University of Szeged
Faculty of Pharmacy
Pharmacognosy Doctoral Program
Program director: Prof. Dr. Judit HOHMANN
Institute of Pharmacognosy
Supervisors: Prof. Dr. Dezső CSUPOR and Dr. Tivadar KISS

Balázs KOVÁCS

Chemical and pharmacological analysis of *Ambrosia artemisiifolia*

Summary of Ph.D. thesis

Complex exam committee:
Head: Prof. Dr. Judit HOHMANN
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Reviewers committee:
Head: Prof. Dr. István ILISZ
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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. **Kovács B**, Szemerédi N, Kúsz N, Kiss T, Csupor-Löffler B, Tsai Y. C, Rácz B, Spengler G & Csupor D.
Antiproliferative and cytotoxic effects of sesquiterpene lactones isolated from *Ambrosia artemisiifolia* on human adenocarcinoma and normal cell lines
Pharmaceutical Biology **2022**, 60(1): 1519
Scopus – Complementary and Alternative Medicine; SJR: Q1 IF: 3.8

II. **Kovács B**, Szemerédi N, Csikós O, Kiss T, Veres K, Spengler G, Csupor-Löffler B & Csupor D.
Chemical composition, antimicrobial and antiproliferative activity of the essential oil from *Ambrosia artemisiifolia* L.
Journal of Essential Oil Research **2024**, 34 (1):42
Scopus - Chemistry; SJR: Q2 IF:2.2 (2023)

III. **Kovács B**, Püski P, Bajtel Á, Ferencz E, Csupor-Löffler B, Csupor D & Kiss T.
Targeted screening and quantification of characteristic sesquiterpene lactones in *Ambrosia artemisiifolia* L. at different growth stages
Plants **2024**, 13 (15): 2053
Scopus – Plant Sciences; SJR: Q1 IF: 4.0 (2023)

The cumulative impact factor of the publications related to the thesis: 10.0

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. MATERIALS AND METHODS

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

The sesquiterpene lactones from aerial parts of *Ambrosia artemisiifolia* were isolated using multistep chromatographic methods, including vacuum liquid chromatography, preparative rotation planar chromatography, preparative HPLC. The extract, fractions and pure compounds were qualitatively analyzed using thin layer chromatography and analytical HPLC. The isolated compounds were identified by means of HPLC and NMR spectroscopy. The isolation process with chromatographical conditions, together with identification and structure elucidation of the compounds are described in paper [I] related to this thesis (i.e. Kovács B et al. *Pharmaceutical Biology* 2022, **60**(1): 1519).

3.2 Extraction of essential oil

The essential oil of ragweed two methods were applied: the conventional hydrodistillation and microwave-assisted essential oil extraction. The components of obtained essential oil were identified using GC-FID and GC-MS analyzes. The methods of essential oil extractions t are published in paper [II] related to this thesis (i.e. Kovács B. et al. *Journal of Essential Oil Research* 2024, **34**(1):42). The yield and content of the essential oils were tested for antibacterial and antiproliferative effects.

3.3 Antibacterial and antiproliferative activity of isolated sesquiterpene lactones and essential oil

The isolated sesquiterpene lactones, and essential oil obtained by hydrostillation and microwave-assisted extraction have been subjected to biological activity investigation. The experimental conditions of antibacterial and antiproliferative activity testing are described in details in papers [I] and [II] related to this thesis.

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

Targeted screening of sesquiterpene lactone in sample sets from two geographical locations were analyzed using HPLC/DAD method. The obtained results were evaluated using various statistical methods. The sample preparation, HPLC/DAD conditions, and statistical analysis methods are described in paper [III] related to this thesis (i.e. Kovács B. et al. *Plants-Basel* 2024, **13**(15): 2053).

4. Results

4.1 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. Based on comparison of NMR and HR-MS spectra with literature data, the following isolated compounds were identified: compound **1** was identified as psilostachyin C (**24**), compound **2** as acetoxydihydrodamsin (**17**), compound **3** as peruvin (**9**), compound **4** as psilostachyin (**25**), compound **5** as 1´-noraltamisin (**61**), compound **6** as psilostachyin B (**27**), and compound **8** as axillarin (**62**).

Compound **7** was identified as a rare *seco*-psilostachyinolide based on NMR and MS experiments, 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.

