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Essential oil composition and *in vitro* biological activity of *Achillea millefolium* L. extracts

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Abstract: The *Achillea* genus is one of the widely used genera to treat various medical ailments. In this study, gas chromatography (GS) and Solid Phase Microextraction (SPME) were used to determine the essential oil composition of the *A. millefolium* L. Human skin fibroblasts (HSF) viability based on spectrophotometrical 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Neutral Red (NR) methods and morphological analysis was performed in *in vitro* cell culture. Free radical scavenging activity of ethanol, ethyl acetate and water extracts of *A. millefolium* L. was also measured. The total oil content in *Achillea* flowers was analyzed only with gas chromatography/mass spectrometry (GC/MS) method. SPME-GC/MS technique revealed that *Achillea* flowers liberate high amounts of monoterpenes (α-pinene; β-pinene; 1,8-cineole). However, proportionally, most of all sesquiterpenes (β-caryophyllene and germacrene D) were found. We also showed that fresh flowers contain much more monoterpenes such as α-pinene or sabinene but fewer amounts of β-pinene than preserved material. MTT analysis indicated that methanol extract from fresh, not dried, plant material and water extract from fresh plants at doses ranging from 25-225 μg/mL had no cytotoxic activity on HSF cells. Methanol extract from dried and freeze herb (doses >75 μg/mL) and ethyl acetate extract from fresh plants (doses >25 μg/mL) inhibited succinyl dehydrogenase activity. In the NR assay, only methanol extract from fresh plant material had no cytotoxic activity. The remainder significantly decreased dye uptake. Moreover, ethyl acetate extract strongly influenced cellular actin filaments composition, and consequently morphology of cells. Analysis of the IC_{50} values revealed that for methanol from fresh herb and water extracts, the IC_{50} values are higher than 250 μg/mL. The lowest IC_{50} was 13 μg/mL, obtained in NR assay for ethyl acetate extract. The highest free radical scavenging activity (1,1-diphenyl-2-picrylhydrazyl (DPPH) method) was found for methanol extract from dried herb at the maximal dose used (175 μg/mL), and was 19.2% higher than control value. When compared to the Trolox standard activity it was equivalent of the 6.8 μg/mL. In conclusion, the results suggest that *A. millefolium* L. extracts may, in limited concentrations, be useful in preparations of topical application and considered for use in clinical dermatology.

Key words: *Achillea millefolium* L., human skin fibroblasts, toxicity, reactive oxygen species, chromatography, Solid Phase Microextraction (SPME)

INTRODUCTION

There is increasing interest in finding natural, biologically active substances from plant material to replace synthetic drugs in the healing of skin wounds in humans. Plants possess many phytochemicals with proved bioactivities, e.g. anti-inflammatory, antioxidant, cytostatic or cytotoxic. The most important genus in the Compositae family (Asteraceae) are the *Achillea* species. The herb *Achillea* is a genous with more than 100 species worldwide [1]. These plants are native to Europe, North America, South Australia and Asia [2]. *A. millefolium* L., known as yarrow, is a perennial herb that has been widely used in folk medicine in many countries [3]. It possesses strong antioxidative, antimicrobial, anticancer, anti-inflammatory or liver protective activities [1, 4]. Therefore, the most popular application of this herb include the healing of skin wounds, menstrual regulation or inflammation, pain, flatulence, dyspepsia, spasmodic diseases and haemorrhages treatment [2, 4]. Phytochemical investigations revealed that such an extensive biological activity of *A. millefolium* L. is related to bioactive components that could be found in this herb. The main constituents of *A. millefolium* L. are essential oils (borneol, chamazulene, α- and β-pinenes, trans-nerolidol, α-thujene, β-myrcene, 1,8-cineole, linalool, geraniol or α-bisabolol), sesquiterpenic lactones and flavonoids (apigenin, luteolin or rutin) [1, 5, 6]. However, the ratio of the components and therefore their health protecting effects are closely associated with geographical and environmental conditions, e.g. a mountainous setting, have a profound effect on the composition of the essential oils.

The aim of this study was to evaluate the essential oil composition and *in vitro* cytotoxic, cytostatic and free radical scavenging properties of methanol and ethyl acetate extracts of *A. millefolium* L. (Asteraceae), collected from the Lublin Region of eastern Poland.

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MATERIALS AND METHODS

Gas chromatography connected with mass spectrometry (GC/MS) analysis and Solid Phase Microextraction (SPME) analytical procedure. The essential oil from *A. millefolium* L. was obtained by means of hydrodistilation with m-xylene in a Derynge apparatus for 3h. The composition of the oil was analyzed using the methods of gas chromatography (GC) and gas chromatography connected with mass spectrometry (GC/MS)

GC/MS analyses were carried out using a GC/MS GCQ (Thermo-Finnigan, USA) fitted with RT-5 (Resteck) capillary column (20 m length, 0.18 mm ID) with 0.2 μm film thickness. A split injection with a ratio 1:50 was used and the volume of injected sample was 1 μL. The initial temperature was 50°C, maintained for 1 min, heating rate 4°C, and final temperature at 280°C.

SPME: The initial temperature was 30°C, maintained for 3 min, heating rate 8°C, and final temperature at 250°C.

The mass spectrometer was fitted with EI source-operated 70 eV, and mass spectra were recorded in the range m/z 35-400 a.m.u. in the full-scan acquisition mode. Identification was based on comparison of obtained mass spectra with those of authentic standards and with spectra of the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) libraries.

Plant enzymes analysis. The selected 17 enzymes, presence and activity existing in *A. millefolium* L. flowers were analyzed using the API ZYM system (Biomèrieux, France). In the study, water solutions of fresh juices were used. Briefly, the juice of freshly collected plant material was diluted 1:10 and 1:100 with aseptic, distilled water. The solutions were filtrated with sterile Acrodisc 0.2 μm (Gelman Sciences) and introduced (65 μL) into microwells containing dehydrated substrates for appropriate enzymes (Table 4). After 4 h incubation at 37°C, one drop of ZYM A reagent (Tris-hydroxymethylaminomethane, 37% hydrochloric acid, sodium lauryl sulphate and water) and one drop of ZYM B (Fast blue BB and 2-methoxyethanol) were added to each well to develop a colour reaction. After 5 min the results were read by comparing them with the standard specimen colour scale sheet enclosed by the manufacturer. As the negative control sample, aseptic distilled water was used. The results were presented as the quantity of hydrolysed substrate (μM/min.).

Table 4 Enzymatic activity of water solutions of fresh juices from *A. millefolium* L.

