



Chemical variability of *Rhododendron tomentosum* (*Ledum palustre*) essential oils and their pro-apoptotic effect on lymphocytes and rheumatoid arthritis synoviocytes

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ABSTRACT

Rhododendron tomentosum (*Ledum palustre*) is an aromatic plant traditionally used for alleviating rheumatic complaints which makes it a potential candidate for a natural drug in rheumatoid arthritis (RA) treatment. However, the effects of plants' volatiles on apoptosis of synovial fibroblasts and infiltrating leucocytes of RA synovia, have not been reported. Volatile fraction of *R. tomentosum* is chemically variable and chemotypes of the plants need to be defined if the oil is to be used for therapeutic purposes. In the presented work, cluster analysis of literature data enabled to define 10 chemotypes of the plant. The volatile fractions of known composition were then tested for bioactivity using a RA-specific *in vitro* models. Essential oils of two wild types (γ -terpineol and palustrol/ledol type) and one *in vitro* chemotype (ledene oxide type) were obtained by hydrodistillation and their bioactivity was tested in two *in vitro* models: I - peripheral blood lymphocytes of healthy volunteers and II - synoviocytes and immune cells isolated from synovia of RA patients. The influence of oils on blood lymphocytes' proliferation and apoptosis rates of synovia-derived cells was determined by flow cytometry. Dose-dependent inhibitory effect of the serial dilutions of *R. tomentosum* oils on proliferation rates of blood lymphocytes was found. At 1:400 dilutions, all the tested oils increased the number of necrotic cells in synovial fibroblasts from RA synovia. Additionally, increased proportions of late apoptotic cells were observed in leucocyte populations subjected to oils at 1:400 dilution.

1. Introduction

Rhododendron tomentosum Harmaja (previously known as *Ledum palustre* L.) is an aromatic bog plant native to central and northern Europe and northern parts of Asia and North America [1,2]. The plant is known for its insect-repellent and therapeutic properties. It has been employed in herbal medicine for a variety of ailments including dyspepsia, cold, bronchitis, tuberculosis and rheumatic diseases [1,2]. From the ethnopharmacological point of view, the anti-rheumatic

activity of *R. tomentosum* is of particular interest since it makes the discussed plant a candidate for a natural drug in the treatment of rheumatoid arthritis (RA). Given the high prevalence and debilitating nature of RA [3–5], as well as lack of fully effective therapies for the disease [3,6,7], there is an urgent need for developing new drugs, characterized with high efficacy and less toxicity. Among these are herbal remedies, characterized with long-standing folk use and low price. Thanks to the complex chemistry, their mode of action is often multi-directional, affecting different disease pathways. The results of

Abbreviations: AHC, agglomerative hierarchical clustering; CFSE, carboxyfluorescein succinimidyl ester; EO, essential oil; FLS, fibroblast-like synoviocytes; GC/MS, gas chromatography/mass spectrometry; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RA, rheumatoid arthritis; SD, standard deviation; SE, standard error; TNF- α , tumor necrosis factor- α ; UPGMA, unweighted pair-group method with arithmetic averages

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Table 1
The percentage content of the predominant compounds in the essential oils of *R. tomentosum* plants grown in different geographical habitats, determined by GC/MS analysis.

Code ^a	Country	Natural habitat	Chemotype ^b	Content in the <i>R. tomentosum</i> essential oils [%] ^c										Source
				Sabinene	β -myrcene	<i>p</i> -cymene	Limonene	α -thujenal	γ -terpineol	Bornyl acetate	Ascaridole	Palustrol	Ledol	
P1	Poland	Miszewko	I	1.5	3.7	4.0	1.4	0.2	8.5	2.0	0.3	6.9	8.1	[17,20]
P2		Lubichowo	I	1.3	0.4	5.3	1.7	0.3	11.8	4.5	0.6	0.8	2.3	[17,20]
F1	Finland	Turku	II	-	5.1	-	-	-	-	-	-	38.9	22.4	[17], dnp ^d
F2		Lohja	II	tr	0.9	tr	tr	-	-	-	-	48.2	32.6	[22]
F3		Vihhti	VII	0.4	-	14.2	0.2	-	-	-	67.3	tr	tr	[22]
S1	Sweden	Uppsala	III	-	21.3	1.0	-	-	-	-	-	22.8	6.1	[23]
L1	Lithuania	Juodupe	II	tr	5.7	0.3	-	-	-	tr	0.1 ^e	42.8	23.9	[24]
L2		Silenai	II	tr	1.2	0.1	3.7	-	-	0.3	0.2 ^e	36.7	30.5	[24]
L3		Samanis	II	0.3	7.5	2.2	-	-	0.6	3.3	5.3 ^e	26.2	21.0	[25]
L4		Rudninkai	II	-	1.9	0.4	-	-	-	0.4	4.0	21.0	36.5	[26]
L5		Sulnys Lake	II	0.3	1.0	6.2	tr	-	-	0.4	16.7 ^e	31.8	23.4	[27]
L6		Sulnys Lake	VI	0.3	0.1	20.3	-	-	-	4.6	20.5 ^e	1.2	5.2	[27]
L7		Sulnys Lake	VII	0.3	0.2	15.3	0.9	0.4	31.2	2.6	24.1 ^e	11.9	11.1	[27]
E1	Estonia	Pudisoo	I	tr	1.3	12.5	0.3	0.3	0.4	0.1	2.7 ^e	15.9	11.8	[28,29]
E2		Jouga	I	tr	1.0	13.9	0.3	1.0	24.7	0.7	2.7 ^e	16.7	12.8	[29]
E3		Ahja	II	-	2.8	0.1	-	0.1	-	-	-	53.5	18.3	[29]
E4		Vana-Vigala	II	-	0.8	1.9	0.1	0.3	2.4	0.3	0.3 ^e	41.0	14.6	[29]
R1	Russia	Shenkursky	II	-	0.7	-	1.2	-	-	-	-	41.1	27.5	[30]
R2		Prokhorokina	II	0.2	14.0	2.1	15.9	-	-	0.2	0.5	31.6	18.1	[31]
R3		Tymsk	IV	0.7	15.3	15.5	10.6	-	-	1.0	0.3	18.7	7.9	[31]
R4		Kiyevskiy	III	-	55.7	0.2	1.3	-	-	-	-	19.6	7.6	[31]
R5		Molodezhny	III	-	46.5	0.3	8.3	-	-	-	0.1	21.1	9.6	[31]
R6		Napas	III	-	35.3	4.7	3.8	-	-	tr	0.2	29.9	10.9	[31]
R7		Tynda	V	33.2	3.2	17.1	0.9	-	-	0.9	1.0	1.8	1.4	[31,32]
R8		Baikal Lake	V	21.9	0.2	12.9	1.6	-	-	1.0	3.0	0.7	0.4	[31,32]
R9		Skovorodino	VII	4.8	0.5	34.3	3.2	-	-	2.9	29.7	tr	tr	[31]
R10		Orotukan	VII	0.4	-	17.0	1.4	-	-	4.2	49.2	0.9	0.3	[31]
R11		Utes	VI	0.5	tr	22.0	5.1	-	-	2.0	8.7	2.3	2.0	[31,32]
R12		Yuzhno-Sakhalinsk	VI	1.0	0.2	51.7	2.5	-	-	1.9	9.8	-	-	[31]
R13		Mirny	VI	1.1	0.2	43.2	2.7	-	-	3.6	21.2	0.2	-	[31]
R14		Svetly	VI	1.1	0.6	44.0	3.6	-	-	1.2	3.4	0.8	1.3	[31]
R15		Kamchatka	VI	0.2	1.2	14.7	7.0	-	-	0.5	-	0.4	0.1	[31]
R16		Niertungri	VI	3.0	0.9	23.6	6.2	-	-	8.8	13.2	2.2	0.3	[31]
R17		Orotukan	VIII	1.6	-	9.1	0.6	-	-	10.8	2.0	0.1	0.9	[31]
R18		Peschaoe Lake	IX	0.3	0.9	3.8	50.3	-	-	0.6	1.4	7.2	3.9	[31,32]
R19		Suiga	IX	0.2	0.2	2.7	22.2	-	-	0.8	2.8	2.0	3.0	[31,32]
R20		Kurlek	IX	0.1	1.0	9.4	27.9	-	-	0.5	2.8	3.7	2.2	[31]
R21		Ust-Galka	IX	0.4	0.7	19.2	48.0	-	-	1.3	1.5	1.8	2.6	[31]
R22		Kolpashovo	IX	0.4	0.8	8.1	21.6	-	-	1.0	1.2	1.3	1.0	[31]
R23		Novy Tevriz	IX	-	0.2	5.0	7.9	-	-	1.0	4.5	0.9	4.5	[31]
R24		Sredny Vasyugan	IX	tr	0.5	8.4	49.9	-	-	1.4	1.8	0.5	2.2	[31]
R25		Myldzhino	IX	0.5	0.8	16.7	38.4	-	-	1.2	1.6	1.2	1.9	[31]
R26		Nyurorka River	IX	0.1	2.5	9.0	35.0	-	-	0.5	2.3	5.8	3.5	[31]
R27		Ust-Chizhapka	IX	0.2	0.5	15.4	26.3	-	-	1.4	3.1	1	1.4	[31]
R28		Staroyugino	IX	0.3	0.5	13.0	33.6	-	-	1.3	2.5	3.4	1.5	[31]
R29		Bol'shaya Griva	IX	0.5	0.6	23.1	24.6	-	-	1.8	2.6	1.1	1.2	[31]
R30		Kindal	IX	0.6	0.6	11.9	31.5	-	-	1.2	3.3	6.8	4.5	[31]
R31		Vertikos	IX	0.8	2.6	7.3	28.3	-	-	0.8	1.2	21.0	10.2	[31]
R32		Toiparovo	IX	0.1	11.3	3.5	35.9	-	-	0.4	0.4	14.1	7.4	[31]