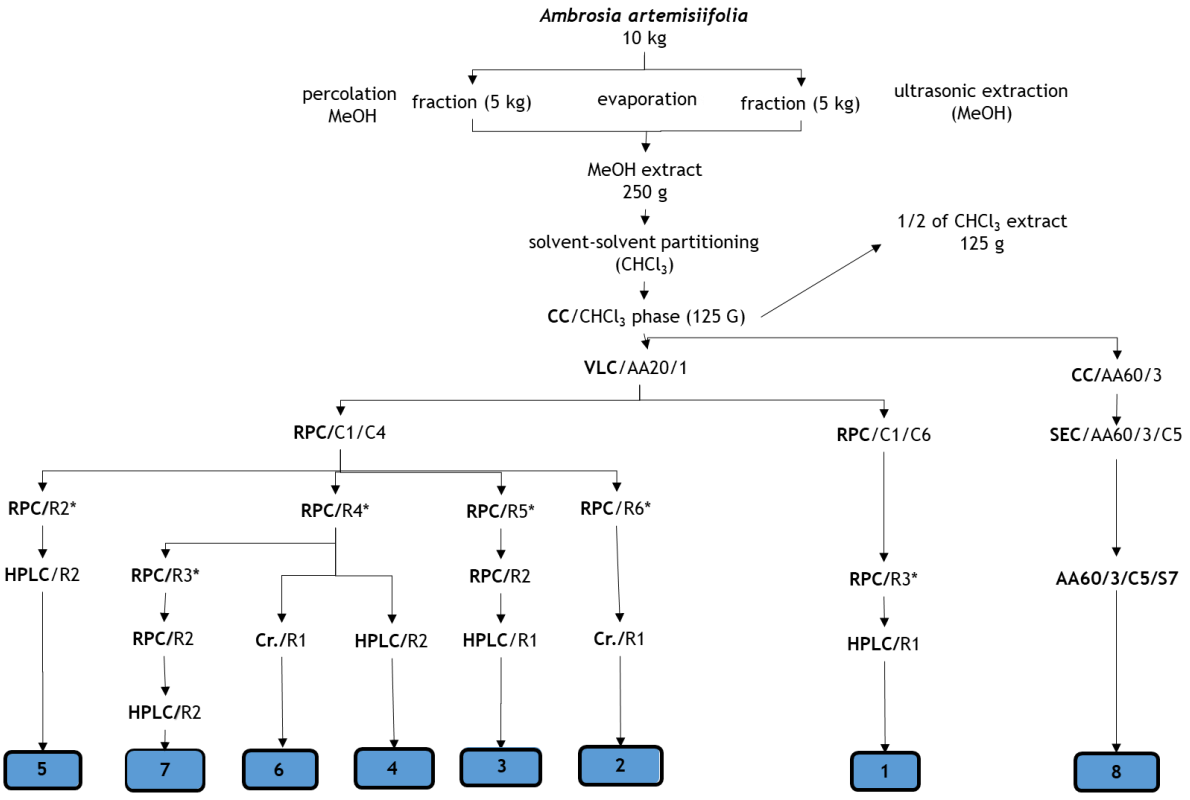

Figure 1. Isolation of sesquiterpene lactones from *Ambrosia artemisiifolia*

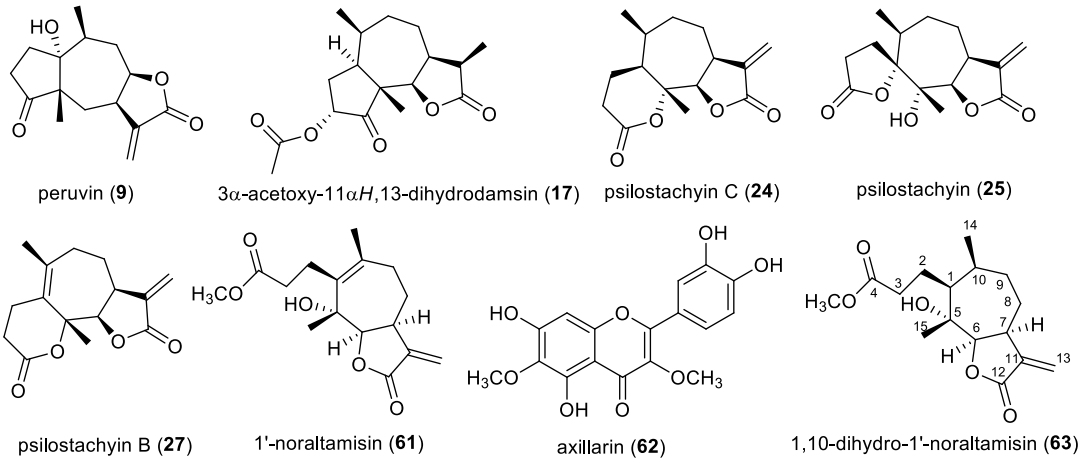

Figure 2. The structure of the isolated compounds from *Ambrosia artemisiifolia*

4.2 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 essential oil components of *A. artemisiifolia* has been examined using GC/FID and GC-MS**.** 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, 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. Components of the cohobated water after hydrodistillation following liquid-liquid separation with *n*-hexane were analyzed. 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.

4.3 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 3**).