No.	Enzyme assayed	Water juice solution (quantity of hydrolyzed substrate in μM/min.)	
		A. millefolium L. flowers	
		1 : 10	1 : 100
0	Control	0	0
1	Alkaline phosphatase	>40	10
2	Esterase (C4)	40	30
3	Esterase lipase (C8)	10	5
4	Lipase (C14)	0	0
5	Leucine arylamidase	20	5
6	Valine arylamidase	0	0
7	Cystine arylamidase	0	0
8	Acidic phosphatase	>40	>40
9	Naphthol-AS-BI-phosphohydrolase	>40	>40
10	α-Galactosidase	30	10
11	β-Galactosidase	30	10
12	β-Glucuronidase	0	0
13	α-Glucosidase	0	0
14	β-Glucosidase	0	0
15	*N*-acetyl-β-glucosaminidase	>40	20
16	α-Mannosidase	30	10
17	α-Fucosidase	0	0

BIOLOGICAL EXPERIMENTS

Establishment of human skin fibroblasts (HSF) cell culture. Freshly excised fragments of human skin were washed twice using RPMI (1640) medium (Gibco™, Paisley, UK) supplemented with antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B) (Gibco), and then placed into wells of 24-well plate. The explants were then overlaid with a warm 1:1 (v/v) mixture of 1% agarose and RPMI 1640 medium. The culture was performed by adding culture medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco) on top of agarose gel, and incubated at 37°C in a humidified 5% CO_2/95% air incubator. Outgrowths of skin fibroblasts were separated and cultured. For experiments, HSF cells obtained from 2 donors were used.

Skin fibroblasts culture. The HSF cells were cultured as monolayers in 25 cm^2 culture flasks (Nunc. Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% FBS (v/v) and antibiotics at 37°C in a humidified atmosphere with 5% CO_2.

For the experiments, the total number of cells was estimated by counting in a haemocytometer. A 100 μL of cell suspension (2×10^4 cells/mL for cells proliferation activity tests or 1×10^5 cells/mL for toxicity tests) was added to appropriate wells of 96-well flat-bottomed microtitre plates (MTT and NR methods). After 24 h of incubation, the medium was discarded and fresh one containing 2% FBS and appropriate plant extract concentrations in 25-225 μg/mL range was added. As a control, HSF cells suspended in 100 μL of culture medium with 2% FBS without plant extracts was used. The total cell number was equivalent to that in the sample wells. Additional controls without cells but containing appropriate plant extract concentrations in 2% FBS culture medium were prepared to exclude non-specific dye reduction (MTT method). As blank control, culture medium with 2% FBS was used.

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Sensitivity of cells to *A. millefolium* L. extracts activity was determined by a standard spectrophotometric MTT assay. Cells grown in 96-well multiplates in 100 μL of culture medium supplemented with 2% FBS were incubated for 3 h with MTT solution (5 mg/mL, 25 μL/well) (Sigma, St. Louis, MO, USA). The yellow tetrazolium salt was metabolized by viable cells to purple crystals of formazan. The crystals were solubilized overnight in a mixture consisting of 10% sodium dodecyl sulfate (SDS) (Sigma) in 0.01 M HCl. The product was quantified spectrophotometrically by absorbance measurement at 570 nm wavelength using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Neutral red (NR) uptake assay. Cells were grown in 96-well multiplates in 100 μL of culture medium (RPMI 1640) supplemented with 2% FBS and various concentrations of

A. millefolium L. extracts (25-225 μg/mL). Subsequently, the medium was discarded and 0.4% NR (Sigma) solution in 2% FBS medium was added to each well. The plate was incubated for 3 h at 37°C in a humidified 5% CO_2/95% air incubator. After incubation, the dye-containing medium was removed, cells fixed with 1% $CaCl_2$ in 4% paraformaldehyde, and thereafter the incorporated dye was solubilized using 1% acetic acetate in 50% ethanol solution (100 μL). The plates were gently shacken for 20 min at room temperature and the extracted dye absorbance was spectrophotometrically measured at 540 nm.

1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radical scavenging test. Free radical scavenging activity of methanol and ethyl acetate extracts of *A. millefolium* L. was measured by the DPPH• assay. This method is based on the ability of antioxidants to reduce the stable dark violet radical DPPH• (Sigma) to the yellow coloured diphenyl-picrylhydrazine. Briefly, 100 μL of DPPH• solution (0.2 mg/mL in ethanol) was added to 100 μL of different plant extract solution concentrations (25-175 μg/mL) and standards. Trolox (Sigma) at increasing concentrations (1-50 μg/mL) was used as a reference for the free radical scavenging activity. After 15 min of incubation at room temperature, the absorbance of the solution was measured at 515 nm. The lower the absorbance, the higher free radical scavenging activity of the plant extracts. The activity of each extract was determined by comparing its absorbance with that of a blank solution (reagents without plant extracts) and standard.

The capability to scavenge DPPH• radical was calculated by the following formula:

DPPH• scavenging effect (%) = $[(X_{control} - X_{extract}/X_{control}) \times 100]$. $X_{control}$ is the absorbance of the control and $X_{extract}$ is the absorbance in the presence of plant extract [7].

Labelling of cytoskeleton F-actin. Cells were incubated in 4-well Lab-Tek chamber slides in 1 mL of culture medium supplemented with 2% FBS and plant extracts. After incubation, cells were rinsed with RPMI 1640 medium and exposed to paraformaldehyde (10%, v/v) solution for 20 min, rinsed 3 times with PBS, exposed to Triton X-100 (0.2%, v/v) solution for 5 min, and rinsed 3 more times with PBS. 0.5 mL PBS containing tetra-methyl-rhodamine-isothiocyanate-phalloidin (TRITC-phalloidin, 1 μg/mL) (Sigma) was added to each well. Incubation in the dark at 37^0C/5% CO_2 for 30 min was carrid out. Observation of the cells was conducted under a fluorescence microscope (Olympus, BX51). Quantitative analysis of fluorescent images was performed by AnalySIS imaging software system.

Argyrophilic nucleolar organizer regions (AgNORs) staining. After incubation in 4-well Lab-Tek II Chamber slides, cells were rinsed with PBS and fixed with absolute ethanol/acetic acid solution (1:1) for 10 min. The silver colloid solution was prepared by 2% gelatin in 1% formic acid mixed 1:2 volumes with 30% aqueous silver nitrate. Cells were immersed in this solution for 5 min at 37°C, rinsed with deionised water and mounted with glycerol. AgNOR proteins were determined using a computer-assisted image analysis system.