(continued on next page)

Table 1 (continued)

Code ^a	Country	Natural habitat	Chemotype ^b	Content in the <i>R. tomentosum</i> essential oils [%] ^c										Source	
				Sabinene	β -myrcene	<i>p</i> -cymene	Limonene	α -thujenol	γ -terpineol	Bornyl acetate	Ascaridole	Palustrol	Ledol		
C1	China	Da Hinggan Mountains	V	27.5	0.4	3.1 ^f	0.1	23.5	-	0.2	-	-	-	-	[33]
C2		Inner Mongolia	VII	6.6	0.1	17.6	0.8	-	-	1.0	-	26.8	-	-	[34]
C3		Changbain Mountains	VIII	1.6	-	16.5 ^g	0.6	-	-	-	-	-	-	1.7	[35]
C4		Inner Mongolia	X	3.2	-	7.1 ^g	-	29.5	-	-	-	-	-	tr	[36]
K1	North Korea	Beckdoo Mountain	V	17.8	0.3	1.2	0.7	-	-	-	-	-	-	-	[37]

^a *R. tomentosum* sampling sites are shown in the map (Fig. 1) and corresponding EOs are indicated in the dendrogram (Fig. 3)

^b Chemotype I - γ -terpineol; II - ledol / palustrol; III - β -myrcene / *p*-cymene / palustrol; IV - β -myrcene / *p*-cymene / palustrol; V - sabinene; VI - *p*-cymene; VII - *p*-cymene / ascaridole; VIII - *p*-cymene / bornyl acetate; IX - limonene; X - α -thujenol.

^c All compounds with a content \geq 20% of the total *R. tomentosum* essential oil composition in at least one examined sample were considered as major compounds. Compounds reflecting the respective chemotypes are written in bold.

^d dnp – own research, data not published.

^e Iso-ascaridol?

^f *O*-cymene?

^g According to authors: benzene. 1,2,4,5-tetramethyl-tr – traces.

the so far conducted studies are encouraging and the anti-arthritic activity of many phytochemicals has been documented [7,8].

So far, only a few studies concerning anti-inflammatory and analgesic properties of *R. tomentosum* have been conducted. These experiments yielded promising results and highlighted the role of flavonoids and essential oil (EO) components in the inhibition of prostaglandin and TNF- α biosynthesis [9,10]. In other studies, both acetic acid-induced writhing and λ -carrageenan-induced paw edema tests in mice and rats confirmed the anti-inflammatory potential of *R. tomentosum* [11,12]. However, the pro-apoptotic activity of this plant toward human lymphocytes or synoviocytes, i.e. the cells which play crucial role in the pathogenesis of RA [3,13–16], has not yet been investigated, thus providing a rationale for the present work.

Another issue to be addressed while searching for the *R. tomentosum*-based anti-RA medication is variable chemical composition of *R. tomentosum* EO which depends strongly on the place of harvesting [1,17]. Such variability is clearly undesirable from therapeutic perspective since it can drastically influence the obtained biological response. Consequently, it would be difficult to standardize a natural drug based on wild-growing *R. tomentosum* plants, especially considering that its habitats are either remote (arctic and subarctic regions) or threatened by overexploitation, as in central Europe [1]. In order to overcome these problems, the project has been launched, aimed at establishing bioreactor cultures of *R. tomentosum* microshoots as a sustainable source of essential oil with stable chemical composition [18,19].

The current research consisted of three parts. First, the data on chemical composition of *R. tomentosum* EOs obtained from various habitats in Europe and Asia was collected. The data which included the results of authors' own studies, as well as literature reports, was subsequently used for hierarchical cluster analysis aimed at defining the EO chemotypes of the plant. The results enabled to evaluate the extent of variability of EO content and discuss its therapeutic considerations. The second part of the study included phytochemical studies targeted at providing EOs with defined composition: samples of wild-growing plants representing different chemotypes were harvested, and so were the bioreactor-grown microshoots of *R. tomentosum*. The volatile fractions were isolated and their composition determined by GC/MS. The third part of the research focused on evaluating pro-apoptotic activity of the obtained EOs toward CD4 and CD8 T cells from peripheral blood samples from healthy volunteers, as well as synovial cells isolated from synovium samples from RA patients undergoing synovectomy. The purpose of the experiments was to establish whether *R. tomentosum* EO affects apoptosis rates of the above cells *in vitro*, which would provide a potential mechanism for its therapeutic action and rationale for further studies concerning anti-RA properties of the plant. Additionally, the study was meant to compare the observed effects with *R. tomentosum* EO originating from *in vitro* biomass, in order to assess whether it retains the bioactivity of the parent plant. Therefore, besides naturally occurring volatile fractions, EO obtained from *R. tomentosum* microshoots was subjected to biological testing.

2. Materials and methods

2.1. Plant materials and essential oil analysis

R. tomentosum shoots were harvested in June 2016 from two different habitats: Miszewko (P1) in the Pomeranian Voivodeship in Poland [17,20], and from Turku (F1) in Southwest Finland [17]. Voucher specimens (no 14252 and 14254, respectively) were deposited in the herbarium of Department of Biology and Pharmaceutical Botany, Medical University of Gdansk. *R. tomentosum* microshoots (IV) were previously established in Department of Pharmacognosy, Medical University of Gdansk from the maternal plant grown in Miszewko (Poland). The microshoots were cultivated for 28 days in temporary immersion RITA® bioreactor (Vitropic, St. Mathieu de Treviers, France)

containing 200 ml liquid Schenk-Hildebrandt medium supplemented with 24.60 μM 2-isopentenyladenine and 592.02 μM adenine, under conditions specified previously [18]. The harvested plant materials were air-dried in shade and stored in sealed containers prior to phytochemical analysis.

The EOs were obtained by hydrodistillation in Deryng apparatus (5 g dried, non-pulverized material, 100 ml of distilled water, 3 h) according to Polish Pharmacopoeia [20,21]. Afterwards, GC/MS analysis of the volatile fractions was performed to establish their chemical compositions, following the methodology reported previously [17]. The main constituents of the EOs from wild-grown plants (P1 and F1 samples) were listed in Table 1. The main constituents of volatile fraction isolated from microshoots were: *p*-cymene 6.9%, alloaromadendrene 5.5%, shyobunone 8.2% and ledene oxide (II) 13.0% [18].

2.2. Hierarchical cluster analysis

A total of 51 chemical compositions of *R. tomentosum* essential oils from the published literature [17,20,22–37], as well as three EOs from authors' own studies (P1, P2, F1) (Table 1) were considered operational taxonomic units. The literature data was gathered with Scopus, PubMed, ScienceDirect, Web of Science and Google Scholar databases using “*Rhododendron tomentosum*”, “*Ledum palustre*”, “essential oil” and “volatile oil” as search terms. To standardize the results, only the bibliographic data concerning the EOs obtained from the plant material (preferably aged shoots) harvested from the specified natural habitats (Fig. 1) from May to October (preferably June and July) and isolated by hydrodistillation or simultaneous distillation-extraction methods, was taken into account. The percentages of 10 major components in the studied EOs (defined as $\geq 20\%$ content in at least one examined sample: sabinene, β -myrcene, *p*-cymene, limonene, α -thujenal, γ -terpineol, bornyl acetate, ascaridole, palustrol, ledol – structures presented in Fig. 2) determined by GC/MS analysis were used to define the chemical relationship between the various *R. tomentosum* samples by agglomerative hierarchical clustering (AHC). The reports on other representatives of the genus, including *R. tomentosum* ssp. *decumbens* and *R. groenlandicum*, were not included in cluster analysis. The results were presented in Fig. 3.

2.3. Lymphocyte proliferation study

2.3.1. Lymphocyte isolation and cell culture

A group of 6 pre-screened, healthy volunteers (all women, the mean age 27 years) was recruited. None of them were taking medication that would influence the immune system. All the individuals involved in the study were informed about the purpose of the experiment and gave their written consent. The project was accepted by the Local Independent Committee for Ethics in Scientific Research at the Medical University of Gdansk (permission number NKEBN/269/2011).

Peripheral blood mononuclear cells (PBMC) were obtained from fasting venous blood samples. Blood was collected into the EDTA-containing Vacutainer™ Tubes (BD Pharmingen, San Diego, USA) and then PBMC were isolated by Histopaque™ (Sigma-Aldrich, St. Louis, USA) flotation. Isolated PBMC were stained supravivally with fluorescent CFSE dye according to the method described by Witkowski [38], resuspended in complete culture medium (RPMI 1640) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (all reagents from Sigma-Aldrich, St. Louis, USA) at $2 \times 10^6 \text{ ml}^{-1}$ and stimulated with immobilized anti-CD3 (BD Pharmingen, San Diego, USA).

Then, the cells were exposed to the EOs isolated from *R. tomentosum* collected in Poland (P1) and in Finland (F1) as well as obtained from the *in vitro* cultures (IV), at three dilutions (1:400, 1:4000 and 1:40000 v/v). The EOs were applied as stock solutions in ethanol, so that the final concentration of the solvent in the culture medium was 0.25%. The EO-treated samples and the controls with only ethanol added were

incubated for 72 and 120 hours at 37°C in 5% CO₂/95% air in an humidified incubator. At the completion of incubation period, the cells were harvested, washed with phosphate buffered saline (PBS), counterstained with fluorochrome-coupled antibodies: RPE-Cy5/anti-CD4 or RPE-Cy5/anti-CD8 (DAKO, Glostrup, Denmark) and analysed by flow cytometry in accordance with the staining procedure described above.

