones

Two human adenocarcinoma cell lines were used in an MTT assay to evaluate the isolated compounds' *in vitro* antiproliferative and cytotoxic effects (the doxorubicin-sensitive Colo 320 and the multidrug-resistant Colo 320 colonic adenocarcinoma cell lines). Acetoxydihydrodamsin (**17**) showed cytotoxic activity (IC_{50} = 7.64 ± 0.37 mM) on the sensitive (Colo 205) cell line after 24 hours of treatment. Among the *seco*-psilostachyinolides, psilostachyin C (**24**) exhibited the most cytotoxic effects (26.6 ± 0.48 mM) on the doxorubicin-sensitive Colo 205 cell line, whereas psilostachyin (**25**) had cytotoxic effects on the multidrug-resistant Colo 320 cell line (17.7 ± 0.20 mM). Psilostachyin B (**27**), which has the similar *seco* derivative structure, did not exhibit any cytotoxic effects on the examined cell lines (**Table 6**). Unlike, the isolated sesquiterpene lactones, the quercetagetin 3,6-dimethyl ether derivative (axillarin (**62**)) had no cytotoxic effects on the examined cell lines after a short exposure (**Table 6**).

Table 6. Cytotoxicity of compounds **1**–**8** isolated from *A. artemisiifolia* (IC_{50} values μM)

Compounds	Cell lines		
	MRC-5	Colo205	Colo320
psilostachyin C (24)	52.69 ± 2.62	26.60 ± 0.48	28.71 ± 0.11
acetoxydihydrodamsin(17)	23.77 ±1.06	7.64 ± 0.37	10.75 ± 0.22
peruvin (9)	11.89 ± 1.45	64.44 ± 1.78	82.37 ± 1.17
psilostachyin (25)	37.57 ± 2.51	24.50 ± 0.45	17.7 ± 0.2
1´-noraltamisin (61)	41.82 ± 2.43	42.43 ± 2.37	40.08 ± 1.31
psilostachyin B (27)	>100	>100	>100
1,10-dihydro-1´-noraltamisin (63)	>100	53.57 ± 0.87	86.59 ± 1.06
axillarin (62)	>100	>100	>100

Values are presented as mean (n=3) and SD of IC_{50} values.

After 72 hours of incubation, acetoxydihydrodamsin (**17**) showed the highest antiproliferative effect on both adenocarcinoma cell lines (IC_{50} values of 5.14±0.55 mM on Colo 205 and 3.67±0.35 mM on Colo 320) (**Table 7**). Axillarin (**62**) had a relatively low dose (IC_{50} value of 4.03 ± 0.56 mM) of inhibition over the proliferation of human embryonal lung fibroblast cells, whereas psilostachyin B (**27**) remained inactive in this experiment as well (**Table 4**). Acetoxydihydrodamsin (**17**) showed selective cytotoxicity against human colonic cancer cell lines compared to human embryonal lung fibroblast cells (selectivity index [SI] = 3.11). Psilostachyin (**25**) and psilostachyin B (**27**) shown

considerable selectivity against the MRC-5 cell line (SI = 3.04 and SI = 4.98), although they demonstrated cell growth inhibitory activity on the Colo320 multidrug resistant cell line (IC_{50} values of 8.78 ± 0.22 mM and 5.29 ± 0.15 mM, respectively).

Table 7. Antiproliferative effects of compounds **1-8** isolated from *A. artemisiifolia* (IC_{50} values μM)

Compounds	Cell lines		
	MRC-5	**Colo205**	**Colo320**
psilostachyin C (24)	35.13 ± 4.03	15.61 ± 0.83	14.66 ± 0.82
acetoxydihydrodamsin (17)	10.96 ± 0.31	5.14 ± 0.55	3.67 ± 0.35
peruvin (9)	26.42 ± 1.30	26.35 ± 1.12	21.19 ± 0.72
psilostachyin (25)	26.36 ± 0.81	10.99 ± 0.56	5.29 ± 0.15
1´-noraltamisin (61)	26.72 ± 0.51	14.37 ± 1.00	8.78 ± 0.22
psilostachyin B (27)	>100	>100	>100
1,10-dihydro-1´-noraltamisin (63)	39.78 ± 0.53	17.01 ± 1.99	34.51 ± 2.07
axillarin (62)	4.03 ± 0.56	66.75 ± 0.96	50.40 ± 2.98

Values are presented as mean (n=3) and SD of IC_{50} values.

5.5.3 The ABCB1 efflux pump (P-glycoprotein) inhibitory activity of sesquiterpene lactones

After evaluating the fluorescence intensity of the cell population, none of the chemicals demonstrated sufficient inhibitory effects based on their FAR values. The studied compounds had FAR values ranging from 0.2 to 1.1.