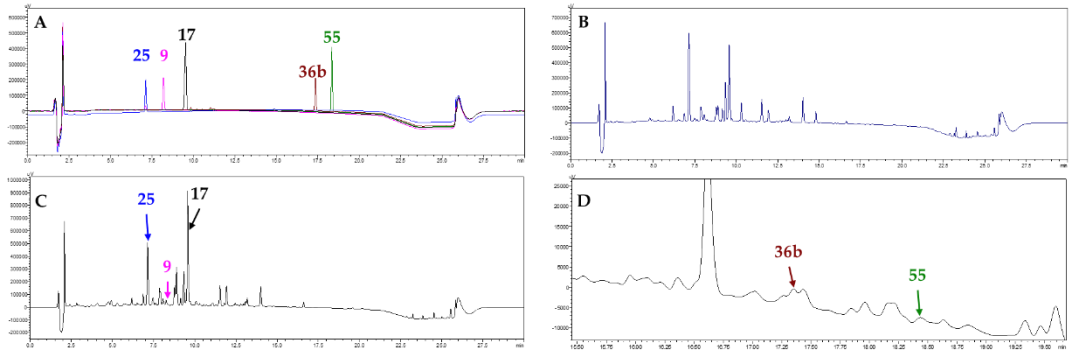

Figure 3. HPLC chromatograms of the reference compounds (**A**) and the crude extract (**B**, **C,** and **D**) at 210 nm. (**25**: psilostachyin, **9**: peruvin, **17**: acetoxydihadryodamsin, **36b**: costunolide, **55**: isoalantolactone).

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 1**).

Table 1. 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
acetoxydihydro-damsin (**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

4.4 Quantification of sesquiterpene lactones

The levels of the marker compounds in aerial parts of ragweed samples collected near Szeged and Nyíri are presented in **Figures 4–5**. 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.

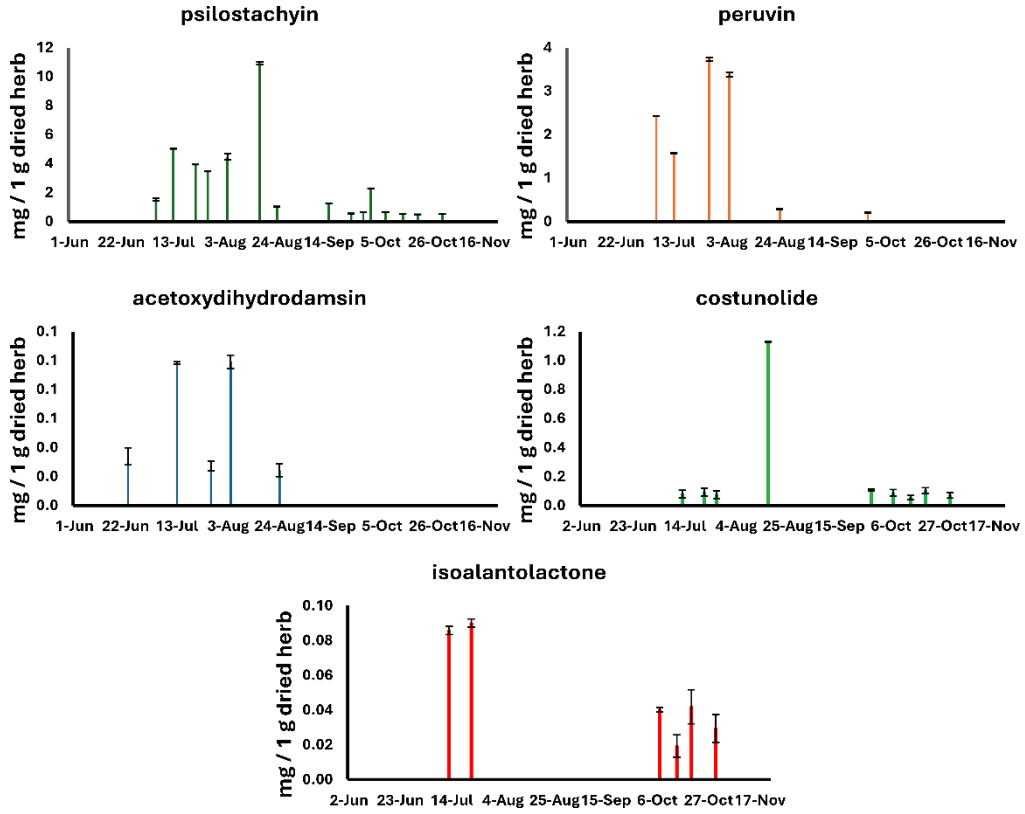

Figure 4. Concentration levels of sesquiterpene lactones in the aerial parts of *A. artemisiifolia* collected near Szeged from June to October.

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, peruvin, and isoalantolacton).

Psilostachyin (**25**), a main component, 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 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 4-5**).

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 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 5**). The concentration of peruvin (**9**) 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 4**).