Statistical analysis. The biological experiments were repeated 3 times. The results were expressed as means ± S.D and the difference tested by Student's t-test. P-values lower than 0.05 were considered statistically significant.

RESULTS

Preparations of *Achillea* species are traditionally used in folk medicine due to their wide spectrum of activities. Extracts of *A. millefolium* L., among others, are good scavengers of active oxygen radicals, and also express cytotoxic or antiproliferative properties. The cytotoxic and anti-proliferative activity of methanol and ethyl acetate extracts of *A. millefolium* L. were measured using Neutral Red (NR) uptake and MTT tests. The experiments with HSF cells seeded at a density of 2×10^4 cells/mL were carried out for 72 h (results read every 24 h) (anti-proliferative activity analysis) and with a density of 1×10^5 cells/mL conducted for only 24 h (cytotoxicity analysis). Two methanol extracts are described: methanol extract obtained from fresh, not dried, plant material and 'methanol old' extract from dried and freeze herb. Ethyl acetate and water extracts were prepared from fresh plant material.

The toxicity of the extracts on HSF cells are shown in Figs. 1a-1b. MTT analysis indicated that methanol and water extracts at doses ranging from 25-225 μg/mL have no cytotoxic activity on HSF cells. Methanol from preserved herb (doses >75 μg/mL) and ethyl acetate (doses >25 μg/mL) extracts inhibited succinyl dehydrogenase activity. The enzyme activity decreased at 225 μg/mL of extracts concentration to 49.2% ± 4.7 and 7% ± 2.5, respectively (Figure 1a). In the NR assay, only methanol extract had no cytotoxic activity. Water, methanol from preserved herb, and ethyl acetate extracts decreased dye uptake to 56.1% ± 11.6 at dose 225 μg/mL, 2.9% ± 2.2 at 175 μg/mL and 4.5% ± 2.6 at 25 μg/mL, respectively (Figure 1b).

IC_{50} values, indicating a concentration of *A. millefolium* L. extracts that inhibited viability of HSF cells by 50% are shown in Table 1. For methanol and water extracts, the IC_{50} values were higher then 250 μg/mL. The lowest IC_{50} was 13 μg/mL obtained in NR assay for ethyl acetate extract.

Figures 2a-c and 3a-c represent the antiproliferative activity of extracts. The MTT test revealed that methanol and water extracts expressed no inhibitory activity on HSF cells proliferation during 72 h of incubation. On the other hand, methanol from preserved plant and ethyl acetate extracts showed strong antiproliferative and cytotoxic activity which increased with the time of incubation. At extracts dose of 225 μg/mL and prolonged time of culture (>24 h), the entire cell population was killed. However, the most intensive antiproliferative activity of these extracts occurred up to 48 h of the experiment. Further incubation did not significantly change the viability of cells (Figures 2a-c). NR assay showed that all tested extracts inhibited HSF cells proliferation. The most potent activity was observed during 48 h of culture. Methanol from preserved herb and ethyl acetate extracts decreased viability of cells to 0% at dose 75 μg/mL. For water and methanol extracts, the viability at concentration

Table 1 A concentration of *A. millefolium* L. extracts that inhibited viability of human skin fibroblasts (HSF) (1×10^5 cells/mL) by 50% (IC_{50}).

Extract	Time of incubation (hours)	IC50 (μg/mL)	
		MTT	NR
Methanol	24	> 250	> 250
Methanol (preserved material)	24	220	80
Ethyl acetate	24	58	13
Water	24	> 250	249

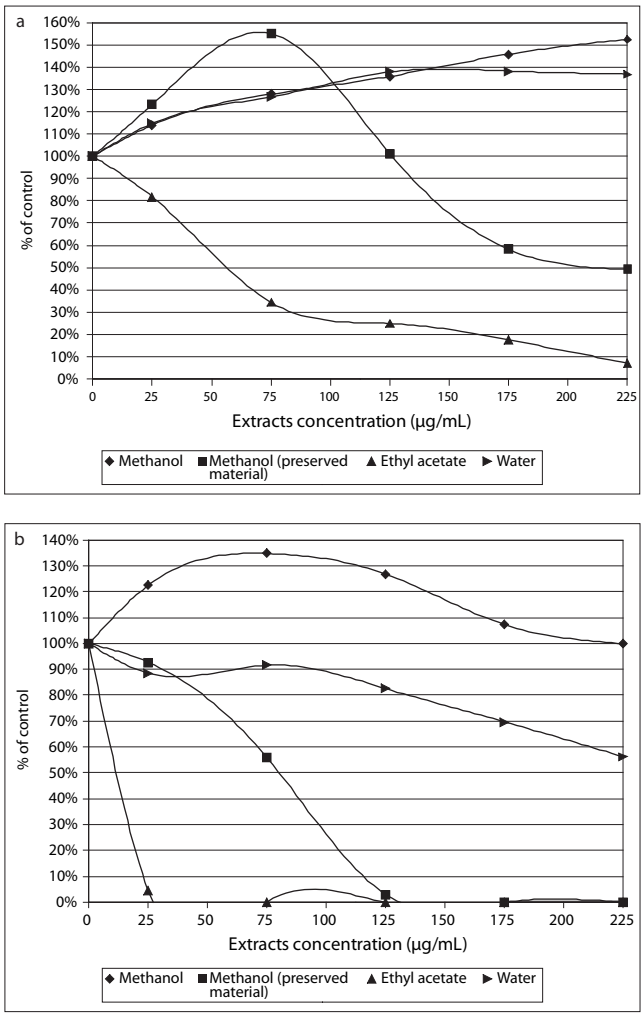

Figure 1 Effect of 24 h treatment of HSF cells (1×10^5 cells/mL) with fresh methanol, methanol, ethyl acetate and water extracts of *A. millefolium* L. The MTT assay (a) and Neutral Red assay (b). Results of the viability tests are expressed as a percentage of the control, arbitrarily set at 100%. The figure shows the average of three independent, representative experiments.

225 µg/mL was 52.5% ± 9.8 and 32.8% ± 4.9, respectively. After a further 24 h of incubation (72 h of the experiment), no significant change in dye uptake was observed (Figs. 3a-c).

Table 2 shows IC_{50} values obtained in MTT and NR assays. Generally, the IC_{50} concentrations obtained in MTT assay were higher than in the NR method.