5.5.4 Drug interactions between sesquiterpene lactones and doxorubicin

ABCB1 transporter-expressing, multidrug-resistant Colo320 colonic cancer cells were used to evaluate the drug interactions between the compounds and doxorubicin. 1,10-dihydro-1´-noraltamisin (**63**) and axillarin (**62**) exhibited synergistic effects with doxorubicin at certain concentrations. Psilostachyin C (**24**), acetoxydihydrodamsin (**17**) and peruvin (**9**) had antagonistic effects at certain concentrations (**Table S3**).

5.5.5 The impact of sesquiterpene lactones on the function of bacterial efflux pumps (EP)

No sesquiterpene lactones showed inhibitory activity on EP function. RFI values were comparable to the untreated sample, ranging around zero.

5.6. Pharmacological activity of extracted essential oil

5.6.1 Antibacterial activity of the essential oil extracted from *A. artemisiifolia*

Table 8 shows the MIC values for the tested essential oil against two Gram-positive and two Gram-negative bacteria. Using the growth microdilution method, we noticed growth inhibition in both Gram-positive and Gram-negative bacteria; the essential oil effectively inhibited the growth of *S. aureus* ATCC 25923 strain (MCI = 0.015%) and the

methicillin-resistant *S. aureus* ATCC 43300 strain (0.25%). The essential oil showed almost no activity against the two Gram-negative bacteria: *E. coli* (MIC = > 1%) *K. pneumoniae* (MIC = > 1%).

Table 8. Minimum inhibitory concentrations (MICs) of the essential oil of *Ambrosia artemisiifolia*

Microorganisms	MIC
***Staphylococcus aureus* ATCC 25923**	0.015%
***Staphylococcus aureus* MRSA ATCC 43300**	0.25%
***Escherichia coli* ATCC 25922**	>1%
***Klebsiella pneumoniae* ATCC 700603**	>1%

Values are presented as mean (n=3) of MIC values.

5.6.2 The effects of the extracted essential oil on the function of bacterial efflux pumps (EP)

The study found out that, *A. artemisiifolia* essential oil did not reduce efflux pump activity on the Gram-negative bacteria *K. pneumoniae* ATCC 700603 strain (**Table 9**). The RFI values were nearly identical to the untreated sample. The RFI values for *E. coli* ATCC 25922 were greater at the same essential oil concentration than compared to the CCP treated control, and a dose-dependent inhibitory activity was observed. In comparison to reserpine, the RFI values for the Gram-positive bacteria were greater in both cases: *S. aures* ATCC 25923 (RFI = 0.68) after treatment with 0.0075% essential oil and MRSA strain (RFI = 0.56) after applying of 0.125% of ragweed essential oil (**Table 10**).

Table 9. Evaluation of the real-time ethidium bromide accumulation assay on the two Gram-negative bacteria strain lines (*E. coli* and *K. pneumoniae*).

Microorganism	EO (%)	CCCP (µM)	RFI
***Escherichia coli* ATCC 25922**	0.50		2.88
	1		3.75
		50	1.79
***Klebsiella pneumoniae* ATCC 700603**	0.50		0.22
	1		0.15
		50	1.11

EO: essential oil, concentrations in percentage; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone. RFI: relative fluorescence index.

Table 10. Evaluation of the real-time ethidium bromide accumulation assay on the two Gram-positive bacteria strain lines (*S. aureus* and the methicillin resistant *S. aureus*).

Microorganism	EO (%)	RES (µM)	RFI
***Staphylococcus aureus* ATCC 25923**	0.0075		0.68
		25	0.42
***Staphylococcus aureus* MRSA ATCC 43300**	0.125		0.56
		25	0.22

EO: essential oil, concentration in percentage by MIC/2 values; RES: reserpine; RFI: relative fluorescence index.

5.6.3 The inhibitory activity of essential oil on biofilm formation of different bacterial strains

Higher dosages (0.5% and 1%) of the essential oil showed no effects on the biofilm formation of the *E. coli* ATCC 25922 strain (**Figure 10**) compared to the positive controls, while the same dosage inhibited *K. pneumoniae* ATCC 600703 strain biofilm formation in a dose-dependent manner (**Figure 10**). The crystal violet experiment revealed that MIC/2 levels did not reduce biofilm formation in *S. aureus* ATCC 25923 (**Figure 11**) and the methicillin-resistant *S. aureus* ATCC 43300 (**Figure 12**).