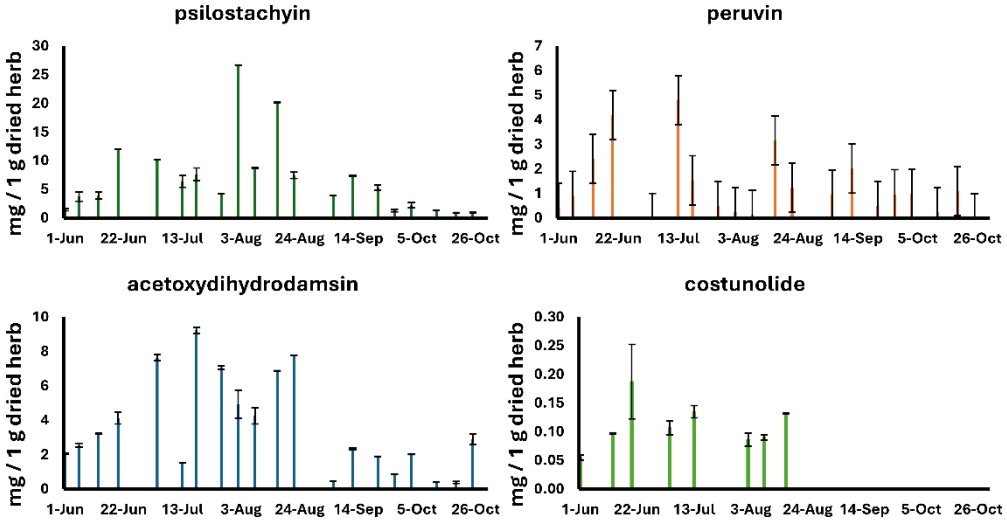

Figure 5. Concentration levels of sesquiterpene lactones in the aerial parts of *A. artemisiifolia* collected near Nyíri from June to October.

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**).

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). Pearson correlation was used to examine the relationship between sesquiterpene lactone concentration levels and climate parameters. 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.

4.5 Pharmacological activities of *Ambrosia* sesquiterpene lactones

4.5.1 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.

4.5.2 Antiproliferative and cytotoxic effects of sesquiterpene lactones

Among the isolated compounds, acetoxydihydrodamsin (**17**) showed cytotoxic activity (IC_{50} = 7.64 ± 0.37 mM) on the sensitive (Colo 205) cell line after 24 hours of treatment. 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 2**). The quercetagetin 3,6-dimethyl ether derivative (axillarin (**62**)) had no cytotoxic effects on the examined cell lines after a short exposure (**Table 2**).

Table 2. 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 3**). 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. 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 3. 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.

4.5.3 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.

4.5.4 Drug interactions between sesquiterpene lactones 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.

4.5.5 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.

4.6. Pharmacological activity of extracted essential oil

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

Table 4 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 4. 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.

4.6.2 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 5**). 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 6**).

Table 5. 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 6. 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.

4.6.3 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 6**) compared to the positive controls, while the same dosage inhibited *K. pneumoniae* ATCC 600703 strain biofilm formation in a dose-dependent manner (**Figure 6**). The crystal violet experiment revealed that MIC/2 levels did not reduce biofilm formation in *S. aureus* ATCC 25923 (**Figure 7**) and the methicillin-resistant *S. aureus* ATCC 43300 (**Figure 8**).

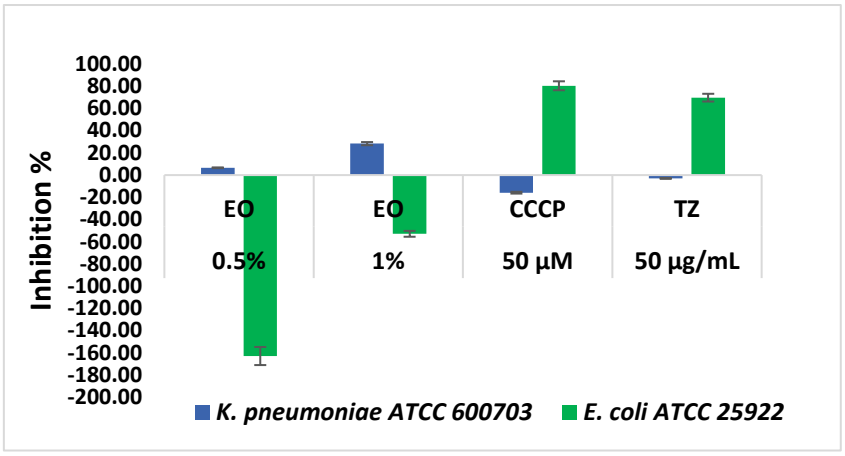

Figure 6. Reduction of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 600703 biofilm formation in the presence of *A. artemisiifolia* EO (EO: essential oil; CCCP: cyanide m-chlorophenylhydrazone; TZ: thioridazine). Bars represent the mean ± SD of four parallel experiments.