A. millefolium L. extracts have also been analyzed for their free radical scavenging activity, using the DPPH test. The highest scavenging activity was found for methanol extract from preserved material at maximal dose used (175 µg/mL) and was 19.2% higher than control value (Table 3). When compared to the Trolox standard activity it was the equivalent of 6.8 µg/mL of that synthetic vitamin concentration. The lowest scavenging effect (6.17% higher then control) was obtained for ethyl acetate extract (Table 3). This was equivalent to 2.2 µg/mL of the Trolox activity.

F-actin cytoskeleton organization and AgNORs deposits were analyzed at 25 µg/mL of each extract concentration.

F-actin filaments organization was analyzed using TRITC-phalloidine fluorescent staining. Control cells are presented in Figure 4. After 24 h incubation of HSF cells with methanol (Figure 4a), methanol from preserved herb (Figure 4b)

Table 2 A concentration of *A. millefolium* L. extracts that inhibited viability of human skin fibroblasts (HSF) (2x10^4 cells/mL) by 50% (IC_{50}).

Extract	Time of incubation (hours)	IC_{50} (µg/mL)	
		MTT	NR
Methanol	24	> 250	> 250
	48	> 250	183
	72	> 250	164
Methanol (preserved material)	24	211	94
	48	140	45
	72	130	47
Ethyl acetate	24	47	16
	48	28	13
	72	22	13
Water	24	> 250	> 250
	48	> 250	225
	72	> 250	246

and water (Figure 4d) extracts there were no cytoskeleton organization changes when compared to control. Ethyl acetate extract strongly influenced the viability of the cells, and therefore morphology, and in consequence cellular actin

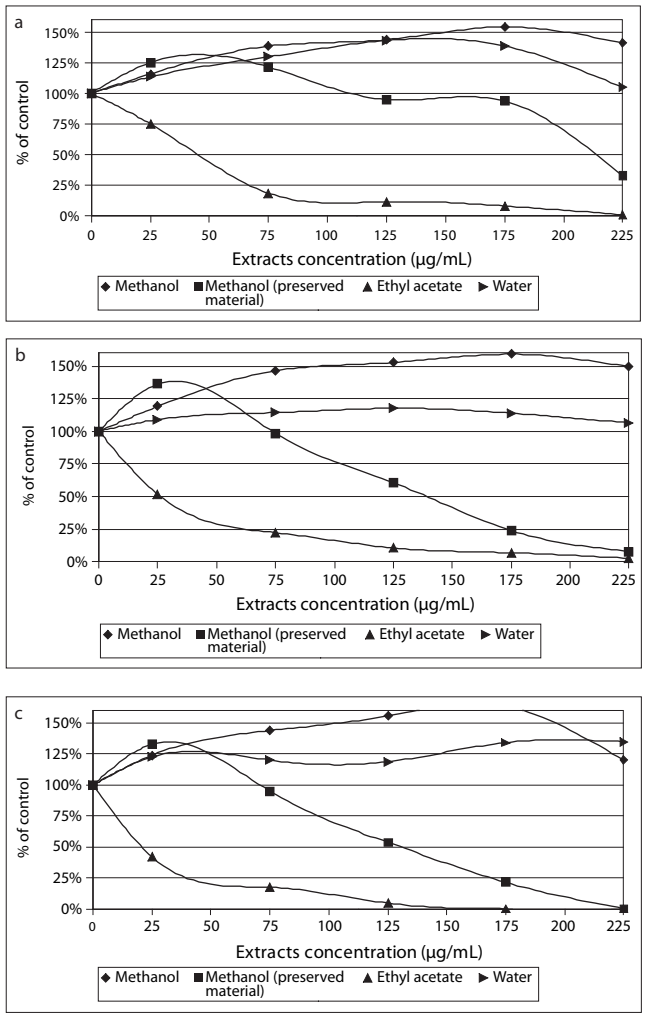

Figure 2 Antiproliferative, dose-dependent effect of fresh methanol, methanol, ethyl acetate and water extracts of *A. millefolium* L. on HSF cells (2 × 10^4 cells/mL) after 24 h (a), 48 h (b) and 72 h (c) of growth. MTT assay. Results of the viability tests are expressed as a percentage of the control, arbitrarily set at 100%. The figure shows the average of three independent, representative experiments.

Table 3 DPPH scavenging effect (%). The % of reduced DPPH· radical by *A. millefolium* L. extracts is compared to the control (0% of reduction).

Extract concentration (µg/mL)	25	75	125	175
Extract	n=3 ±S.D.	n=3 ±S.D.	n=3 ±S.D.	n=3 ±S.D.
Methanol	3.46±0.49	7.41±0.37	12.22±0.74	16.11±0.43
Methanol (preserved material)	3.58±0.49	9.2±0.43	13.95±0.49	19.2±0.62
Ethyl acetate	2.59±0.8	3.89±0.31	5.56±1.17	6.17±0.25
Water	1.3±0.49	5.12±0.19	11.05±0.8	14.38±0.74

filaments composition was also to a large extent destroyed (Figure 4c).

In control cells, there were many interphase AgNORs deposits (Figure 5). Methanol (Figure 5a), methanol from preserved plant (Figure 5b) and water (Figure 5d) extracts did not influence the number and size of interphase NORs. Ethyl acetate extract after 24 h of incubation destroyed HSF cells; however, some nucleolin debris could be observed (Figure 5c).