2.3.2. Flow cytometry analysis

Phenotyping and analysis of effects of EOs on proliferation rate of the healthy PBMC were performed using the FACScan™ system (BD Biosciences, San Jose, USA). At least 3×10^4 cells were acquired from each sample using the CellQuest software of the instrument and then analyzed using the Cyflogic v. 1.2.1 software. During analysis, the lymphocytes were first identified by forward and side scatter gating and then by CD4 and CD8 expression, respectively (with further identification of the EOs' effect on the T-cell subpopulation according to the staining scheme used). The pattern of staining with the CFSE corresponding to nondividing cells which retained all of the marker, and to those dividing, which exhibited the 1:2 dilution of the fluorochrome with each consecutive division, was then visualized as fluorescence histograms (Fig. 4). The proportions of proliferating CD4⁺ or CD8⁺ T cells in control and EO-treated samples was then calculated as ratio of the number of cells which diluted the CFSE at least 1 in 2 (i.e., underwent at least one division) to the total number of either CD4⁺ or CD8⁺ lymphocytes in the analyzed sample. The effect of *R. tomentosum* EOs on CD4⁺ and CD8⁺ cell proliferation after 72 and 120 h was shown in Fig. 5.

2.4. Synoviocyte study

2.4.1. Synoviocytes isolation and cell culture

In order to assess the influence of different *R. tomentosum* EOs on the viability of the cellular components of human inflamed synovium, fragments of synovial/pannus tissue were obtained from 3 patients (all women, age 36 to 46 years) at the time of hand joint synovectomy. All patients fulfilled the American College of Rheumatology revised criteria for diagnosis of RA [39]. Patients were treated with steroids, methotrexate and nonsteroid anti-inflammatory drugs. The study was conducted in accordance with the Declaration of Helsinki and the experimental protocol was approved by the Independent Bioethics Commission for Research, Medical University of Gdansk. Tissue fragments were immediately processed for the release of single cells according to protocol given in our previous papers [40,41]. Briefly, the material was first cut into small (5–10 mm³) pieces and placed in sterile PBS, with attention given to the removal of as much fat tissue as possible. A mechanical tissue disruptor (Medimachine™, BD Biosciences) was then used for mincing of the tissue and cell release. The remaining tissue debris, fibers and multicellular clumps were then removed from the crude cell suspension by filtration through the Filcon 50™ filters (BD Biosciences). Single cells were then transferred to the complete culture medium formulated as above and cultured at 1 million cells per well for 48 hours (37°C, 5% CO₂) in 24-well culture plates, with either 1:400 or 1:4000 or 1:40000 v/v dilution (as in the case of lymphocyte studies) of one of the EOs (F1, P1 or IV) or with analogous concentration of absolute ethanol as control.

2.4.2. Flow cytometric assessment of the effect of EOs on survival and death of synovial cells

After completion of the incubation with (or without) EOs, the adherent cells were released from the surface of culture wells with help of a tissue scraper. In order to facilitate their release, the plates were incubated for 15 minutes on ice. Released cells were washed with cold PBS, counted and stained with FITC-anti-CD55 (fibroblast-like synoviocytes, FLS), APC-anti-CD14 (monocytes/macrophages) and V450-anti-CD3 (T cells).

The proportions of living, early and late apoptotic, and necrotic cells

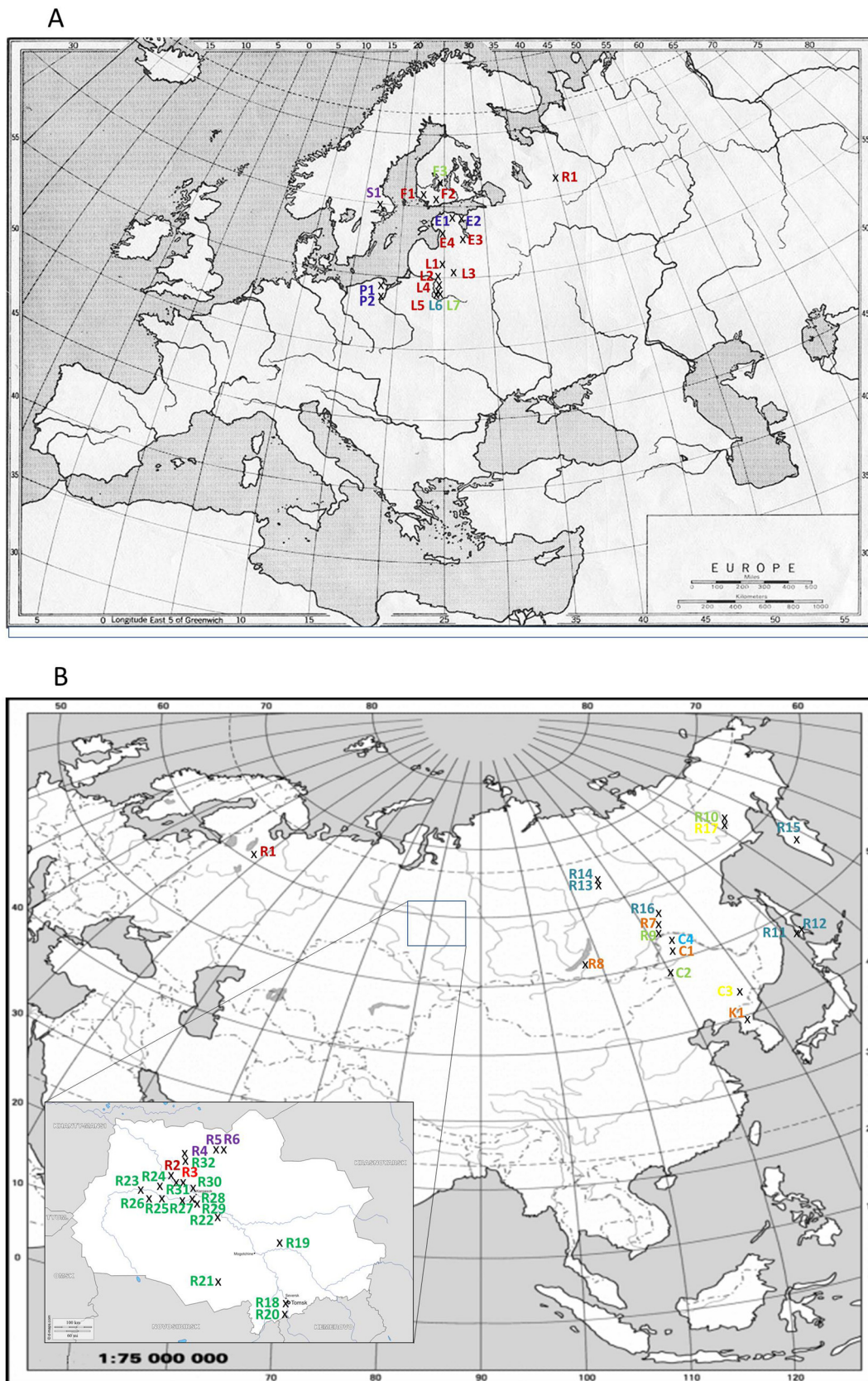


Fig. 1. Geographical distribution of the fifty-four *R. tomentosum* sampling sites in Europe (A) and Asia (B), according to the published literature and own studies (detailed description is presented in Table 1). The inset shows Tomsk Oblast in Russia.

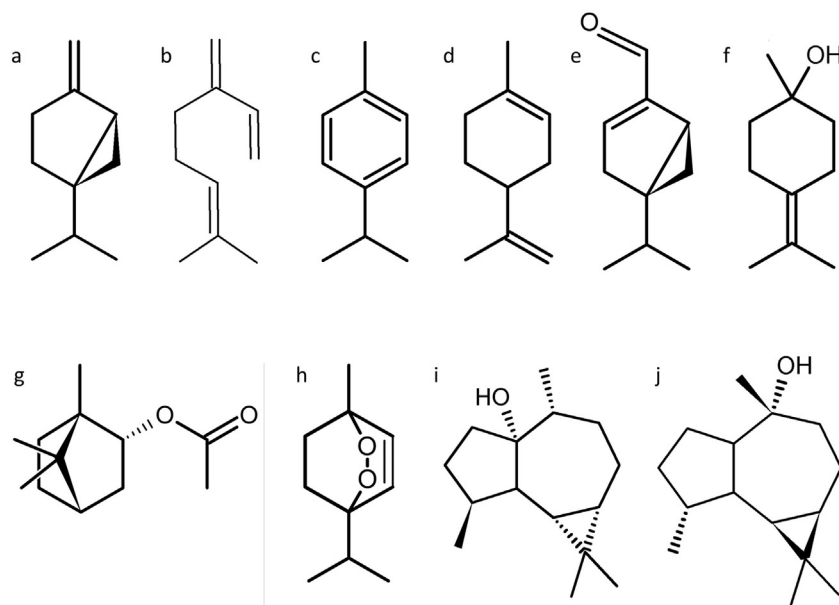


Fig. 2. The major compounds determined in the chemical profiles of the essential oils of *R. tomentosum* plants growing in Europe and Asia, which were the basis for designation of ten chemotypes: a) sabinene, b) β -myrcene, c) *p*-cymene, d) limonene, e) α -thujenal, f) γ -terpineol, g) bornyl acetate, h) ascaridole, i) palustrol, j) ledol.

were evaluated in the FLS, monocyte and T cell populations by staining with PE-annexin V and 7-aminoactinomycin D (7-AAD) in accordance with manufacturer's protocol. All staining reagents were from BD Pharmingen, San Diego, USA.