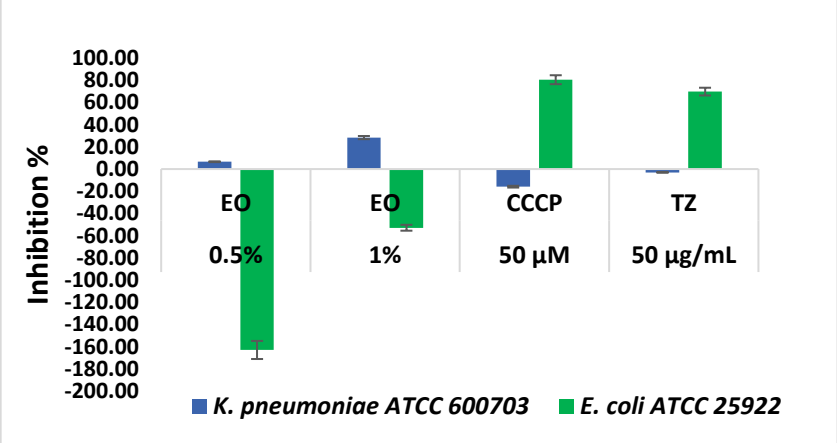


Figure 10. Reduction of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 600703 biofilm formation in the presence of *A. artemisiifolia* EO (EO: essential oil; CCCP: cyanide m-chlorophenylhydrazone; TZ: thioridazine). Bars represent the mean ± SD of four parallel experiments.

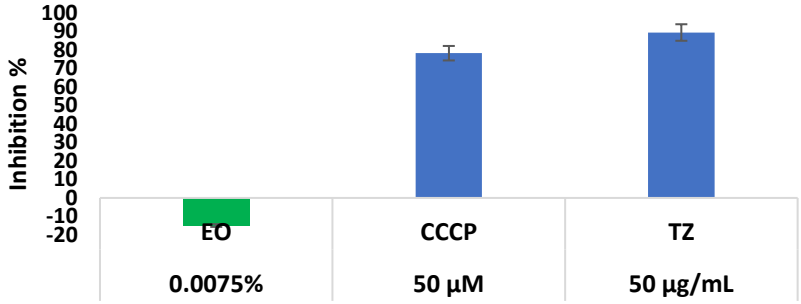


Figure 11. Reduction of *S. aureus* ATCC 25923 biofilm formation in the presence of *A. artemisiifolia* EO (EO: essential oil; CCCP: cyanide *m*-chlorophenylhydrazone; TZ: thioridazine). Bars represent the mean ± SD of four parallel experiments.

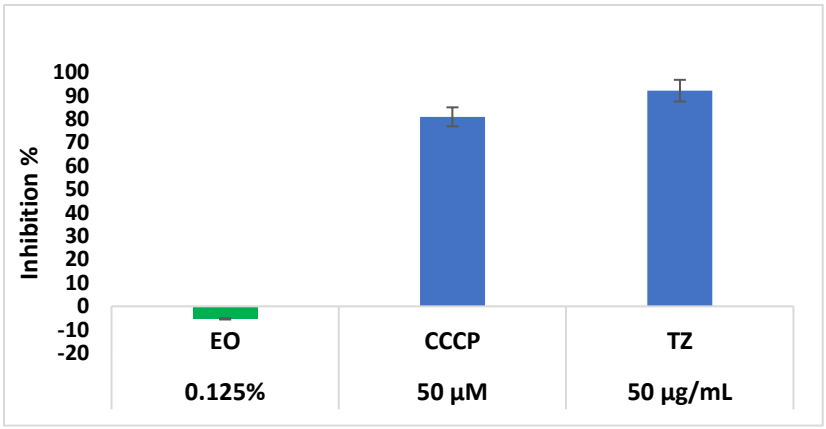


Figure 12. Reduction of *S. aureus* ATCC 43300 biofilm formation in the presence of *A. artemisiifolia* EO (EO: essential oil; CCCP: cyanide m-chlorophenylhydrazone; TZ: thioridazine). Bars represent the mean ± SD of four parallel experiments.

5.5.4 Cytotoxic and antiproliferative activity of ragweed essential oil

The EO had a strong cytotoxic effect on the MDR (Colo 320) and Colo 205 cell lines, as indicated by the IC_{50} values established after 24 hours of incubation (IC_{50} values: 0.0130% and 0.015%, respectively). In case of MCF-7 cell line, a short exposure to the plant essential oil resulted IC_{50} values of 0.1605%. Plant essential oil treatment of the A549 lung cancer cell line resulted low inhibition concentrations with IC_{50} values of 0.0413%, respectively. On the human embryonal lung fibroblast cell line MRC-5, the IC_{50} value was a magnitude higher (0.104%) (**Table 11**).

Table 11. Cytotoxic effects as determined in IC_{50} values after treating different cancer cell lines and normal cell lines with the essential oil and the positive control for 24 h.

	A549 AP	Colo 320 AP	Colo 205 AP	MCF-7 AP	MRC-5
Essential oil	0.0413±0.0005 (%)	0.0103±0.0008 (%)	0.015±0.001 (%)	0.1605±0.0091 (%)	0.104±0.002 (%)
Doxorubicin	8.62 (µM)	4.03±0.89 (µM)	3.05±0.49 (µM)	2.11±0.08 (µM)	2.57±0.01 (µM)
Cisplatin	>100 (µM)	13.32±0.49 (µM)	34.94±26.39 (µM)	17.68±0.31 (µM)	34.24±0.54 (µM)

IC_{50} values represent the mean ± SD of four parallel experiments.