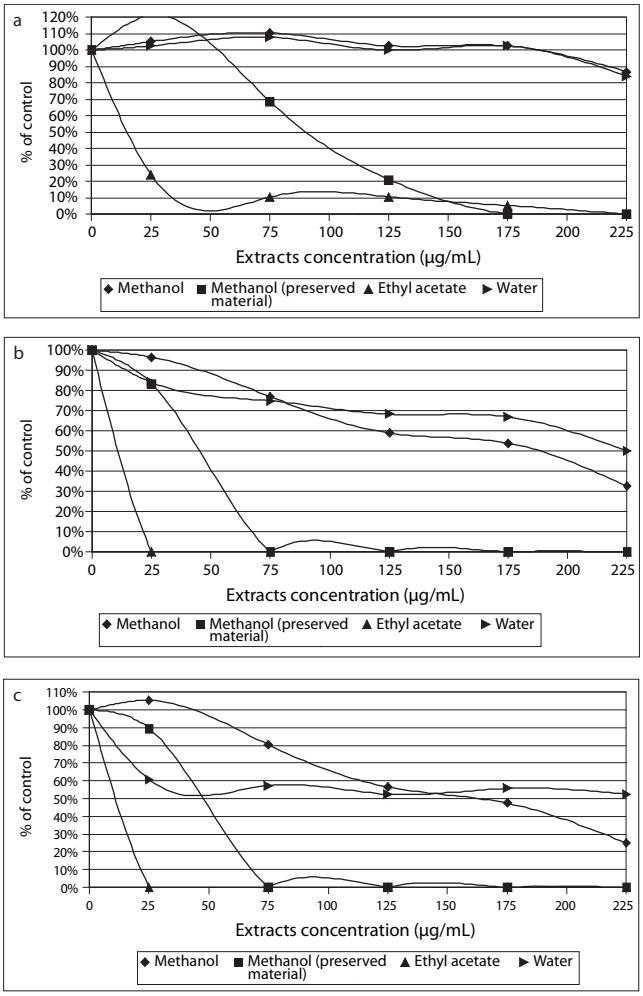

Figure 3 Antiproliferative, dose-dependent effect of fresh methanol, methanol, ethyl acetate and water extracts of *A. millefolium* L. on HSF cells (2×10^4 cells/mL) after 24 h (a), 48 h (b) and 72 h (c) of growth. Neutral Red (NR) assay. Results of the viability tests are expressed as a percentage of the control, arbitrarily set at 100%. The figure shows the average of three independent, representative experiments.

Table 5 The composition of the essential oil of *A. millefolium* L. Samples were analyzed using SPME method.

No.	Compound	Retention index	Retention time (min.)	Quantity [%]
1	α-pinene	940	9.00	7.1
2	β-pinene	981	9.93	3.7
3	1,8-cineole	1038	11.13	6.6
4	cis-sabinene hydrate	1075	-	-
5	β-linalool	1107	-	-
6	trans-pinocarveol	1152	-	-
7	camphor	1155	13.45	2.1
8	α-copaene	1387	17.52	1.1
9	β-elemene	1401	17.76	3.6
10	β-caryophyllene	1434	18.27	11.1
11	trans-α-Bergamotene	1444	18.43	3.6
12	α-humulene	1469	18.82	1.9
13	γ-muurolene	1477	18.95	3.3
14	γ-gurjunene	1489	19.13	1.9
15	germacrene D	1496	19.24	18.2
16	γ-cadinene	1528	19.73	1.4
17	δ-cadinene	1536	19.85	1.2
18	(E)-nerolidol	1570	20.36	1.0
19	caryophyllene oxide	-	20.84	2.4
20	viridiflorol	-	20.97	3.5
21	C16	1599	20.78	1.9
22	C17	-	21.46	1.7
23	unknown	-	21.60	3.2
24	unknown	-	21.87	1.7
25	unknown	-	22.19	2.2
26	unknown	-	22.26	3.0
27	C20	-	23.52	1.6

HPTLC and HPLC analysis combined with densitometry revealed that methanol extracts contain phenolic acids and flavonoides. In ethyl acetate extract, mainly phenolic acids and karotenoides were found.

The activity of the 17 enzymes, analyzed in *A. millefolium* L. with API ZYM system, is shown in Table 4. The highest activity (even at 1:100 dilution) was observed for acidic phosphatase and naphthol-AS-BI-phosphohydrolase (>40 µM). High activities (dilution 1:10) which decreased at dilution 1:100 were obtained for alkaline phosphatase, esterase (C4) and *N*-acetyl-β-glucosaminidase. Lower but detectable activities were found for esterase lipase (C8), leucine arylamidase, α- and β-galactosidase and α-mannosidase. The activity of other tested enzymes was not detectable.

The analysis of volatile essential oils was performed by using a combination of solid-phase microextraction (SPME) with gas chromatography (GC) and mass spectrometry (MS). Moreover, the total oil content in *Achillea* flowers was analyzed only with GC/MS method. SPME-GC/MS technique revealed that *Achillea* flowers liberate high amounts of monoterpenes (α-pinene; β-pinene; 1,8-cineole). However, proportionally, most of all sesquiterpenes (β-caryophyllene and germacrene D) were found. There were also unknown n-alkans with n=16, 17 and 18 carbon atoms, eluated (Table 5). The GC/MS analysis of the total essential oil content was performed with fresh *Achillea* flowers (flowers II), collected in 2007 and stored for one year (collection 2006) (flowers I). We showed that fresh flowers contain much more monoterpenes, such as α-pinene or sabinene, but less amounts of β-pinene than preserved material. Flowers I were also proportionally abundant with sesquiterpenes, such as β-caryophyllene and its oxide and (E)-nerolidol. There were also found high amounts of chamuzalen, which was not detected in flowers II (Table 6).