Flow cytometry data acquisition was performed using the 8-color FACSVerse™ cytometer (BD Biosciences). At least 10^5 events were recorded from each sample. The analysis was performed using the Flowing Software v. 2.5.1 (Perttu Terho, Turku Centre for Biotechnology, University of Turku, Finland). The following gating strategy was adopted: first, lymphocytes (smallest, low granularity), monocyte/macrophages (larger, medium granularity) and FLS (largest, medium-high granularity) were identified in forward scatter (FSC) and side scatter (SSC) plots. Then, cells exhibiting the phenotypes corresponding to T cells ($CD3^+ CD14^+ CD55^{low}$), monocyte/macrophages ($CD3^+ CD14^+ CD55^{low}$) and the FLS ($CD3^+ CD14^+ CD55^+$) were identified

within the original scatter gates. Next, doublets and larger aggregates of cells were eliminated from further analysis by plotting the forward scatter area versus forward scatter height for each cellular population defined as above and subtracting the events with $FSC-A > FSC-H$. Within each population of such defined single T cells, FLS and monocyte/macrophages, the cells negative for both Annexin V and 7-AAD staining ($An-7AAD^-$) were considered alive, those staining with annexin but not with 7-AAD ($An+7AAD^-$) – early apoptotic, those staining with both annexin and 7AAD ($An+7AAD^+$) – late apoptotic and finally those staining predominantly with 7AAD ($An-7AAD^+$) were considered necrotic. Results were shown as the proportions of these four sub-population in the synovial T cells, FLS and monocyte/macrophages (Fig. 6).

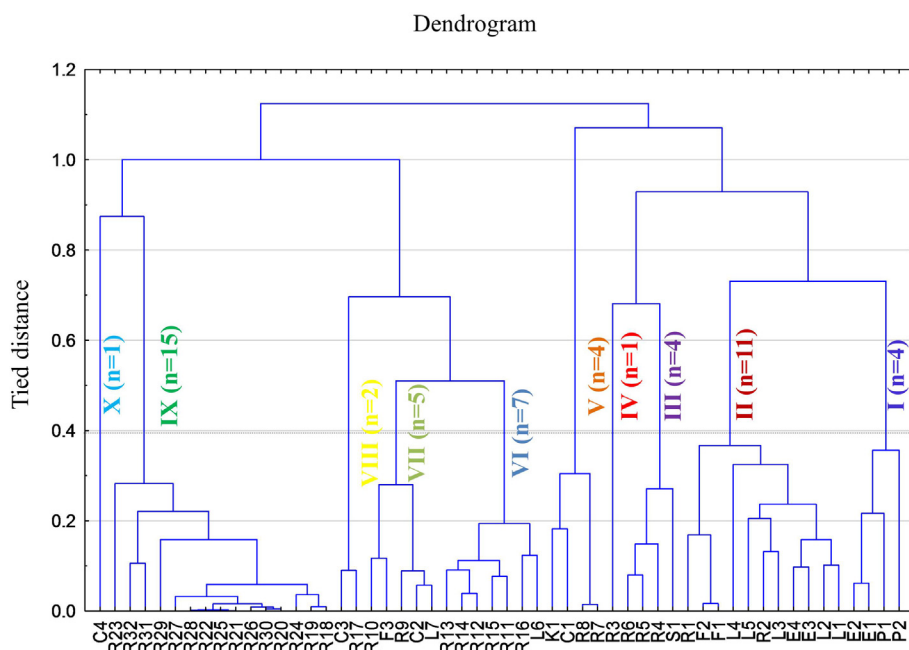


Fig. 3. Dendrogram obtained from the agglomerative hierarchical cluster analysis (AHC) of fifty-four *R. tomentosum* essential oil compositions. AHC formed ten clusters (I – X)* (n - number of samples in every cluster).

* Chemotype I - γ -terpineol; II - ledol / palustrol; III - β -myrcene / palustrol; IV - β -myrcene / *p*-cymene / palustrol; V - sabinene; VI - *p*-cymene; VII - *p*-cymene / ascaridole; VIII - *p*-cymene / bornyl acetate; IX - limonene; X - α -thujenal

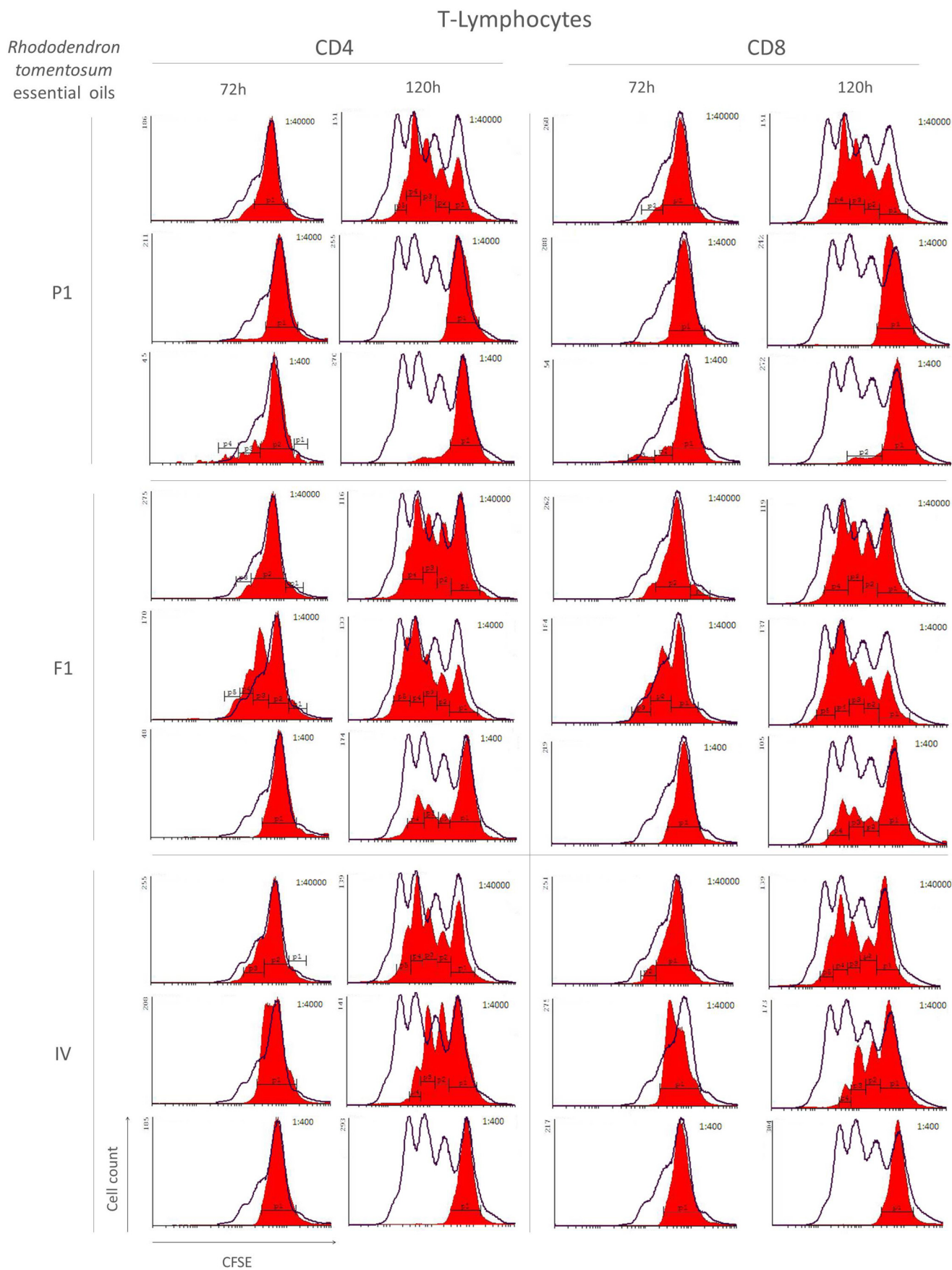


Fig. 4. Representative histograms showing the proliferation patterns of CD4+ and CD8+ T cells, illustrated by the CFSE dilution and obtained after 72 and 120 hours of stimulation with serial dilutions of the *R. tomentosum* essential oils* (1:400-1:40000 v/v).

* P1, F1, IV - *R. tomentosum* EOs obtained from the shoots collected in Miszewko in Poland, in Turku in Finland and from *in vitro* cultures, respectively