The essential oil of ragweed exhibited potent antiproliferative activity against the two human colonic cancer cell lines, according to our experimental results of the in vivo antiproliferative activity using the MTT assay. **Table 12** demonstrate that, with a starting concentration of 1% of *A. artemisiifolia* essential oil, the serially diluted oil had an IC_{50} value of 0.054% on the doxorubicin-sensitive Colo 205 cells. For the MDR Colo 320 cells, the concentration required to inhibit 50% of cell proliferation was even lower (0.008%). Cell proliferation was reduced when the MCF-7 breast cancer cell line was

treated with the plant essential oil, resulted low IC_{50} values (0.099%). The adenocarcinoma human alveolar basal epithelial cells were incubated for 72 hours, and the plant essential oil had an IC_{50} values of 0.3086% (**Table 12**).

Table 12. Antiproliferative effects as determined in IC_{50} values after treating different cancer cell lines and normal cell lines with the essential oil and the positive control for 72 h.

	A549 AP	**Colo 320 AP**	**Colo 205 AP**	**MCF-7 AP**	**MRC-5**
Essential oil	0.3086±0.0235 (%)	0.008±0.000 (%)	0.054±0.005 (%)	0.099±0.011 (%)	0.091±0.002 (%)
Doxorubicin	0.060±0.01	0.22±0.03	0.48±0.03	0.02±0.01	0.31±0.03
Cisplatin	3.16±0.05	3.68±0.14	28.82±2.51	10.85±0.05	1.66±0.06

IC_{50} values represent the mean ± SD of four parallel experiments.

6. Discussion

The aims of the present work were to isolate sesquiterpene lactones from the aerial parts of *A. artemisiifolia*, to extract essential oil form the aerial parts and to investigate their pharmacological activity, and last but not least to measure the sesquiterpene lactone content of different ragweed samples. For the isolation, we used different chromatographic techniques, such as open column chromatography, preparative rotation planar chromatography, medium pressure chromatography and high-performance liquid chromatography. To determine the sesquiterpene content of ragweed samples, we used an analytical HPLC instrument after the sample preparation.

From the MeoH extract, eight compounds were isolated: psilostachyin C (**24**), acetoxydihydrodamsin (**17**), peruvin (**9**), psilostachyin (**25**), 1′-noraltamisin (**61**), psilostachyin B (**27**) and the flavonoid axillarin (**62**). After NMR data comparison we found out that 1,10-dihydro-1′-noraltamisin (**63**) was obtained for the first time from *A. artemisiifolia*. Bearing an open ring system, it belongs to a rare class of *seco*-psilostachyinolides. According to literature data, the biosynthetic pathways of this type of *seco* derivatives are localized in two different places inside plant cells: the mevalonic acid (MVA) pathway in cytoplasm or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in the plastids [62]. The precursor in both metabolic pathways, 2*E*,6*E*-farnesyl pyrophosphate (FPP), outcomes of the addition of a molecule of IPP onto GPP. The cyclization of FPP resulted the cyclodecadiene-type product, which is the skeleta of this open ring system, or the geometrical isomer at C-2 (2*E*,6*E*-FPP) of FPP, or of nerolidyl pyrophosphate, by nucleophilic attack on the distal double bond which leads to the germacradienyl cation, from here other lactone skeletal types may arise [63]. Only a few number of these kinds of *seco* derivatives from the genus *Ambrosia* have been documented in the literature so far. Only one Mexican population of *Ambrosia confertiflora* DC (collected in North Zacatecas) was found to contain 1′-noraltamisin (**61**) [60]. Two more sesquiterpene lactones with similar structures are altamisin from *A. peruviana* (a collection form Central America) [64] and altamisic acid from *A. tenuifolia* (a collection from North Central Argentina) [58]. Stefanovic et al. [65] identified 4-oxo-3,4-ambrosan-6,12-olide-3-oic acid from a Yugoslavian collection of *A. artermisiifolia*; Taglialatela-Scafati et al. [57] also discovered the same sesquiterpene from an Italian plant sample.

To assess the cytotoxic and antiproliferative activity of the isolated sesquiterpene lactones, we build up two different experimental setups of MTT assay: a short-term (24

h) treatment of a relatively higher number of cells (cytotoxic activity) and a long-term (72 h) treatment if a lower cell number (cytostatic or antiproliferative activity). After a short exposure period, acetoxydihydrodamsin (**17**) showed the most potent cytotoxic effects on Sensitive (Colo 205) cell line. 1´-noraltamisin (**61**) and psilostachyin (**25**) the two *seco*-psilostachyinolides possessed significant antiproliferative activity of the multidrug-resistant Colo 320 cell line and showed moderate selectivity against human embryonal lung fibroblast cell line (**Table S3**). The cytotoxicity of sesquiterpene lactones is associated with the existence of an exocyclic double bond adjacent to the γ-lactone ring [66] which may be crucial for the alkylation of nucleophilic sites of target enzymes. However, acetoxydihydrodamsin (**17**) is lack of this moiety, and bears an acetyl group at C-3 demonstrating that the presence of an α,β-unsaturated carbonyl group is not crucial for the inhibition of cell proliferation. The isolated quercetagetin 3,6-dimethyl ether axillarin (**62**) had strong antiproliferative activity with relatively low concentration on MRC-5 cell line and showed no selectivity toward them.