Figure 4

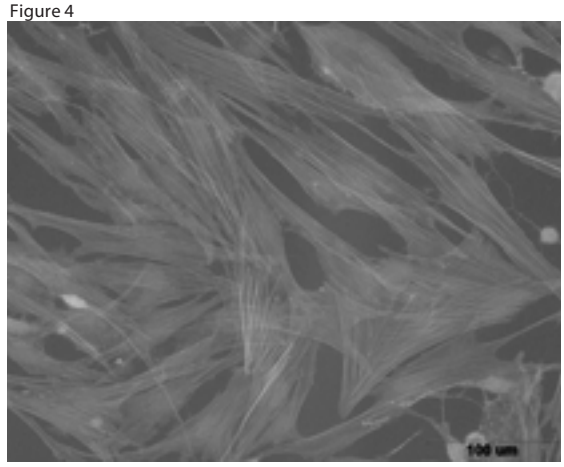

Figure 4a

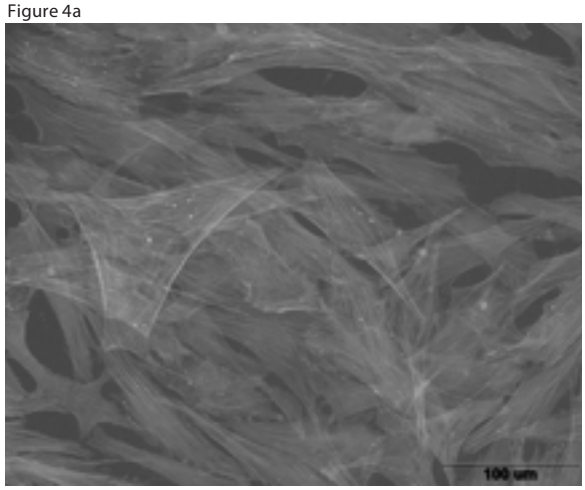

Figure 4b

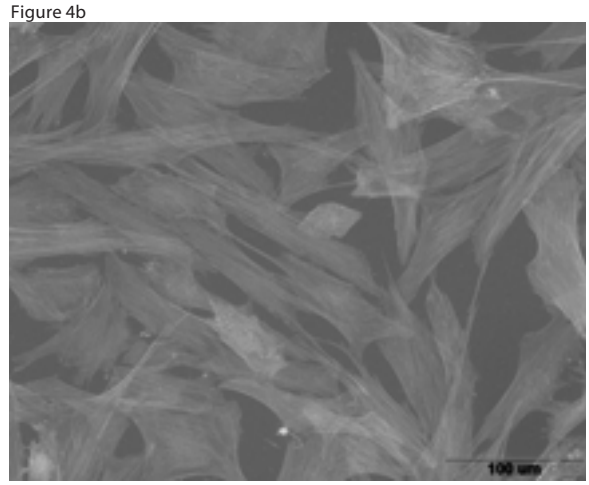

Figure 4c

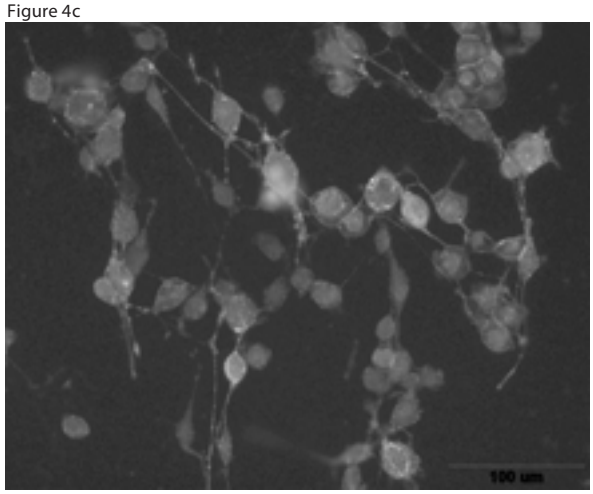

Figure 4d

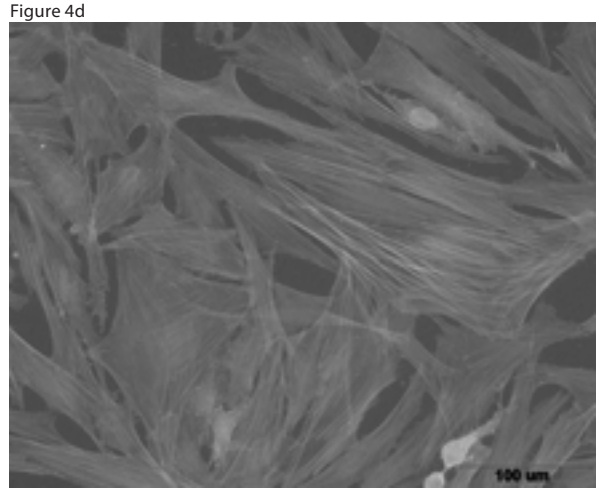

Figure 4 Cytoskeleton organization in HSF cells. Control sample. TRITC-phalloidin fluorescent staining. Bar 100 µm. 24 h of incubation of HSF cells with fresh methanol, (4a), methanol (4b) and water (4d) extracts of *A. millefolium* L. brought no cytoskeleton organization changes when compared to control. Ethyl acetate extract strongly influenced cells morphology, and composition of cellular actin filaments was seriously destroyed (4c). Bar 100 µm.