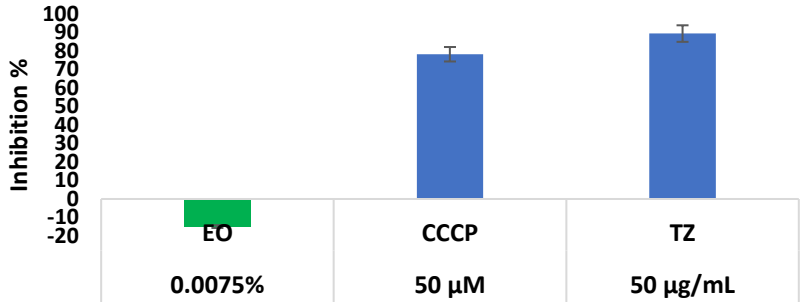

Figure 7. Reduction of *S. aureus* ATCC 25923 biofilm formation in the presence of *A. artemisiifolia* EO (EO: essential oil; CCCP: cyanide *m*-chlorophenylhydrazone; TZ: thioridazine). Bars represent the mean ± SD of four parallel experiments.

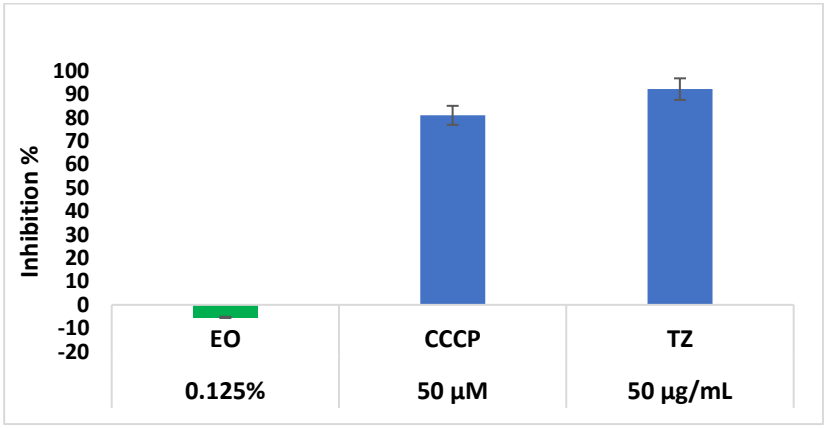

Figure 8. Reduction of *S. aureus* ATCC 43300 biofilm formation in the presence of *A. artemisiifolia* EO (EO: essential oil; CCCP: cyanide m-chlorophenylhydrazone; TZ: thioridazine). Bars represent the mean ± SD of four parallel experiments.

4.5.4 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 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 7**).

Table 7. 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 MTT assay results . **Table 8** demonstrate that, with a starting concentration of 1% 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 8**).

Table 8. 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.

5. 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 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, 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 the *seco*-psilostachyinolides.

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**) possessed significant antiproliferative activity of the multidrug-resistant Colo 320 cell line, and showed moderate selectivity against human embryonal lung fibroblast cell line. The cytotoxicity of sesquiterpene lactones is associated with the existence of an exocyclic double bond adjacent to the γ-lactone ring 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. 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, altering the NF-κB signaling pathway, cell cycle checkpoint inhibition, provoking apoptosis and cell cycle arrest [70]. 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. 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. 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. 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, 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. Geoclimatic factors may impact the secondary metabolites of ragweed, leading to variations in sesquiterpene lactone profiles across samples taken from different areas. It is 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 (e. g. Novara, Italy). The sesquiterpene content of the plant may differ regarding the vegetation period and the collection place etc., yet some studies described the isolation of characteristic *Ambrosia* sesquiterpenes that are widely distributed in species of different origins. 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 result to early senescence or cell death. Water stress is one of the major environmental issue, which can alter plant development and production. It has been reported that 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 etc.) was negatively modulated by the water deficit stress, and they also observed that the glandular trichome density and size was decreased. 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. The analyzed target metabolites reached their peak during the full-bloom period, from mid-July to mid-August, in both collection places.

The essential oil of ragweed, unlike some other species (e.g. chamomile, yarrow), is not used in medicine, and there is little data on its composition. We used two different techniques for the extraction of ragweed essential oil: hydrodistillation and microwave-assisted extraction. The first technique yielded 0.13% (v/w) which is comparable to a Chines collection, but a Serbian collection near Belgrade where the geo-climatic conditions are almost the same, somehow exceeded our results with a 0.18% yield. 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. The essential oil extracted from *A. artemisiifolia* in Korean collection, also showed differences in quantities and in chemical components using two different extraction techniques: headspace-solid-phase microextraction and simultaneous distillation-extraction. The two above mentioned extraction techniques let the detection of sesquiterpene hydrocarbons (67.58%), 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.