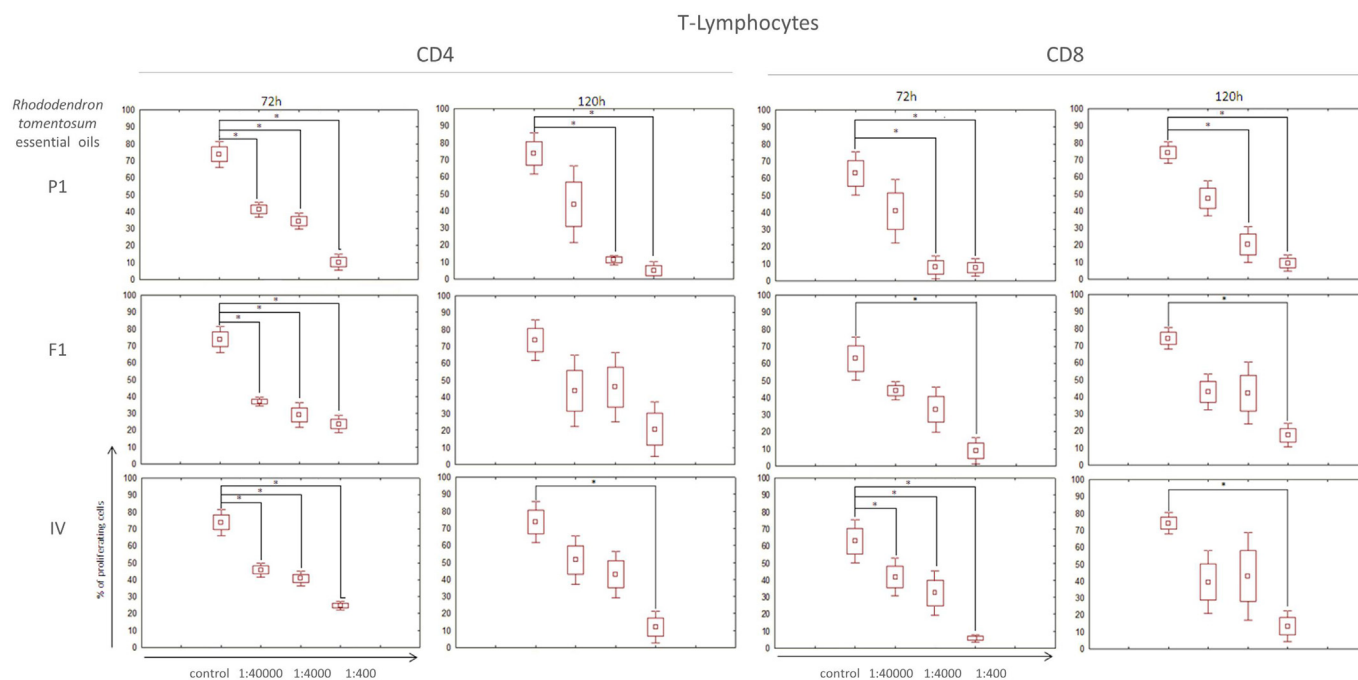


Fig. 5. The effect of the *R. tomentosum* essential oils, applied in serial dilutions (1:400–1:40000 v/v), on the proliferation of CFSE-labeled CD4+ and CD8+ T cells after 72 and 120 hours, analyzed by flow cytometry. P1, F1, IV correspond to *R. tomentosum* EOs obtained from the shoots collected in Miszewko in Poland, in Turku in Finland and from *in vitro* cultures, respectively. Box-and-whisker plots depict mean proportions of dividing cells (central point), SD (box) and SE (whiskers). (*) mark statistical significance at $p < 0.05$, using non-parametric Mann-Whitney U-test.*

2.5. Statistical analysis

2.5.1. Hierarchical cluster analysis

AHC analysis was performed using the Dell Statistica data analysis software system (Dell Inc. 2016, version 13, software.dell.com). 1- r Pearson correlation was selected as a measure of similarity, and the unweighted pair-group method with arithmetic averages (UPGMA) was used for cluster definition (Fig. 3).

2.5.2. Lymphocyte proliferation and synoviocyte survival/death study

Statistical analyses were performed with STATISTICA 8™ (StatSoft, Poland) using non-parametric Mann-Whitney U-test. Values are shown either in the box-and-whisker diagram with marked means, standard errors (SE) and standard deviation (SD) (Fig. 5) or as bars (means) and standard errors (SE, whiskers) (Fig. 6).

3. Results and discussion

3.1. Chemotypes of *R. tomentosum* essential oils

To identify and characterize the various chemotypes of *R. tomentosum* in Europe and Asia, the percentage contents of the main constituents of its EOs (Table 1) obtained from the plant materials collected in different geographical locations (Fig. 1) were submitted to cluster analysis. The resulting dendrogram separated fifty-four *R. tomentosum* populations into ten distinct chemotypes (Fig. 2). European ecotypes formed mainly cluster I and II. First of them was characterized by high concentrations of γ -terpineol followed by a slightly smaller content of *p*-cymene, palustrol and ledol, while the second one was composed mainly of palustrol (21.0%–53.5%) and ledol (14.6%–36.5%, Table 1). The two last-mentioned aromadendrane derivatives, named after the plant (i.e. *Ledum palustre* L.), were supposed to be the most distinctive for the studied species. However, as the results of AHC revealed, the so-called typical chemotype II was recognized only in 1 sample (R2) outside Europe, indicating a large variability of this plant material worldwide. This issue was mentioned already by Belousova

et al. [32], who defined four new chemotypes in Russia, with predominance of limonene, *p*-cymene, sabinene and β -myrcene. This point of view is generally consistent with our observations. Furthermore, our research showed that β -myrcene is usually accompanied by palustrol, as in cluster III, which was identified in Europe (S1) as well as in Russia (R4-R6). Chemotype IV, similar to the previous one but with simultaneous high content of *p*-cymene, was represented only by one individual EO (R3) obtained from *R. tomentosum* collected in Tomsk Oblast. Four populations from the eastern part of Russia, China and North Korea (R7, R8, C1, K1) formed chemotype V, as their volatile fractions were rich in sabinene. Clusters VI, VII and VIII, also identified mainly in the Far East Asia, were abundant in another aromatic monoterpene, *p*-cymene. Additionally, in the last two of them ascaridole and bornyl acetate were determined as the major components, respectively. The high amount of limonene (7.9%–50.3%) differentiated the Tomsk Oblast populations as a distinct chemotype IX which comprised the fifteen reported EOs. According to the cluster analysis, *R. tomentosum* collected in Inner Mongolia (C4) distinguished from other ecotypes, having α -thujenal as the primary compound (chemotype X).

It should be noted that the defined chemotypes were not always in accordance with the geographical origins of the discussed plant. For instance, each of three populations (L5-L7) collected from the limited area of Sulnys Lake in Lithuania, where the distances between sampling sites were only 1–1.5 km [27] represented different cluster (II, VI and VII, respectively). For plant materials collected in southern Finland (F1-F3), Orotukan in eastern Russia (R10, R17) and Inner Mongolia region in China (C1, C2, C4) this phenomenon was also observed. It seems that especially chemotype VII, characterized by EO rich in *p*-cymene and ascaridole, can be found independently from latitude and longitude which is probably related with genetic factors. The designation of as many as ten *R. tomentosum* chemotypes in Europe and Asia confirmed the common knowledge that chemical composition of a volatile fraction within a species is variable and can be affected not only by geographical origin, but also genetic characteristics, physiological and environmental conditions as well as the analytical procedures [42]. Such diversity in the EOs' profiles offers a wide range for selection of desired chemotypes

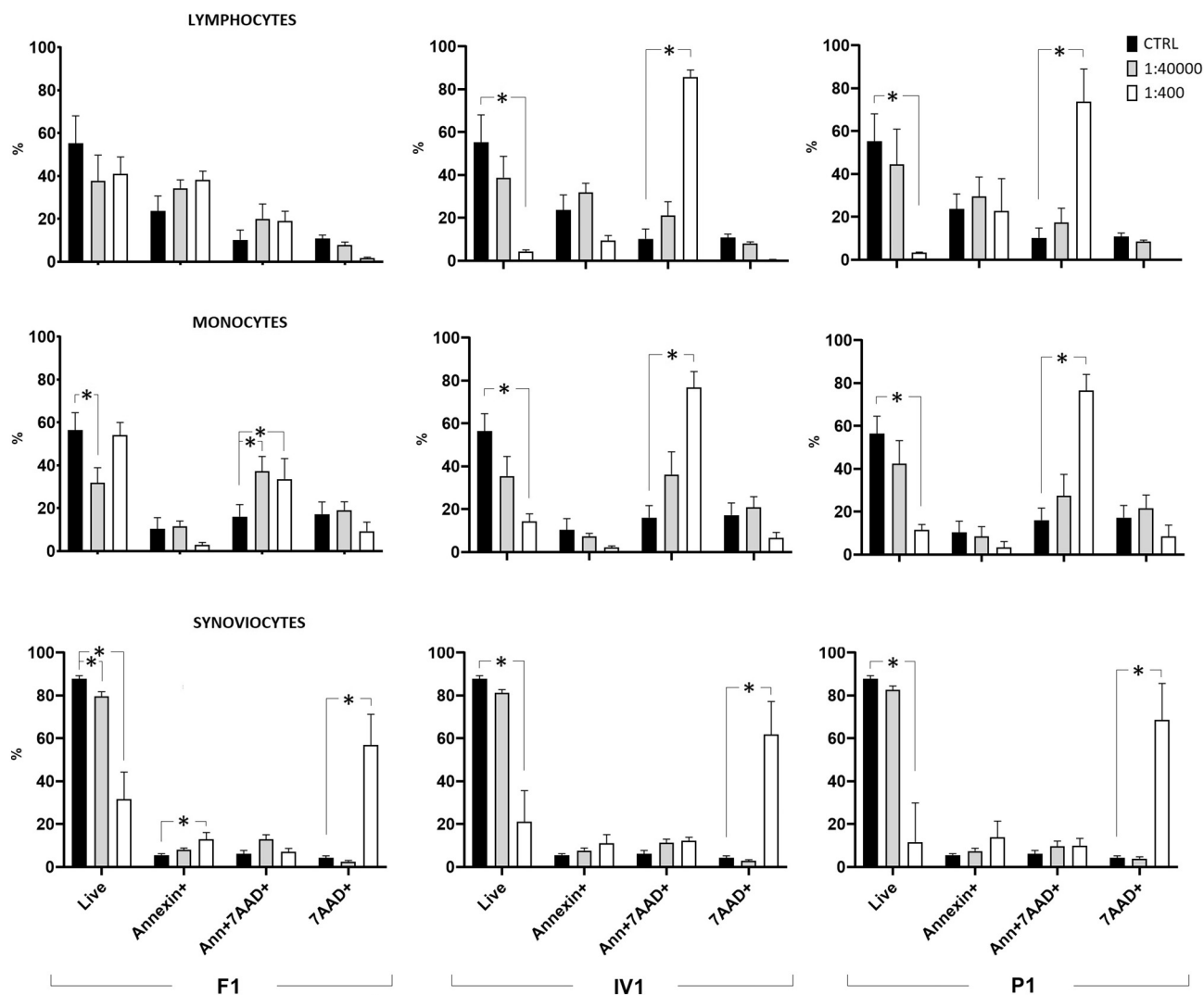


Fig. 6. The effect of the *R. tomentosum* essential oils F1, P1 and IV1, applied in serial dilutions (1:400-1:40000 v/v), on the survival, apoptosis and necrosis of synovial T cells (LYMPHOCYTES), monocyte/macrophages (MONOCYTES) and fibroblast-like synoviocytes (SYNOVIOCYTES) assessed using Annexin V/7AAD staining as described in the Materials and Methods. Mean proportions of living (Live), early apoptotic (Annexin +), late apoptotic (Ann + 7AAD +) and necrotic (7AAD +) cells are shown as bars, and SEM values are indicated as whiskers. The asterisks (*) mark statistical significance at $p < 0.05$, using non-parametric Mann-Whitney U-test. Results for intermediate EO dilution (1:4000 v/v) are not shown for clarity.

for specific medicinal or agricultural purposes. For instance, for cough treatment the chemotypes with large content of ledol should be chosen, as this compound was previously reported to exhibit antitussive properties. However, too high ledol concentrations are considered to be toxic, causing dizziness and nausea [1]. For this reason, there is a need to control the amount of ledol in the plant material, especially in case of chemotypes I and II [17]. In Russian *R. tomentosum* populations, the content of ledol is usually low, which is probably the cause that this plant can still be found in the Pharmacopoeia of the USSR as „*Cornus Ledi palustri*” [43]. On the contrary, in European countries it finds application only in folk medicine.

