Polish J. of Environ. Stud. Vol. 17, No. 4 (2008), 569-580

Original Research

Lamium Album Extracts Express Free Radical Scavenging and Cytotoxic Activities

R. Paduch [1]*, G. Matysik [2], M. Wójciak–Kosior [2], M. Kandefer–Szerszeń [1], A. Skalska–Kamińska [2], M. Nowak–Kryska [2], P. Niedziela [3]

[1] Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[2] Department of Chemistry, Laboratory of Planar Chromatography, Medical University, Staszica 6, 20-081 Lublin, Poland
[3] Stationary care facility, Kolejowy Hospital, Department of Surgery, Kruczkowskiego 21, 20-468 Lublin, Poland

Received: 29 October, 2007
Accepted: 23 February, 2008

Abstract

Lamium album, commonly known as "white dead nettle," is a perennial herb widely used in folk medicine. The present paper presents the toxic, anti-proliferative, and free radical ($DPPH^{\cdot}$) scavenging activities of methanol and ethyl acetate extracts of that plant. In order to determine the biologically active compounds, the plant extracts were separated by high performance thin layer chromatography (HPTLC) on silica gel Si 60 F_{254} and high performance liquid chromatography (HPLC) combined with densitometry. Methanol extract was rich mainly with flavonoids and phenolic acids. Ethyl acetate extract contained mainly triterpenes.

Both extracts showed no toxic effects against normal human skin fibroblasts (HSF) in the range of applied concentrations (25–225 μg/ml). Anti-proliferative activity revealed that methanol extract expressed lower inhibitory properties than ethyl acetate one. The MTT test was, however, less sensitive than Neutral Red (NR) assay. Ethyl acetate extract did not exhibit $DPPH^{\cdot}$ radical scavenging activity. Methanol extract reduced the radical of about 29% at the highest applied concentration (225 μg/ml). Both extracts slightly influenced cellular cytoskeleton organization and amount, and size of agyrophilic nucleolar organizer regions (AgNOR) protein deposits.

These findings suggest that extracts of *Lamium album* exhibit potential usefulness in preparation of new natural formulations.

Keywords: *Lamii albi* Flos, chromatography, human skin fibroblasts, anti-proliferative activity, free radical scavenging properties

Introduction

Plants produce many phytochemicals, expressing differentiated antioxidant, cytotoxic, cytostatic, anti-inflammatory or anti-microbial activities. Antioxidant properties are closely associated with the prevention of free radical-induced lipid oxidation and thereby destructive and irreversible damage to the components of cell membranes [1]. Many epidemiological and experimental studies revealed that phenolic compounds are responsible for antioxidant activity of plant extracts. They are involved in reducing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [2]. They are also shown to inhibit various types of oxidizing enzymes [3].

*e-mail: rpaduch@biotop.umcs.lublin.pl

Natural antioxidants have also been found to inhibit apoptosis because apoptotic cell death was initially thought to be mediated by oxidative stress [4]. On the other hand, the wide spectrum of biologically active substances, found in plants, show anti-proliferative or even cytotoxic properties against normal human cells. In vitro tests revealed that, depending on used eluents, selected plant extracts contained differentiated sets of substances which expressed cytotoxic, cytostatic or proliferation-promoting activities in cell cultures [5].

Lamium album is a perennial herb which is widely used in folk medicine. It belongs to the *Lamiaceae* family, which is known as a source of plant species containing large amounts of phenolic acids [6]. Beside phenolic compounds, *Lamium album* contains such substances as iridoids, flavonoides, triterpenes, fatty acids, polysaccharides, saponines, phytoecdysteroids, amines, essential oils, tannins and mucilage [5, 7]. Such diversity of compounds is, therefore, responsible for high biological activity of that plant. It is well known that *Lamium album* demonstrates anti-septic, anti-inflammatory and bacteriostatic activities [5, 7]. For that reason this plant is widely used in folk wound-healing therapies.

In vitro bioassays have been extensively used to analyze biological activities of medicinal plants. This study evaluates free radical scavenging, cytotoxic and cytostatic activities of methanol and ethyl acetate extracts of *Lamium album* on human normal skin fibroblasts (HSF). The aim of this work was to find the relationship between free radical scavenging and cytotoxic properties of *Lamium album* extracts and scientifically evaluate their potential usefulness in modern wound-healing therapies.