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 etc. Because of its anti-inflammatory, antibacterial antioxidant activities, this essential oil has a great scientific interest. Chamomile (*Matricaria recutita* L.) has also been used for medicinal purposes, and the main essential oil components (α-bisabolol, and azulenes) have antimicrobial and antioxidant activities.

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. 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 *K. pneumoniae*. The 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. Other studies investigated the effects of some

essential oil components (thymol, carvacrol, eugenol) showed similar results on *Salmonella thyphimurium* (Gram-negative bacteria). Plant essential oils may have the capability to reduce biofilm formation by affecting several systems involved in its growth. In our biofilm inhibition assay, we used two important biofilm producing bacteria: *E. coli* and *S. aureus*. 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. In case when the cell membrane loses its integrity, which results leakage of the cell components, and lead to cell death. 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, and in these conditions the planktonic cells are still present, however the efficiency reduced as the sessile population increased. 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 cyclic terpenes 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.

Acknowledgement

I would like to express my greatest attitude to my supervisors, Dezső Csupor and Tivadar Kiss, for leading my PhD work with inestimable professional knowledge, and who have supported and tirelessly helped me throughout this journey. I will always be grateful to them.

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enjoyable and memorable.

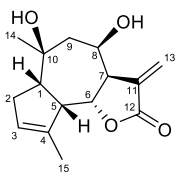
I am very thankful to Bajtelné Hmelyvoszki Zsuzsanna, for collecting and providing *Ambrosia artemisiifolia* samples; to Róbert Berkecz, for helping me with the LC-MS measurements; to Norbert Kúsz and to Yu-Chi Tsai for the NMR measurements and evaluations; to Orsolya Csikós (Pepszint Ltd, Szeged) for the microwave-assisted extraction; to Katalin Veres for the GC and GC-MS analysis and Nikoletta Szemerédi and Gabriella Spengler (Department of Medical Microbiology, Albert Szent-Györgyi Health center and Albert Szent-Györgyi Medical School, University of Szeged) for the cytotoxic and antiproliferative investigations.

I am very grateful to my previous supervisor, László Fodorpataki, for inspiring me to learn science and who helped me to start research work during my BSc studies.

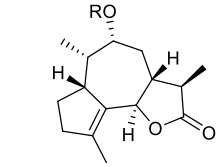
Finally, I would like to extend my special thanks to my parents, to my sister, relatives and friends. I could not have carried out this work without their support and love.

Structures

Sesquiterpene lactones isolated from *Ambrosia artemisiifolia*

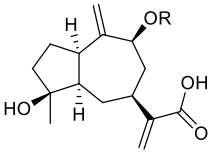


cumambrin B (**1**)



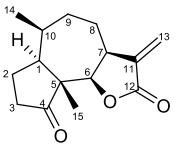
1β,7β,9β,10β,13αH-guaia-4(5)-en-12,6β-olide-9-*O*-β-D-glucoside (**2**)

R = Glc

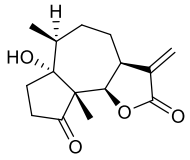


4β-hydroxy-1α,5α,7α,9αH-guaia-10(14),11(13)-dien-12-acid 9-*O*-β-D-glucoside (**3**)

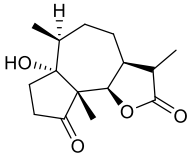
R = Glc



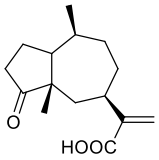
damsin (**4**)



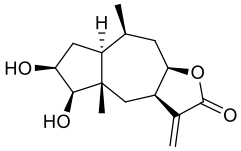
coronopilin (**5**)



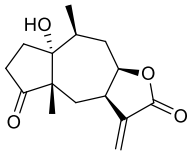
dihydrocoronopilin (**6**)



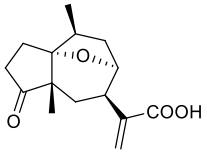
damsinic acid (**7**)



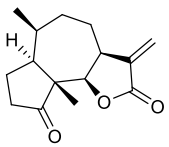
cumanin (**8**)



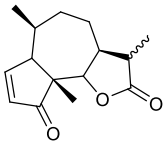
peruvin (**9**)



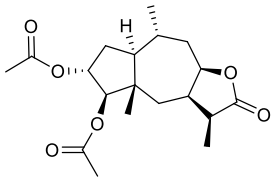
ambrosic acid (**10**)



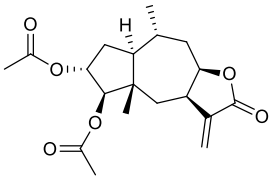
amborsin(**11**)



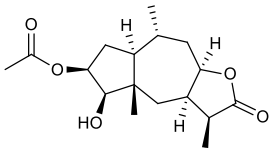
artesovin (**12**)



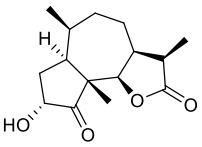
3-deacetoxylhymenalone (**13**)