The insect-and tick-repellent properties of *R. tomentosum* EO have been associated with the presence of large amounts of β -myrcene, palustrol and ledol, thus pointing at chemotypes III and IV as promising repellent agents [23,24]. However, it was suggested that other EO constituents such as *p*-cymene, terpinyl acetate, sabinene, α - and β -pinene, bornyl acetate, β -phellandrene and camphene also contribute to the observed effects [44].

The presence of high amounts of sabinene in the volatile fraction (chemotype V) has been linked to strong antioxidant activity, as in the case of North Korean *R. tomentosum* [37]. However, so far the

compounds directly responsible for anti-inflammatory properties of the discussed plant remain unknown.

The cluster analysis conducted did not include the North American representatives of the genus, namely *R. groenlandicum* (also referred to as *L. palustre* ssp. *groenlandicum*) and *R. tomentosum* ssp. *decumbens* (aka *R. tomentosum* ssp. *subarcticum*) [45]. As opposed to *R. tomentosum* of Eurasian origin, the reports concerning chromatographic analysis of essential oil obtained from the above plants are scarce. The work by Schantz and Hiltunen [46] included GC analysis of *R. groenlandicum* and *R. tomentosum* ssp. *decumbens* obtained from unspecified location in Alaska. The volatile fractions of both plants were dominated by germacrone ($\geq 40\%$), a sesquiterpene constituent not found in substantial amounts in Eurasian specimens. The analysis also revealed the presence of large amounts of allo-aromadendrene and myrtenal (ca. 15-20% in total), however, methods available at the time did not enable to separate these compounds [46]. In the current work, myrtenal was not identified as a major constituent in any of the cluster groups whereas allo-aromadendrene was found only in essential oil from *in vitro*-grown shoots of *R. tomentosum* [18]. The analyses of *R. groenlandicum* from Quebec, conducted by Belleau and Collin [47], confirmed the presence of germacrone, however, it was shown to be abundant only during the

flowering period. Other major constituents included sabinene, α -pinene and terpinen-4-ol (up to over 22, 16 and 9%, respectively) [47]. *R. tomentosum* ssp. *decumbens* was extensively studied by Baldwin [45] who analyzed essential oil composition of specimens collected from different locations in Alaska. The volatile fractions contained chiefly cymene (47-62%) and varying amounts of α - and β -pinene and terpinen-4-ol. Contrary to previous reports, no germacrone was found in essential oil samples. Since the volatiles were extracted using headspace method [45], the results are not directly comparable to the data included in hierarchical clustering. From a therapeutic point of view, the presence of germacrone may be of interest given its anti-inflammatory and anticancer properties [48]. Still, it is not clear whereas germacrone-rich *R. tomentosum* plants should be considered a distinct chemotype or the accumulation of this compound is associated only with flowering period in some populations.

3.2. Anti-proliferative activity of *R. tomentosum* essential oils toward healthy peripheral blood CD4+ and CD8+ T lymphocytes

In our work, the anti-proliferative effects of the selected *R. tomentosum* EOs, obtained from the plant material collected in Miszewko in Poland (P1), Turku in Finland (F1), and from *in vitro* cultures (IV), toward CD4+ and CD8+ T lymphocytes were tested, each in three concentrations representing decimal dilutions of the respective samples. P1 oil represented chemotype I, where γ -terpineol predominated, F1 belonged to chemotype II with highly pronounced presence of ledol and palustrol (Table 1), while IV was characterized by unique chemical composition, with ledene oxide (II) as the major constituent [18,19]. Due to the fact that females are statistically three times more often affected by autoimmune diseases than men [49,50], only women were included in our study.

The influence of *R. tomentosum* EOs on proliferating peripheral blood CD4+ and CD8+ cells after 72 and 120 hours is shown in Figs. 4 and 5. All of the studied EOs caused the decrease in the proliferation of anti-CD3-stimulated T cells, independently from the chemotype. As it was depicted, most of the used volatile fractions had similar suppressive effects on CD4+ as well as CD8+ cells (Figs. 4 and 5). Both of aforementioned subpopulations of lymphocytes are known to be involved in the pathogenesis of RA, but their precise role has not been investigated in detail yet. It is known that activated CD4+ T cells differentiate into specialized effector cells and regulate the local inflammation and infiltration of the rheumatoid synovium, while CD8+ T cells contribute to tissue damage and high production of TNF- α and IFN- γ [51,52]. The statistically significant, dose-dependent effect of the serial dilutions of *R. tomentosum* EOs on proliferating lymphocytes was observed in each variant except F1 after 120 hours and IV in CD8+ cell population after 120 hours (Fig. 4). Moreover, it was demonstrated that P1 EO was characterized with the strongest activity among all studied volatile fractions. In this case, the number of divided cells decreased from about 70% in the control variants to 10% after 72 hours and 5% after 120 hours in CD4+ cell population and to 8% in CD8+ cell population, at 1:400 concentration. In comparison, F1 and IV EOs inhibited the proliferation of CD4+ cells by about 45% after 72 hours in respect to the control. Approximate values are reproducible in other options.

So far, to the best of the authors' knowledge, no other research on the influence of *R. tomentosum* EO on the human lymphocytes *ex vivo* has so far been conducted. The effects of the plant on different cell lines were reported, however, none of the studies concerned the EO specifically. The cytotoxicity of the discussed plant, collected in Moscow Region, was previously tested on cultured human lymphoblastoid cell line Raji. In this study, the crude ethanol extract of *R. tomentosum* was one of the most active among the examined 61 plant species. Interestingly, the isolated ledol showed low growth-inhibiting effect at relatively high concentrations (10–50 μ g/mL), scarcely approaching the activity profile of cyclophosphamide [53]. In another study, cytotoxic properties of methylene chloride (99%) and methanol (71%) extracts of

R. tomentosum collected in Russia were demonstrated with mouse leukemia L1210 cells as a target [54]. Recently, proapoptotic activity of the organic extracts prepared from Alaskan plant material against acute myeloid leukemia cells was determined, with indication of ursolic acid as potentially responsible for the effect [55].

3.3. Pro-apoptotic activity of *R. tomentosum* essential oils toward synoviocytes

The main battlefield in rheumatoid arthritis-affected joints is their synovium, which becomes infiltrated by active T and B lymphocytes, and monocyte/macrophages. Due to interplay between the cytokines and other growth factors released from these cells, the fibroblast like synoviocytes, or FLS, respond by proliferating and secretion of excessive amounts of matrix, which forms the so called pannus filling the joint cavity and reducing its mobility. Our results described above, showing the anti-proliferative effect of *R. tomentosum* EOs on peripheral blood T cells suggested, that the similar, potentially desirable effect may occur for synovia-infiltrating lymphocytes and possibly other cells infiltrating or developing in the pannus. In this case we did not assess proliferation, but instead examined the pro-apoptotic effect of the studied EOs on synovial fibroblasts and infiltrating lymphocytes and monocyte/macrophages (Fig. 6). We have demonstrated that indeed, all concentrations of the EOs tested increased the proportions of late apoptotic (An⁺7AAD⁺) T cells. This increase was statistically significant for the highest concentrations of P1 and IV1 EOs used, but the trend was present also for F1 EO (Fig. 6, upper row). Lack of statistical significance of differences for lower EO concentrations (with the results from tests where EO was added as 1:4000 not shown for clarity) is likely due to low number of synovial samples tested (3) and relatively broad spread of the results. Still, we can conclude that anti-proliferative effects on human T cells exerted by the *R. tomentosum* EOs is due to its apoptosis-inducing activity. Interestingly, a very similar effect, i.e. the significant increase of late apoptotic (An⁺7AAD⁺) cells was seen in CD14+ monocyte/macrophage population exposed to high concentration (1:400) of each of the three EOs tested (Fig. 6, middle row). In this case, the exposure to F1 EO at 1:40000 had also produced a significant increase of the proportion of late apoptotic monocyte/macrophages. Together, these results may indicate a possibility of variable and indirect anti-inflammatory effect of the *R. tomentosum* EOs. On the other hand, 48-hour exposure of the synovial fibroblasts (FLS) to higher concentration of each of the three EOs had uniformly lead to significant increase in the proportion of necrotic (An⁺7AAD⁺) cells, at the expense of the cells remaining alive (An⁻7AAD⁻) (Fig. 6, bottom row). The results obtained for lower concentrations of the EOs were not significant. Also this observation, if confirmed on a larger group, may show the beneficial, anti-proliferative effect of the studied EOs on excessively proliferating FLS in RA synovia. Further experiments would determine if the effect of the EOs on FLS is due to direct toxicity or accelerated (as compared to both T cells and monocytes) progression through early and late apoptosis.