After investigating the P-glycoprotein (ABCB1) inhibitory activity of the isolated compounds, we concluded that this type of secondary metabolites have different targets inside cancer cells: inhibition of DNA methylation [67], altering the NF-κB signaling pathway [66], cell cycle checkpoint inhibition [33], provoking apoptosis and cell cycle arrest [68]. Considering the fact that *Ambrosia* sesquiterpene lactones possessing different biological activities (cytotoxic, antiproliferative effects etc.) with multiple targets inside cancer cells which lead to apoptosis or cell death as mentioned above, these secondary metabolites might be appropriate for novel drug development in the future. However, it is also necessary to assess the possible cytotoxic effects of this type of terpenes, the selectivity toward normal cell lines and the interaction with other drugs. There are not enough data in the literature about the possible harmful effects of the sesquiterpene lactones in the case of long-term consumption of ragweed or any other related *Ambrosia* species. Therefore, either focusing on the possible utilization of the plant, or on the safe application of its secondary metabolites for different purposes, we have to take into consideration how the plant sesquiterpene lactone content might change in different vegetation periods.

For assessing the quantitative changes of sesquiterpene lactones, plant samples in different phenotypes were collected from two different places in Hungary (northeast and southeast). The plant samples from the northeast part of Hungary were covered much denser foliage with dense glandular trichome layer, and during the blooming period more

dense composite inflorescence. On the contrary, plant samples from southeast of Hungary had less-dense foliage and capitulum and higher stems. Since in Asteraceae plants, the sesquiterpene lactone synthesis take place in the smooth endoplasmic reticula of capitate glandular trichomes (CGTs), then it is then secreted in the extracellular and subcuticular space [69], this may explain the observed differences in sesquiterpene lactone profile from the two collection sites. The key enzymes of sesquiterpene biosynthesis are located in the smooth endoplasmic reticulum of CGT secretory cells located on the leaf surface [70]. Connecting to the above-mentioned observations, the sesquiterpene lactone content may correlate with the development stage of aboveground parts (foliage density and trichome coverage) of ragweed. Geoclimatic factors may impact the secondary metabolites of ragweed, leading to variations in sesquiterpene lactone profiles across samples taken from different areas. Some authors hypothesized that due to the great genetic variation in *A. artemisiifolia*, the collection of the plant even from the same places afforded different phytochemical profiles (Novara, Italy). Taglialatela-Scafati and his co-workers described different sesquiterpene profiles with hydroxydihydrodamsin as the main compounds from the first collection, while isabelin as the main compound in the third collection [57]. Another collection from the same place showed different chemical profile: Božičević and his colleagues [71] pointed out that the ragweed pollen did not contain sesquiterpenes presented in the leaves and flowers as Taglialatela-Scafati at al. reported previously. Despite all of that, the sesquiterpene content of the plant may differ regarding the vegetation period, the collection place etc., some studies described the isolation of characteristic *Ambrosia* sesquiterpenes that are widely distributed in species of different origins. Psilostachyin (**24**) was isolated from ragweed samples collected in Australia [72], Argentina [73] and in the US [74]. Others reported similar results: the collection of ragweed samples from Poland [75] and Italy [57] led the isolation of peruvin (**9**) and acetoxydihydrodamsin (**17**), two characteristic sesquiterpenes of the genus. Furthermore, geoclimatic elements also have impact on the sesquiterpene lactone content of *A. artemisiifolia*. Different environmental stresses may lead to significantly reduced growth as well as physiological and yield responses, which may resulting to early senescence or cell death [76]. Water stress is one of the major environmental issues, which can alter plant development and production. A study revealed the impact of water deficit treatment on a phylogenetically close species *Artemisia annua* L.: the authors noted that the main sesquiterpene content (artemisinin, artennuin-B, artemisinic acid, dihydroartemisinic acid) was negatively modulated by the water deficit stress, and they

also observed that the glandular trichome density and size was decreased [77]. These observations correlate with ours: samples collected near Szeged had lower glandular trichome density compared to ragweed samples from Nyíri. Our findings also correspond with previous research on the development of metabolites in *A. annua*, indicating a peak in sesquiterpene concentration during complete flowering stage [78]. The HPLC method developed by our group pointed out that ragweed has a high variability in terms of its sesquiterpene lactone profile. The analyzed target metabolites reached their peak during the full-bloom period, from mid-July to mid-August, in both collection places. Our findings are quite similar to those published in other studies [73].