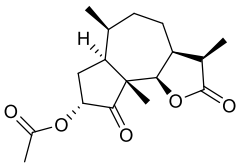
3α,4β-diacetylhymenoratin (**14**)



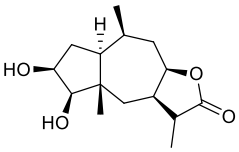
3β-acetoxy-4β-hydroxy-1α,7α,10β,11α*H*-pseudoguaia-12,8β-olide (**15**)



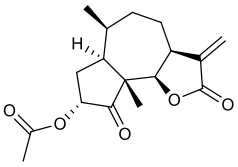
3α-hydroxy-11α*H*,13-dihydrodamsin (**16**)



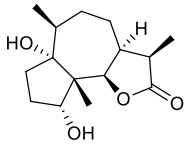
acetoxydihydrodamsin (**17**)



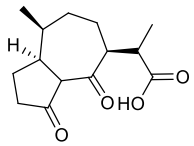
dihydrocumanin (**18**)



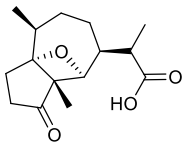
3-acetoxydamsin (**19**)



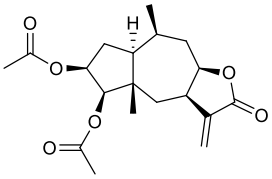
tetrahydrocoronopilin (**20**)



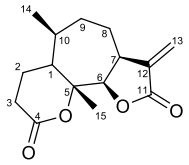
6-oxadamsinic acid (**21**)



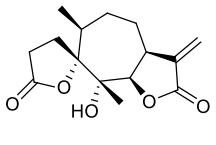
ambroxatane (**22**)



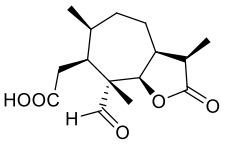
cumanin diacetate (**23**)



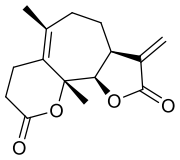
psilostachyin C (**24**)



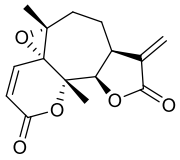
psilostachyin (**25**)



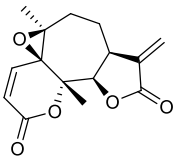
4-oxo-3,4-*seco*-ambrosan-6,12-olide-3-oic acid (**26**)



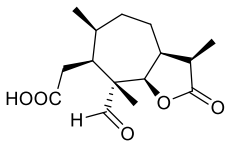
psilostachyin B (**27**)



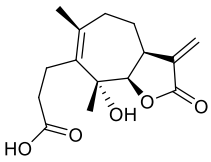
paulitin (**28**)



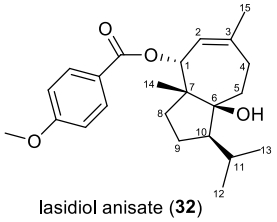
isopaulitin (**29**)



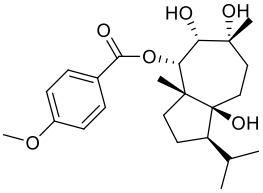
4-oxo-3,4-*seco*-ambrosan-6,12-olide-3-oic acid (**30**)



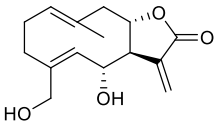
altamisic acid (**31**)



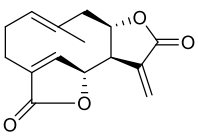
lasidiol anisate (**32**)



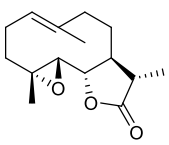
2,3-dihydro-2,3-dihydroxylasidiol 1-anisate (**33**)



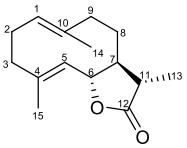
artemisiifolin (**34**)



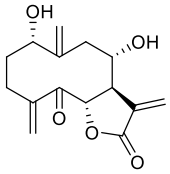
isabelin (**35**)



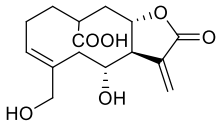
dihydroparthenolide (**36**)



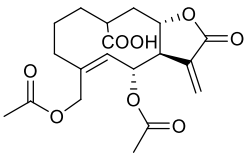
costunolide (**36b**)



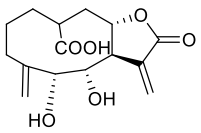
5-oxo-8α-hydroxy-1-epiartemorin (**37**)



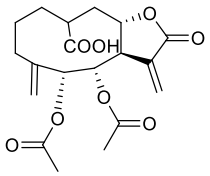
artemisiifolinic acid (**38**)



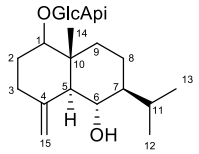
diacetyl-artemisiifolinic acid (**39**)