4. Conclusions

In conclusion, exploring the phytochemical diversity of the EOs obtained from *R. tomentosum* collected in different geographical locations allows to distinguish ten various chemotypes, which provides important information for exploiting their potentials in the pharmaceutical industry. The study revealed substantial variability of EO chemistry which is a key issue to be addressed when designing biological experiments. Moreover, *R. tomentosum* was evaluated as a source of potential antiarthritic drugs. EOs of the plant were demonstrated to have anti-proliferative and pro-apoptotic activity toward CD4 and CD8 T cells as well as toward synovia-infiltrating monocyte/macrophages and fibroblast-like synovial cells. Since increased proliferation of these cells is critical for RA development, the obtained results can be

considered promising. However, *R. tomentosum* EOs of different origin provided different biological responses, thus emphasizing the need to use volatile fraction of defined chemistry. In this regard, microshoot cultures of *R. tomentosum* offer some advantages: since they are grown in controlled environment, the yield and composition of essential oil are expected to be stable. Moreover, positive effects obtained with the use of EO from the microshoots prove that this type of biomass can provide biologically active constituents.

Further experiments are required in order to establish the lowest effective concentration of the EO, as well as the molecular mechanisms of their anti-proliferative and pro-apoptotic activities. Still, the results of the study show potentially interesting effects of the volatile fractions isolated from *R. tomentosum* plants collected from the different habitats on the proliferation of human T lymphocytes.

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Author contributions

AJ prepared the essential oils samples, gathered the literature data for AHC analysis, participated in lymphocytes proliferation study, analyzed the results and wrote the manuscript. AK, KC-F and AD conducted experiments on synoviocytes. AM-R and KC-F performed lymphocyte experiments. PL prepared synovial tissue samples. AB carried out AHC analysis. BZ conducted GC analysis of essential oils. ML, JM-W and EB conceptualized the study, analyzed the data and edited the manuscript.

Declaration of Competing Interest

All co-authors agree on the contents of this manuscript and consent to submit it. The manuscript has not been submitted to any other journal for simultaneous consideration. The authors declare that they have no conflict of interest.

References

- [1] A. Dampc, M. Luczkiewicz, *Rhododendron tomentosum* (*Ledum palustre*). A review of traditional use based on current research, *Fitoterapia* 85 (2013) 130–143, <https://doi.org/10.1016/j.fitote.2013.01.013>.
- [2] A. Dampc, M. Luczkiewicz, Labrador tea – the aromatic beverage and spice: a review of origin, processing and safety, *J. Sci. Food Agric.* 95 (2015) 1577–1583, <https://doi.org/10.1002/jsfa.6889>.
- [3] L.D. Kumar, R. Karthik, N. Gayathri, T. Sivasudha, Advancement in contemporary diagnostic and therapeutic approaches for rheumatoid arthritis, *Biomed. Pharmacother.* 79 (2016) 52–61, <https://doi.org/10.1016/j.biopha.2016.02.001>.
- [4] S.E. Gabriel, K. Michaud, Epidemiological studies in incidence, prevalence, mortality, and comorbidity of the rheumatic diseases, *Arthritis Res. Ther.* 11 (2009) 229, <https://doi.org/10.1186/ar2669>.
- [5] W. Burton, A. Morrison, R. Maclean, E. Ruderman, Systematic review of studies of productivity loss due to rheumatoid arthritis, *Occup. Med.* 56 (2006) 18–27, <https://doi.org/10.1093/occmed/kqi171>.
- [6] C. Deighton, R. O'Mahony, J. Tosh, C. Turner, M. Rudolf, Management of rheumatoid arthritis: summary of NICE guidance, *Br. Med. J.* 338 (2009) b702, <https://doi.org/10.1136/bmj.b702>.
- [7] S.S. Laev, N.F. Salakhutdinov, Anti-arthritis agents: Progress and potential, *Bioorg. Med. Chem.* 23 (2015) 3059–3080, <https://doi.org/10.1016/j.bmc.2015.05.010>.
- [8] C.L. Yang, T.C. Or, M.H. Ho, A.S. Lau, Scientific basis of botanical medicine as alternative remedies for rheumatoid arthritis, *Clin. Rev. Allergy Immunol.* 44 (2013) 284–300, <https://doi.org/10.1007/s12016-012-8329-8>.
- [9] H. Tunón, C. Olavsdotter, L. Bohlin, Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF-induced exocytosis, *J. Ethnopharmacol.* 48 (1995) 61–76, [https://doi.org/10.1016/0378-8741\(95\)01285-L](https://doi.org/10.1016/0378-8741(95)01285-L).
- [10] P. Black, A. Saleem, A. Dunford, J. Guerrero-Analco, B. Walshe-Roussel, P. Haddad, A. Cuerrier, J.T. Arnason, Seasonal variation of phenolic constituents and medicinal activities of northern labrador tea, *Rhododendron tomentosum* ssp. *subarcticum*, an inuit and cree first nations traditional medicine, *Planta Med.* 77 (2011) 1655–1662, <https://doi.org/10.1055/s-0030-1270968>.
- [11] S. Baananou, E. Bagdonaitė, B. Marongiu, A. Piras, S. Porcedda, D. Falconieri, N.A. Boughattas, Supercritical CO₂ extract and essential oil of aerial part of *Ledum palustre* L. - chemical composition and anti-inflammatory activity, *Nat. Prod. Res.* 29 (2015) 999–1005, <https://doi.org/10.1080/14786419.2014.965167>.
- [12] M. Zhang, J.-J. Zhao, W.-B. Wu, Y. Zhao, X.-L. Zhu, W.-J. Qu, Analgesic and Anti-inflammatory activities of extracts from *Ledum palustre* L. in mice, *Nat. Prod. Res. Dev.* 22 (2010) 326–329.
- [13] C.N. Tran, S.K. Lundy, D.A. Fox, Synovial biology and T cells in rheumatoid arthritis, *Pathophysiology* 12 (2005) 183–189, <https://doi.org/10.1016/j.pathophys.2005.07.005>.
- [14] M. Mellado, L. Martínez-Munoz, G. Cascio, P. Lucas, J.L. Pablos, J.M. Rodríguez-Frade, T cell migration in rheumatoid arthritis, *Front. Immunol.* 6 (2015) 384, <https://doi.org/10.3389/fimmu.2015.00384>.
- [15] E. Neumann, S. Lefevre, B. Zimmermann, S. Gay, U. Müller-Ladner, Rheumatoid arthritis progression mediated by activated synovial fibroblasts, *Trends Mol. Med.* 16 (2010) 458–468, <https://doi.org/10.1016/j.molmed.2010.07.004>.
- [16] H. Liu, R.M. Pope, The role of apoptosis in rheumatoid arthritis, *Curr. Opin. Pharmacol.* 3 (2003) 317–322, [https://doi.org/10.1016/S1471-4892\(03\)00037-7](https://doi.org/10.1016/S1471-4892(03)00037-7).
- [17] A. Jesionek, L. Poblocka-Olech, B. Zabiegala, A. Bucinski, M. Krauze-Baranowska, M. Luczkiewicz, Validated HPTLC method for determination of ledol and alloaromadendrene in the essential oil fractions of *Rhododendron tomentosum* plants and *in vitro* cultures and bioautography for their activity screening, *J. Chromatogr. B Biomed. Sci. Appl.* 1086 (2018) 63–72, <https://doi.org/10.1016/j.jchromb.2018.04.006>.
- [18] A. Jesionek, A. Kokotkiewicz, P. Włodarska, B. Zabiegala, A. Bucinski, M. Luczkiewicz, Bioreactor shoot cultures of *Rhododendron tomentosum* (*Ledum palustre*) for a large-scale production of bioactive volatile compounds, *Plant Cell Tissue Organ Cult.* 131 (2017) 51–64, <https://doi.org/10.1007/s11240-017-1261-0>.
- [19] A. Jesionek, A. Kokotkiewicz, A. Krolicka, B. Zabiegala, M. Luczkiewicz, Elicitation strategies for the improvement of essential oil content in *Rhododendron tomentosum* (*Ledum palustre*) bioreactor-grown microshoots, *Ind. Crop. Prod.* 123 (2018) 461–469, <https://doi.org/10.1016/j.indcrop.2018.07.013>.
- [20] A. Jesionek, B. Zabiegala, A. Bucinski, M. Luczkiewicz, From harvesting to distillation – effect of analytical procedures on the yield and chemical composition of *Rhododendron tomentosum* (*Ledum palustre*) essential oil, *Acta Pol. Pharm.* 76 (2019) 83–92, <https://doi.org/10.32383/appdr/93932>.
- [21] *Farmakopea Polska VI*, Polskie Towarzystwo Farmaceutyczne, Warszawa, 2002.
- [22] Y. Holm, I. Laakso, H. Harmaja, R. Hiltunen, The essential oils of *Ledum palustre* L.: an ascaridole chemotype, in: 24th International Symposium on Essential Oils, Technische Universität Berlin, Berlin, July, vols. 21–24 p. 50.
- [23] T.G. Jaenson, K. Pålsson, A.K. Borg-Karlson, Evaluation of extracts and oils of tick-repellent plants from Sweden, *Med. Vet. Entomol.* 19 (2005) 345–352, <https://doi.org/10.1111/j.1365-2915.2005.00578.x>.
- [24] R. Butkienė, V. Šakočiūtė, D. Latvėnaitė, D. Mockutė, Composition of young and aged shoot essential oils of the wild *Ledum palustre* L., *Chemija* 19 (2008) 19–24.
- [25] R. Butkienė, D. Mockutė, The variability of the essential oil composition of wild *Ledum palustre* L. shoots during vegetation period, *J. Essent. Oil Res.* 23 (2011) 9–13, <https://doi.org/10.1080/10412905.2011.9700423>.
- [26] A. Judzentiene, R. Butkiene, J. Budiene, F. Tomi, J. Casanova, Composition of seed essential oils of *Rhododendron tomentosum*, *Nat. Prod. Commun.* 7 (2012) 227–230.
- [27] A. Judzentiene, J. Budiene, A. Misunas, R. Butkiene, Variation in essential oil composition of *Rhododendron tomentosum* gathered in limited population (in Eastern Lithuania), *Chemija* 23 (2012) 131–135.
- [28] T. Grešušnikova, K. Järvan, A. Orav, M. Koel, Comparative analysis of the composition of the essential oil from the shoots, leaves and stems of the wild *Ledum palustre* L. from Estonia, *Procedia Chem.* 2 (2010) 168–173, <https://doi.org/10.1016/j.proche.2009.12.024>.
- [29] A. Raal, A. Orav, T. Gretchushnikova, Composition of the essential oil of the *Rhododendron tomentosum* Harmaja from Estonia, *Nat. Prod. Res.* 28 (2014) 1091–1098, <https://doi.org/10.1080/14786419.2014.907287>.
- [30] N.V. Ulyanovskii, D.S. Kosyakov, S.A. Pokryshkin, K.G. Bogolitsyn, O.S. Ulyanovskaya, Study of volatile compounds composition of *Ledum palustre* L. using the method of thermodesorption gas chromatography - mass spectrometry, *Khim. Rastit. Syria* 4 (2014) 153–161, <https://doi.org/10.14258/jcprn.201404201>.
- [31] N.I. Belousova, V.A. Khan, A.V. Tkachev, Khimicheskiy sostav efirnogo masla balgulinikov, *Khim. Rastit. Syria* 3 (1999) 5–38.
- [32] N.I. Belousova, V.A. Khan, T.P. Berezovskaya, Intraspecies chemical variability of the essential oil of *Ledum palustre*, *Chem. Nat. Compd.* 26 (1990) 398–405, <https://doi.org/10.1007/BF00598991>.
- [33] Q. Zhao, Q. Ding, G. Yuan, F. Xu, B. Li, L. Wang, J. Ouyang, Comparison of the essential oil composition of wild *Rhododendron tomentosum* stems, leaves, and flowers in bloom and non-bloom periods from Northeast China, *J. Essent. Oil Res.* 19 (2016) 1216–1223, <https://doi.org/10.1080/0972060X.2016.1141065>.
- [34] Y. Ueyama, S. Hashimoto, H. Nii, K. Furukawa, Constituents of the essential oil from *Ledum palustre* L. var. *angustum* N. Busch, *Nippon Nogeikagaku Kaishi* 63 (1989) 849–851, <https://doi.org/10.1271/nogeikagaku1924.63.849>.
- [35] G. Qu, B. Li, H. Zhao, F. Du, G. Sun, R. Wang, Chemical composition and application as biomaterials of essential oils from *Ledum palustre* of changbai mountain China, *Appl. Mech. Mater.* 568–570 (2014) 1907–1910, <https://doi.org/10.4028/www.scientific.net/AMM.568-570.1907>.
- [36] L.L. Zhang, H.X. Wang, Y.M. Wang, M. Xu, X.Y. Hu, Diurnal effects on chinese wild *Ledum palustre* L. essential oil yields and composition, *J. Anal. Sci. Methods Instrum.* 7 (2017) 47–55, <https://doi.org/10.4236/jasmi.2017.72005>.
- [37] D.-M. Kim, B.-W. Nam, Extracts and Essential Oil of *Ledum palustre* L. Leaves and their antioxidant and antimicrobial activities, *J. Food Sci. Nutr.* 11 (2006) 100–104,