Figure 5

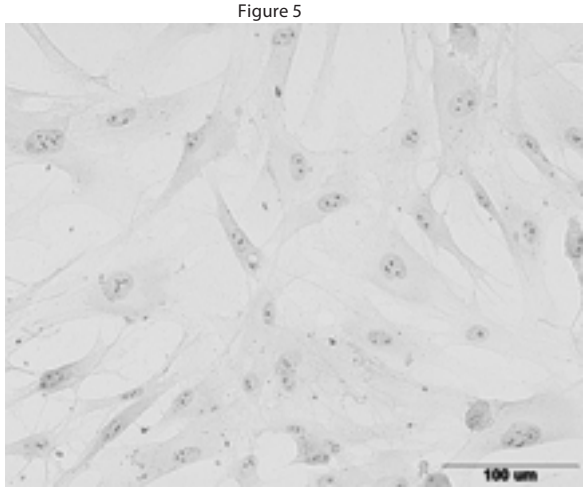

Figure 5a

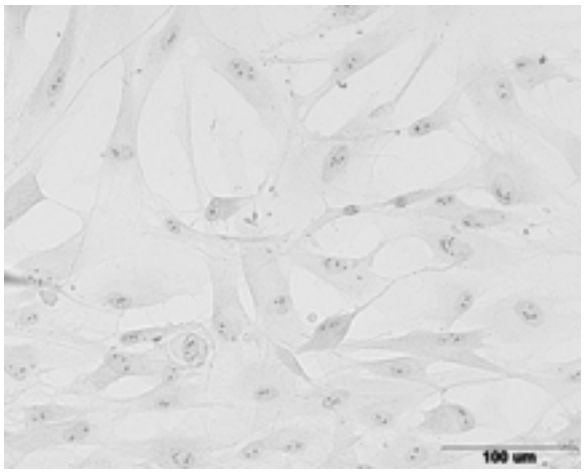

Figure 5b

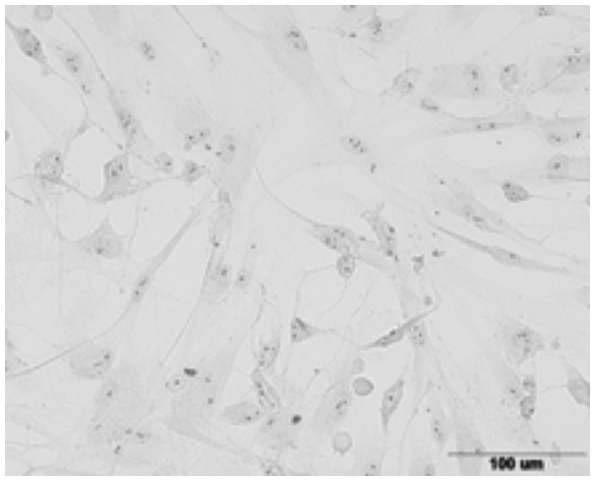

Figure 5c

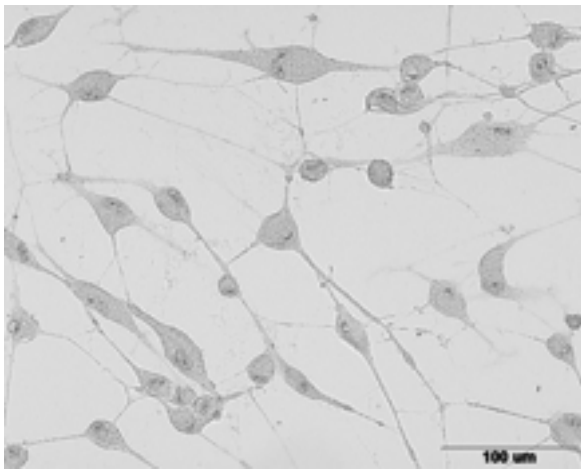

Figure 5d

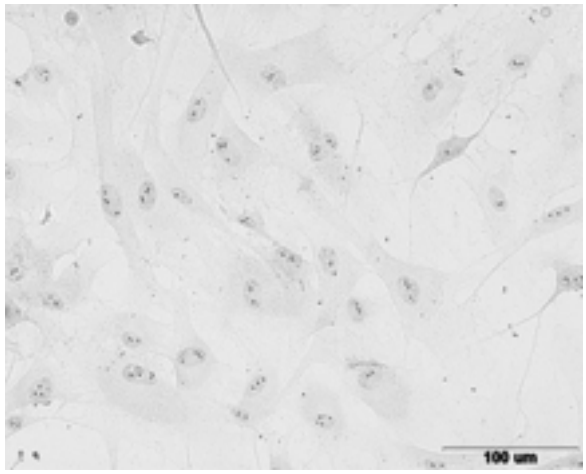

Figure 5 Nuclei of HSF cells silver-stained for the AgNOR proteins. Control sample. Bar 200 µm. Many interphase AgNORs deposits can be seen. Fresh methanol (5a), methanol (5b) and water (5d) extracts of *A. millefolium* L. did not influence the number and size of interphase NORs. Ethyl acetate extract destroyed HSF cells; however, some nucleolin debris can be observed (5c).

DISCUSSION

Naturally occurring substances in higher plants have been recognized as having many effects on biological systems, e.g. cytotoxic, antiproliferative, antioxidant or free radical scavenging activities. In the presented work, methanol, ethyl acetate and water extracts of *A. millefolium* L. flowers harvested from the Lublin Region of eastern Poland were analyzed. Cytotoxic and antiproliferative activities were tested using 2 methods with different specificity profiles: MTT (succinyl dehydrogenase activity analysis in living cells) and neutral red (NR) (measuring the dye uptake by functional lysosomes of living cells). The results of both methods were comparable in general activity, but in NR assay the cytotoxic and antiproliferative effects of the extracts were stronger than those obtained in the MTT test. This may suggest that the profound effect of plant extracts was directed at the lysosomal membranes as the general main site of action of many toxic substances, and consequently change the viability of the cells, and to a lesser extent the metabolism (mitochondrial activity) of HSF cells. This observation results from the specific features of the substances comprising the plant extracts. There are many compounds with high biological activities, e.g. terpenes, flavonoids and essential oils.

Substances of plant origin are in widespread use in many products of topical application. Therefore, there should be extensive research on their precutaneous absorption and toxicity. This report presents the analysis of *A. millefolium* L. composition and potential biological activity of the plant compounds.

Table 6 The composition of the essential oil of *A. millefolium* L. Samples were analyzed using GC/MS method.
flowers I – one year stored *Achillea* flowers, flowers II – fresh *Achillea* flowers.

Plant material				Flowers I	Flowers II
No.	Compound	Retention index	Retention time (min.)	Quantity [%]	Quantity [%]
1	α-thujene	928	6.25	0.3	0.3
2	α-pinene	934	6.42	3.8	18.5
3	camphene	948	6.84	0.8	1.6
4	2,4(10)-thujadiene	955	7.02	0.0	0.0
5	sabinene	974	7.58	2.9	8.8
6	β-pinene	978	7.69	13.2	6.1
7	2,3-dehydro-1,8-cineole	987	7.94	-	0.4
8	β-myrcene	993	8.11	0.3	-
9	2-pentyl-furan	993	8.11	0.2	-
10	α-terpinene	1018	8.91	0.3	0.4
11	p-cymene	1026	9.18	0.5	0.2
12	limonene	1029	9.30	0.4	0.3
13	1,8-cineole	1032	9.40	15.6	13.7
14	(Z)-β-ocimene	1039	9.62	0.1	0.2
15	(E)-β-ocimene	1019	8.94	-	0.2
16	phenylacetaldehyde	1049	9.96	0.2	-
17	γ-terpinene	1059	10.30	0.6	0.9
18	cis-sabinene hydrate	1069	10.62	0.1	0.4
19	trans-sabinene hydrate	1101	11.69	-	0.3
20	β-linalool	1102	11.72	0.3	0.1
21	hotrienol	1108	11.92	-	0.2
22	α-thujone	1108	11.94	-	-
23	β-thujone	1119	12.29	-	-
24	α-campholenal	1130	12.68	0.1	0.3
25	trans-pinocarveol	1141	13.05	0.6	1.4
26	camphor	1146	13.23	3.7	4.3
27	pinocarvone	1165	13.88	0.7	1.9
28	borneol	1168	14.00	2.2	3.3
29	p-mentha-1,5-dien-8-ol	1170	14.05	0.5	0.3
30	menthol	1176	14.25	-	-
31	terpinen-4-ol	1179	14.38	1.4	1.9
32	p-cymen-8-ol	1190	14.74	0.0	-
33	α-terpineol	1194	14.87	2.8	1.3
34	myrtenal	1200	15.08	0.4	1.3
35	verbenone	1214	15.56	0.1	0.1
36	piperitone	1259	17.07	0.3	-
37	cis-chrysanthenyl acetate	1264	17.25	-	0.2
38	bornyl acetate	1288	18.05	0.8	1.2
39	geranyl formate	1293	18.23	0.1	-
40	thymol	1299	18.43	0.1	-
41	eugenol	1363	20.51	0.2	0.1
42	α-copaene	1377	20.98	0.2	0.1
43	decanoic acid	1385	21.22	1.0	-
44	β-bourbonene	1388	21.31	0.2	0.1
45	β-elemene	1401	-	-	-
46	β-caryophyllene	1422	22.40	6.3	1.1
47	β-copaene	1431	22.68	0.1	0.0
48	trans-α-Bergamotene	1444	-	-	-
49	α-humulene	1456	23.45	0.9	0.2
50	γ-muurolene	1480	24.19	0.2	-
51	γ-gurjunene	1489	-	-	-
52	germacrene D	1484	24.31	2.2	3.0
53	γ-humulene	1484	24.31	-	0.5
54	ar-curcumene	1486	24.36	0.0	-
55	epi-cubebol	1498	24.73	0.2	0.2
56	α-muurolene	1503	24.90	0.1	0.1
57	γ-cadinene	1528	-	-	-
58	δ-cadinene	1527	25.59	0.6	0.8
59	(E)-nerolidol	1567	26.78	4.1	0.1
60	germacrene-D-4-ol	1581	27.19	-	0.5
61	spathulenol	1584	27.26	0.8	0.4
62	caryophyllene oxide	1589	27.41	3.9	1.3
63	viridiflorol	1597	27.66	0.8	0.6
64	β-oplopenone	1616	28.18	-	1.0
65	C16	1600	-	-	-
66	humulene epoxide II = 1,2-humulenepoxide	1615	28.15	0.5	-
67	γ-eudesmol +cis-asarone	1629	28.56	1.1	-
68	γ-eudesmol	1630	28.57	-	0.1
69	unknown	1644	28.96	0.4	0.2
70	epi-α-cadinol = τ-cadinol	1647	29.05	0.7	2.4
71	α-cadinol	1661	29.45	1.8	2.3
72	unknown	1679	29.94	0.4	-
73	unknown	1694	30.35	0.7	3.2
74	unknown	1697	30.45	-	1.2
75	C17	1700	-	-	-
76	pentadecanal	1717	30.98	0.2	-
77	chamazulene	1741	31.63	12.6	-
78	bisabolone isomer	1751	31.88	-	2.7
79	pentadecanone, 6,10,14-trimethyl- 2-; = hexahydrofarensyl acetone = phytone	1846	34.35	0.9	-
80	palmitic acid methyl ester	1927	36.38	0.1	-
81	C20	2000	-	-	-
82	linoleic acid methyl ester	2096	40.36	0.1	0.1
83	linolenic acid methyl ester	2103	40.51	-	0.1
84	C23	2298	44.74	0.4	0.2
85	C25	2498	48.75	0.2	0.2