The essential oil of ragweed, unlike some other species (e.g. chamomile, yarrow) of the Asteraceae family, is not used in medicine, and there is little data on its composition. We used two different techniques for the extraction of *A. artemisiifolia* essential oil: hydrodistillation and microwave-assisted extraction. The first technique yielded 0.13% (v/w) which is comparable to what Han at al. reported from a Chines collection [16], but a Serbian collection near Belgrade where the geo-climatic conditions are almost the same. somehow exceeded our results with a 0.18% yield [15]. The GC and GC-MS analysis of the extracted ragweed essential oil revealed some differences in composition using the above mentioned two techniques. The microwave-assisted extraction provided significantly higher amount from the oxygenated sesquiterpenes (31.91%), and also higher quantities of germacrane D (25.22%), the main essential component, than the conventional hydrodistillation. Son and his colleagues after investigated the essential oil extracted from *A. artemisiifolia* in Korean collection, they also reported differences in quantities and in chemical components using two different extraction techniques: headspace-solid-phase microextraction and simultaneous distillation-extraction [17]. The two above mentioned extraction techniques let the detection of sesquiterpene hydrocarbons (67.58%), as the most abundant essential oil components, which is comparable to our results with germacrane D (32.92%) as the major compound. Other authors using the same hydrodistillation technique reported similar results in the sesquiterpene components, as germacrane D (24.1%) the major compound from a Serbian collection, near Belgrade [15]. The minimum inhibitory concentration on the two Gram-positive bacterial strains (methicillin-susceptible *S. aureus* (MIC=0.015) and MRSA (MIC=0.25%)) showed similarity what other reports found (MIC: 0.025-0.4%) [15].

The essential oils of the Asteraceae species may be of perspective not only for their anti-inflammatory but also for their antimicrobial effects. Some species, such as *Achillea* and *Matricaria*, have been used for various purposes: yarrow (*Achillea millefolium* L.) has been used to treat bruises, sprains, and swollen tissues, to heal wounds, and provide relief from rashes and itching [79]. Because of its anti-inflammatory, antibacterial antioxidant activities, this essential oil has a great scientific interest [81, 82]. Chamomile (*Matricaria recutita* L.) has also been used for medicinal purposes [82], and the main essential oil components (α-bisabolol, its oxides and azulenes) have antiphlogistic, antimicrobial and antioxidant activities [83].

In our efflux pump inhibitory assay, the EP inhibitory activity of the extracted essential oil was tested in *E. coli*, *K. pneumoniae* strains and drug resistant and sensitive *S. aureus* strains. In case of Gram-positive bacteria, ragweed essential oil increased EB accumulation in methicillin-sensitive and resistant *S. aureus* strains. The tested essential oil expressed stronger efflux pump inhibition on the *E. coli* strain, however there were no efflux pump inhibitory effects on the other Gram-negative bacteria, *K. pneumoniae*. We hypothesized that a possible explanation for this may be that Gram-negative bacteria have different sensibilities to efflux pump inhibitors, which can be explained by their different cell wall structure [84]. Other studies investigated the effects of some essential oil components (thymol, carvacrol, eugenol) showed similar results on *Salmonella thyphimurium* (Gram-negative bacteria). It was hypothesized that the above-mentioned phenolic compounds due to their hydrophobic nature make the bacterial membrane more permeable, also increase the sensitivity to antibiotics by blocking the efflux pump [85]. Plant essential oils may have the capability to reduce biofilm formation by affecting several systems involved in its growth [86]. In our biofilm inhibition assay, we used two important biofilm producing bacteria: *E. coli* and *S. aureus,* both are clinically relevant for causing hospital- and community-acquired infections. In the case of sensitive and resistant *S. aureus* strains the tested essential oil at sub-MIC concentrations (MIC/2 or lower) showed no antibiofilm activity. While using higher concentrations (0.5% and 1%) the plant essential oil showed inhibition on biofilm formation of *K. pneumoniae*. Ragweed essential oil contains terpenoids as main components (cyclic terpenes, terpene alcohols). These naturally occurring constituents target the bacterial cell wall and the cytoplasmic membrane [87]. In case when the cell membrane loses its integrity, which results leakage of the cell components, and lead to cell death. A different explanation might be that the damaged cell wall loses its capability to attach the surface and, in this way, the bacterial

cells are not able to form biofilm [88]. Gram-negative bacteria are known to be more resistant to essential oils than Gram-positive ones, but contrary to that, we pointed out that none of the Gram-positive bacterial strains showed sensitivity against ragweed essential oil. Moreover, plant essential oils rich in monoterpenes and phenylpropanoids had high efficacy in bacterial biofilm inhibition, especially in the phases of biofilm development, in these conditions the planktonic cells are still present, however the efficiency reduced as the sessile population increased [89]. However, our findings resulted the contrary to these data, no biofilm inhibition was observed on the tested bacterial strains, one possible explanation for that is the low concentration of the above-mentioned secondary metabolites in the extracted essential oil. Future studies need to be performed to explore the molecular mechanisms behind the biofilm inhibitory activity of the plant essential oil and to explore its particular components activity which may give us more detailed insights of these effects.