- <https://doi.org/10.3746/jfn.2006.11.2.100>.
- [38] J. Witkowski, Advanced application of CFSE for cellular tracking, *Curr. Protoc. Cytom.* 9 (2008), <https://doi.org/10.1002/0471142956.cy0925s44> 9.25.
- [39] F.C. Arnett, S.M. Edworthy, D.A. Bloch, D.J. McShane, J.F. Fries, N.S. Cooper, L.A. Healey, S.R. Kaplan, M.H. Liang, H.S. Luthra, T.A. Medsger, D.M. Mitchell, D.H. Neustadt, R.S. Pinals, J.G. Schaller, J.T. Sharp, R.L. Wilder, G.G. Hunder, The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis, *Arthritis Rheum.* 31 (1988) 315–324, <https://doi.org/10.1002/art.1780310302>.
- [40] A. Kokotkiewicz, M. Luczkiewicz, J. Pawlowska, P. Luczkiewicz, P. Sowinski, J. Witkowski, E. Bryl, A. Bucinski, Isolation of xanthone and benzophenone derivatives from *Cyclopia genistoides* (L.) vent. (honeybush) and their pro-apoptotic activity on synoviocytes from patients with rheumatoid arthritis, *Fitoterapia* 90 (2013) 199–208, <https://doi.org/10.1016/j.fitote.2013.07.020>.
- [41] I. Henc, A. Kokotkiewicz, P. Luczkiewicz, E. Bryl, M. Luczkiewicz, J.M. Witkowski, Naturally occurring xanthone and benzophenone derivatives exert significant anti-proliferative and proapoptotic effects in vitro on synovial fibroblasts and macrophages from rheumatoid arthritis patients, *Int. Immunopharmacol.* 49 (2017) 148–154, <https://doi.org/10.1016/j.intimp.2017.05.034>.
- [42] A.C. Figueiredo, J.G. Barroso, L.G. Pedro, J.J.C. Scheffer, Factors affecting secondary metabolite production in plants: volatile components and essential oils, *Flavour Fragrance J.* 23 (2008) 213–226, <https://doi.org/10.1002/ffj.1875>.
- [43] A.N. Shikov, O.N. Pozharitskaya, V.G. Makarov, H. Wagner, R. Verpoorte, M. Heinrich, Medicinal Plants of the Russian Pharmacopoeia; their history and applications, *J. Ethnopharmacol.* 154 (2014) 481–536, <https://doi.org/10.1016/j.jep.2014.04.007>.
- [44] T.G.T. Jaenson, K. Palsson, A.-K. Borg-Karlson, Evaluation of Extracts and Oils of mosquito (Diptera: Culicidae) repellent plants from Sweden and Guinea-Bissau, *J. Med. Entomol.* 43 (2006) 113–119, <https://doi.org/10.1093/jmedent/43.1.113>.
- [45] T.A. Baldwin, Effects of Resource Limitation on the Essential Oilproduction in *Ledum palustre* ssp. *Decumbens* (Ait.) Hultén, FIU Electronic Theses and Dissertations, vol. 1382, Florida International University, Miami, 2003, <https://doi.org/10.25148/etd.FI14050419>.
- [46] M. von Schantz, R. Hiltunen, Über die Zusammensetzung des ätherischen Öles von *Ledum palustre* L. einschließlich der geographischen Rassen ssp. *groenlandicum* (Oeder) Hult. und ssp. *decumbens* (Ait.) hult., *Sci. Pharm.* 39 (1971) 137–146.
- [47] F. Belleau, G. Collin, Composition of the essential oil of *Ledum groenlandicum*, *Phytochemistry* 33 (1993) 117–121, [https://doi.org/10.1016/0031-9422\(93\)85406-H](https://doi.org/10.1016/0031-9422(93)85406-H).
- [48] B.B. Aggarwall, W. Yuan, S. Li, S.C. Gupta, Curcumin-free turmeric exhibits anti-inflammatory and anticancer activities: identification of novel components of turmeric, *Mol. Nutr. Food Res.* 57 (2013) 1529–1542, <https://doi.org/10.1002/mnfr.201200838>.
- [49] R.F. van Vollenhoven, Sex differences in rheumatoid arthritis: more than meets the eye, *BMC Med.* 7 (2009) 12, <https://doi.org/10.1186/1741-7015-7-12>.
- [50] A. Jura-Poltorak, K. Olczyk, Diagnostics and assessment of rheumatoid arthritis activity, *J. Lab. Diagn.* 47 (2011) 431–438.
- [51] A. Skapenko, J. Leipe, P.E. Lipsky, H. Schulze-Koops, The role of the T cell in auto-immune inflammation, *Arthritis Res. Ther.* 7 (2005) S4–S14, <https://doi.org/10.1186/ar1703>.
- [52] J. Pawlowska, A. Mikosik, M. Soroczynska-Cybula, A. Jozwik, P. Luczkiewicz, S. Mazurkiewicz, A. Lorzczynski, J.M. Witkowski, E. Bryl, Different distribution of CD4 and CD8 T cells in synovial membrane and peripheral blood of rheumatoid arthritis and osteoarthritis patients, *Folia Histochem. Cytobiol.* 47 (2009) 1–6, <https://doi.org/10.2478/v10042-009-0117-9>.
- [53] N.A. Spiridonov, D.A. Kononov, V.V. Arkhipov, Cytotoxicity of some Russian ethnomedicinal plants and plant compounds, *Phytother. Res.* 19 (2005) 428–432, <https://doi.org/10.1002/ptr.1616>.
- [54] E.A. Goun, V.M. Petrichenko, S.U. Solodnikov, T.V. Suhinina, M.A. Kline, G. Cunningham, C. Nguyen, H. Miles, Anticancer and antithrombin activity of Russian plants, *J. Ethnopharmacol.* 81 (2002) 337–342, [https://doi.org/10.1016/S0378-8741\(02\)00116-2](https://doi.org/10.1016/S0378-8741(02)00116-2).
- [55] C.M. McGill, P.L. Tomco, R.M. Ondrasik, K.C. Belknap, G.K. Dwyer, D.J. Quinlan, T.A. Kircher, C.P. Andam, T.J. Brown, D.F. Claxton, B.M. Barth, Therapeutic effect of Northern Labrador tea extracts for acute myeloid leukemia, *Phytother. Res.* 32 (2018) 1636–1641, <https://doi.org/10.1002/ptr.6091>.