The correctness of choosing human skin fibroblasts (HSF cells) for our work was supported by experiments by Dijoux et al., who showed that human keratinocytes appear to be less useful than fibroblasts for screening purposes [8].

The *Achillea* extracts are a rich source of terpenes. It is a well-known fact that this group of substances demonstrate strong biological activities in the form of anti-microbial, anti-parasitic, anti-viral, anti-fungal or immunomodulatory activities [9]. However, essential oils have also been shown to have a toxic effects on human cells. Hayes et al. demonstrated that lemon myrtle oil was toxic to human skin fibroblasts. They also found that the oil provoked significant loss in the cellular functioning of skin, especially loss of integrity, cellular necrosis or vacuolation, when compared to control skin [10]. We also obtained similar results of toxicity with ethyl acetate extracts of *Achillea* flowers. There were changes in cytoskeleton organization, its destruction, or significant alterations in cell morphology. These results may be due to the solvent effects of essential oils. Their hydrophobic compounds may exert a direct effect on cellular membranes, changing the phospholipid bilayer properties leading to disruption of cellular volume [11]. Moreover, phenolic compounds of essential oils influence hydrophobically cell membrane, trigger an oxygen burst, and in consequence make the bilayer unstable [12]. Moreover, camphor has been reported to have antioxidant activity. It also reduces the toxicity of phenol and parachlorophenol. However, Soekanto et al. revealed that camphor itself show cytotoxicty in cell cultures [13]. It is used in skin lotions for its analgesic and anti-pruritic properties. But when per oral overdosed, symptoms of toxicity occur rapidly and may lead even to coma and death caused of respiratory tract failures [14]. In our tests, camphor was one of the most dominant compound in *A. millefolium* L. flowers. Therefore, toxicity observed in cell cultures may be partially caused by camphor activity.

Reactive oxygen intermediates generated by metabolism of essential oils may also cause cytotoxic effects, cell injury and death. In our study, compounds of ethyl acetate extract exhibited the least free radical scavenging activity. Simultaneously, the highest cytotoxic effects were observed. Therefore, free radicals should be considered as one of the main causes of cell death after incubation with that extract. On the other hand, methanol extract from preserved herb possesses relatively higher radicals scavenging activity. Generally, the highest scavenger capacity can be found in polar extracts, such as the methanolic ones [15].

The GC/MS and SPME analyses of the essential oil in *A. millefolium* L. flowers led to identification of their major components (Tables 4 and 5). The composition was dominated by the presence of monoterpenes, a group of substances known for their antioxidative or anti-inflammatory activities. Lourens et al. revealed that extracts of the *Helichrysum* species contain mainly 1,8-cineole and α-pinene exert free radicals scavenging activity [16]. Moreover, caryophyllene, germacene-D and *Origanum vulgare* ssp. *Vulgare* plants were also found to have antioxidant activity [17]. In our study, α-pinene, β-pinene, 1,8-cineole, terpinen-4-ol, α-terpineol, borneol, (E)-nerolidol, β-caryophyllene, caryophyllene oxide or germacene-D made the main core of compounds in *Achillea* flowers, demonstrating radicals scavenger activity.

We also showed that fresh and preserved plant material contain different amounts of main mono- and sesquiterpenes. This is related to essential oil evaporation from preserved material, even when stored at low temperature or substances conversion. Moreover, Farhat et al. showed major seasonal changes in the composition of the oil [18]. Therefore, the concentration and composition of the oil in our tests may resulted from seasonal and year differences during the collection of plants.

Biochemical pathways are closely related to specific enzymes catalyzing each step of substances conversion in living cells. In our tests, we showed that some enzymes from *A. millefolium* L. express strong substrate conversion activity. Such biologically active substances may imply that the therapeutic effect may be related to the quantity and activity of plant enzymes that are present in the preparations [19]. Moreover, plant enzymes or essential oils may influence the activity of animal cells or their enzymes. It has been shown that plant extracts may inhibit animal acetylcholinesterase (AChE), serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) or alkaline phosphatase (ALP) activity [15, 20]. Moreover, β-myrcene has been shown to inhibit enzymes of cytochrome P4502B subfamily [21]. In consequence, slight changes in the biochemical chain may lead to disruption of cellular metabolism and cell death.

Essential oils and their components of plant derivation have many applications in medicine and industry. Topically applied, they may exert beneficial features of potential toxicity on epidermal cells barrier integrity or deeper tissue, especially skin fibroblasts. However, we have shown that extracts from *A. millefolium* L. also exhibit promising biological activities on human skin fibroblasts, but a comprehensive evaluation, especially of the toxicity effects, should be unertaken before larger scale application.

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