Although the investigated *Ambrosia* species is an invasive and allergenic weed, and the extensive and long-standing use of common ragweed as a medicinal herb has not been proven by the available data, the isolated sesquiterpenes and the extracted essential oil possess considerable cytotoxic and antiproliferative activity on different cancer cell lines. However, further studies are still required to examine toxicological profiles of the plant secondary metabolites and that of the essential oil and to evaluate the positive features that can lead to the medicinal use of this plant.

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Supplementary information

Table S1. The ^{1}H and ^{13}C-NMR data of 1,10-dihydro-1'-noraltamisin (**63**)

C/H	δ_H,[a] mult. (*J* in Hz)	δ_C,[b] type
1	1.59, m	53.0, CH
2	1.46, m; 1.92, m	21.8, CH_2
3	2.35, m (2H)	36.4, CH_2
4		175.9, C
5		77.1, C
6	4.55, d (9.5)	89.6, CH
7	3.49, m	41.9, CH
8	1.86, m; 2.23, m	36.3, CH_2
9	1.46, m; 1.59, m	29.6, CH_2
10	2.32, m	34.0, CH
11		141.0, C
12		172.6, C
13	5.59, d (3.5); 6.23, d (3.5)	121.7, CH_2
14	0.94, d (7.0, 3H)	19.1, CH_3
15	1.27, s (3H)	20.6, CH_3
OCH_3	3.65, s (3H)	52.0, CH_3

[a] 500 MHz, CD_3OD. [b] 125 MHz, CD_3OD.

Table S2. Chemical composition of the essential oil isolated from the aerial parts of *A. artemisiifolia*

No.	Compounds	RI	A %	B %	C %
1	*n*-hexanal	847	0.05	-	0.52
2	2-heptanone	884	-	-	0.44
3	santolinatriene	900	0.14	-	-
4	α-pinene	930	3.08	2.24	-
5	norbornane	947	2.79	-	-
6	benzaldehyde	959	-	-	0.57
7	sabibene	967	tr.	-	-
8	1-octen-3-ol	973	-	-	1.94
9	β-pinene	976	1.09	1.22	-
10	sulcatone (6-methyl-5-hepten-2-one)	979	-	-	1.38
11	myrcene	985	1.04	1.07	-
12	limonene	1027	3.12	3.82	-
13	*trans*-β-ocimene	1041	2.89	-	-
14	*cis*-linalool oxide	1065	-	-	1.97
15	*trans*-linalool oxide	1082	-	-	2.78
16	linalool	1097	0.27	-	1.61
17	α-pinene oxide	1107	-	-	2.06
18	*trans*-p-mentha-2,8-dien-1-ol	1118	-	-	1.34
19	*cis*-p-mentha-2,8-dien-1-ol	1133	-	-	1.06
20	*cis*-limonene oxide	1134	0.27	-	-
21	*trans*-pinocarveol	1137	0.14	-	5.40
22	*trans*-verbenol	1142	1.29	tr.	18.07
23	camphor	1149	tr.	tr.	-

No.	Compounds	RI	A %	B %	C %
24	lavandulol	1161	0.53	tr.	tr.
25	borneol	1172	4.84	7.17	36.67
26	terpinen-4-ol	1177	tr.	tr.	-
27	α-terpineol	1192	-	-	1.67
28	myrtenal	1194	4.35	-	-
29	verbenone	1204	-	-	4.04
30	trans-carveol	1217	0.23	-	2.84
31	carvone	1242	0.24	-	-
32	bornyl acetate	1282	4.07	8.76	-
33	thymol	1286	-	5.46	-
34	carvacrol	1294	-	2.02	-
35	δ-elemene	1327	0.35	-	-
36	eugenol	1348	0.11	-	1.91
37	cyclosativene	1363	0.14	-	-
38	α-copaene	1370	0.34	tr.	-
39	β-cubebene	1382	1.00	-	-
40	α-guaiene	1401	0.13	-	-
41	β-caryophyllene	1414	2.93	3.69	-
42	β-copaene	1430	0.14	-	-
43	α-cis-beragmotene	1425	2.82	-	-
44	trans-geranylacetone	1444	1.58	tr.	-
45	α-humulen	1448	1.38	1.73	-
46	cis-muurola-4(15),5-diene	1455	0.13	-	-
47	γ-curcumene	1470	0.78	1.06	-
48	germacrane D	1474	18.81	25.22	-
49	β-trans-bergamotene	1477	1.87	tr.	-
50	bicyclogermacrene	1490	1.77	0.55	-
51	α-muurolene	1493	1.85	-	-
52	lavandulyl-2-methyl-butyrate	1498	0.31	2.18	-
53	β-bisabolene	1503	4.01	5.76	-
54	δ-cadinene	1513	1.62	tr.	-
55	β-sesquiphellandrene	1518	0.20	-	-
56	*trans*-nerolidol	1559	0.87	-	-
57	spathulenol	1568	7.74	5.27	1.82
58	caryophyllene oxide	1574	7.30	6.73	-
59	viridiflorol	1577	0.30	-	-
60	globulol	1581	0.32	-	-
61	salvial-4(14)-en-1-one	1587	0.71	-	-
62	guaiol	1592	0.82	-	-
63	humulene epoxide	1605	2.47	1.55	-
64	rosifoliol	1608	0.53	-	-
65	junenol	1617	2.56	-	-
66	eudesmol	1619	0.97		