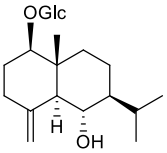
isoartemisiifolinic acid (**40**)



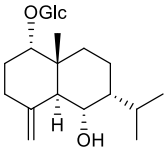
diacetyl-isoartemisiifolinic acid (**41**)



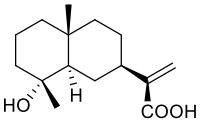
1β,6α-dihydroxyeudesman-4(15)-ene-1-*O*-β-D-apiofuranosyl(1"-6')-*O*-β-D-glucopyranoside (**42**)



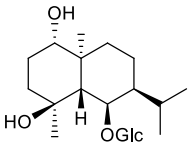
1β,6α-dihydroxyeudesman-4(15)-ene-1-*O*-β-D-glucopyranoside (**43**)



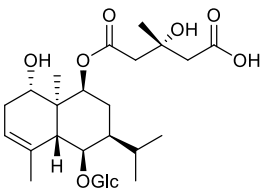
7-epi-eudesm-4(15)-ene-1α,6α-diol 1-*O*-β-D-glucopyranoside (**44**)



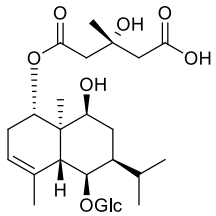
ilicic acid (**45**)



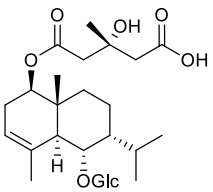
pumilaside A (**46**)



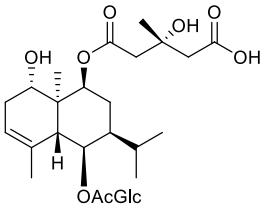
1α,6β,9β-trihydroxy-5,10-bis-epi-eudesm-3-ene-9-*O*-[(*S*)-3"-hydroxy-3"-methylglutaryl]-6-*O*-β-D-glucopyranoside (**47**)



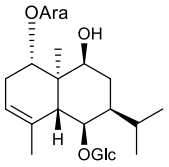
1α,6β,9β-trihydroxy-5,10-bis-epi-eudesm-3-ene-1-*O*-[(*S*)-3"-hydroxy-3"-methylglutaryl]-6-*O*-β-D-glucopyranoside (**48**)



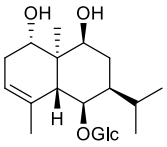
1β,6α-dihydroxy-7-epi-eudesm-3-ene-1-*O*-[(*S*)-3"-hdroxy-3"-methylglutaryl]-6-*O*-β-D-glucopyranoside (**49**)



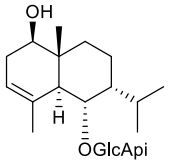
1α,6β,9β-trihydroxy-5,10-bis-epi-eudesm-3-ene-9-*O*-[(*S*)-3"-hydroxy-3"-methylglutaryl]-6-*O*-(6'-*O*-acetyl)-β-D-glucopyranoside (**50**)



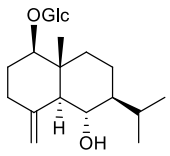
1α,6β,9β-trihydroxy-5,10-bis-epi-eudesm-3-ene-1-*O*-α-L-arabinopyranosyl-6-*O*-β-D-glucopyranoside (**51**)



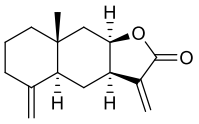
1α,6β,9β-trihydroxy-5,10-bis-epi-eudesm-3-ene-6-*O*-β-D-glucopyranoside (**52**)



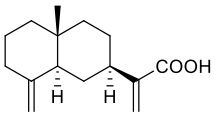
1β,6α-dihydroxy-7-epi-eudesm-3-ene-6-*O*-β-D-apiofuranosyl-(1⟶6)-β-D-glucopyranoside (**53**)



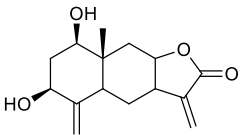
1β,6α-dihydroxyeudesman-4(15)-ene-1-*O*-β-D-glucopyranoside (**54**)



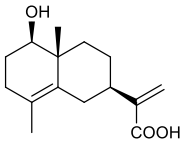
isoalantolactone (**55**)



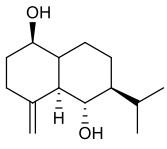
costic acid (**56**)



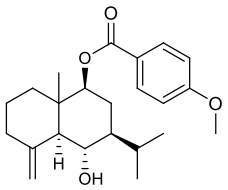
granilin (**57**)



1β-hydroxyeudesma-4,11(13)-dien-12-oic acid (**58**)



1β,6α-dihydroxyeudesm-4(15)-ene (**59**)



6α-hydroxyeudesm-4(15)-ene-9β-*O*-anisate (**60**)