No.	Compounds	RI	A %	B %	C %
67	napth-1-ol	1637	0.11	-	-
68	α-muurolol	1639	0.26	-	-
69	α-cadinol	1647	0.25	-	-
70	Selin-11-en-α-ol	1655	0.97	-	-
71	intermedeol	1660	0.26	-	-
72	germacra-4(15),5,10(14)-trien-1-alpha-ol	1682	1.04	-	-
73	phytone	1833	0.71	-	-
	Totally identified (%)		91.19	94.63	90.60

RI: retention indices calculated against C8 to C32 *n*-alkanes on a ZB-5MS column. A: essential oil distilled from air-dried plant; B: essential oil from microwave-assisted extraction; C: cohobated water extracted with *n*-hexane. tr: trace element.

Table S3. The drug interactions between the compounds and doxorubicin

Compounds	Starting concentration	Ratio*	CI at ED_{50}**	SD	Type of interaction
psilostachyin C (24)	50 μM	5.8:1	3.71	0.73	strong antagonism
		11.6:1	3.34	0.43	strong antagonism
		23.2:1	2.55	0.31	antagonism
		46.4:1	1.13	0.07	slight antagonism
		92.8:1	1.47	0.28	antagonism
		185:1	1.34	0.28	moderate antagonism
acetoxydihydrodamsin (17)	15 μM	1.74:1	2.45	0.48	antagonism
		3.48:1	2.37	0.16	antagonism
		6.96:1	1.48	0.22	antagonism
		13.92.1	1.55	0.15	antagonism
		27.84:1	1.22	0.23	moderate antagonism
		55.68:1	1.34	0.29	moderate antagonism
peruvin (9)	65 μM	7.54:1	1.28	0.16	moderate antagonism
		15.08:1	1.7	0.23	antagonism
		30.16:1	2.34	0.3	antagonism
		60.32.1	1.4	0.024	moderate antagonism
		120.64:1	1.7	0.11	antagonism
		241.28:1	1.01	0.18	additive effect
psilostachyin (25)	25 μM	2.9:1	0.95	0.06	additive effect
		5.8:1	1.44	0.32	moderate antagonism
		11.6:1	0.67	0.14	synergism
		23.2:1	2.26	0.42	antagonism
		46.4:1	1.39	0.07	moderate antagonism
		92.2:1	1.16	0.21	slight antagonism
1´-noraltamisin (61)	30 μM	3.48:1	2.62	0.62	antagonism
		6.69:1	1.02	0.072	additive effect
		13.92:1	0.88	0.043	slight synergism
		27.84:1	1.09	0.039	additive effect
		55.68:1	1.33	0.036	moderate antagonism
		111.36:1	0.94	0.28	additive effect
psilostachyin B (27)	100 μM	11.6:1	0.4	0.14	Syn.
		23.2:1	0.34	0.06	Syn.
		46.4:1	2.6	0.36	Antagonism
		92.8:1	1.13	0.09	Slight antagonism
		185.6:1	1.4	0.2	Moderate ant.
		371.2.1	1.5	0.16	Antagonism
1,10-dihydro-1´-noraltamisin (63)	100 μM	11.6:1	0.4	0.14	syn.
		23.2:1	0.34	0.06	syn.
		46.4:1	2.6	0.36	antagonism
		92.8:1	1.13	0.09	slight antagonism
		185.6:1	1.4	0.2	moderate ant.
		371.2:1	1.5	0.16	antagonism
axillarin (62)	150 μM	17.42:1	0.1	0.03	very strong syn.
		34.84:1	0.79	0.02	moderate syn.
		69.68:1	0.74	0.03	moderate syn.
		139.36:1	0.74	0.05	moderate syn.
		278.72:1	0.75	0.7	moderate syn.
		557.44:1	0.51	0.05	syn.

*Ratio: the best combination ratio between compounds and doxorubicin

**CI at ED_{50}: combination index value at the 50% growth inhibition dose

Combination index (CI): 0–0.1: very strong synergism, 0.1–0.3: strong synergism, 0.3–0.7: synergism, 0.7–0.85: moderate synergism, 0.85–0.9: slight synergism, 0.9–1.1: additive effect, 1.1–1.2: slight antagonism, 1.2–1.45: moderate antagonism, 1.45–3.3: antagonism, 3.3–10: strong antagonism, >10: very strong antagonism