Materials and Methods

Plant Material

Samples of *Lamium album* were collected in the Botanical Garden of the M. Curie-Skłodowska University, Lublin, Poland, in July 2006. The identify of the plant was confirmed by the Department of Pharmaceutical Botany, Medical University, Lublin, Poland. (A voucher specimen is deposited in the Department of Pharmaceutical Botany, Medical University, Lublin, Poland.) After collection the flowers of *Lamium album* were washed and dried at 40ºC.

Methanol and ethyl acetate extracts of *Lamium album* were prepared by heating 20 g of plant material with 300 ml of solvent for 5 h at 60ºC under reflux. Each of these extracts was concentrated under reduced pressure at 30ºC to 100 ml.

Methanol and ethyl acetate extracts were used for determination of pentacyclic triterpenes and phenolic acids. For phenolic acid analysis, 100 ml of the examined extract was evaporated to dryness, washed with hot water (50 ml) and cooled in a refrigerator for 24 h. The tar-like precipitates, containing ballasts, were filtered and washed with distilled water. The filtrate was defatted by double extraction with petroleum ether (30 ml samples) and next the aqueous solution was extracted 10 times with diethyl ether (20 ml). The combined ether extracts were evaporated to 100 ml volume and were extracted ten times with 10 ml portions of 5% aqueous $NaHCO_3$ to transform the phenolic acids into water-soluble natrium salts. The combined aqueous fractions were acidified with 36% HCl to pH 3 and again extracted ten times with 10 ml portions of diethyl ether. The ether extracts were dried with anhydrous Na_2SO_4. After evaporation of the solvent the dry fraction of free phenolic acids was obtained [8, 9].

For cell culture, 10 ml of each extract was evaporated to dryness. Part of the dry residue was dissolved in dimethylsulfoxide (DMSO) to obtain a concentration of 100 mg/ml (stock solution). The final quantity of DMSO in the highest of applied plant extract concentration did not exceed 0.25%. Such concentration of DMSO in culture medium had no influence on HSF cell viability, as was shown in our previous tests.

Chromatographic Analysis

The analysis of phenolic acids and triterpenes in investigated extracts was carried out by high performance thin layer chromatography. Additionally, phenolic acids were examined by high performance liquid chromatography.

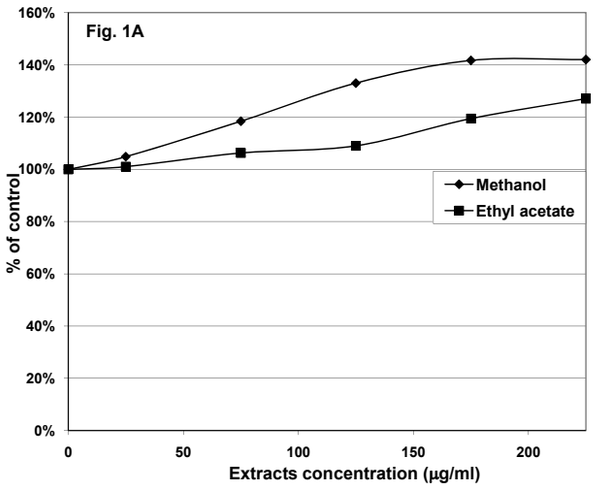
The separation of the components of extracts was performed on 10 cm x 10 cm HPTLC plates coated with silica gel Si60 F_{254} (E. Merck, Darmstadt, Germany). Before use, the plates were washed with methanol and dried for 20 min. at room temperature. The standards of triterpenes and phenolic acids (Sigma Chemical Co., St. Louis, USA) were dissolved in methanol (0.01%). 5 µl samples of standards of triterpenes, phenolic acids and extracts were spotted by means of an AS 30 automatic applicator (Desaga, Heidelberg, Germany) under nitrogen at 2.5 atm. Chromatograms were developed in horizontal Teflon DS chambers (Chromdes, Lublin, Poland) on a distance of 8.5 cm. Densitograms were obtained by use of a Desaga CD 60 densitometer controlled by a Pentium computer. For qualitative purposes, linear scans were obtained with slit dimension of 0.1 mm x 2 mm at λ=530 nm for triterpenes and λ=254 nm for phenolic acids.

All chromatographic systems were chosen experimentally. For triterpenes, mobile phase consisted of toluene, acetone and 85% formic acid (4.5 : 0.5 : 0.1, v/v/v) was used. The location of the spots was determined after spraying with 10% ethanolic H_2SO_4 (98%) and heating at 120ºC for 3 min in daylight or UV λ = 366 nm [10, 11]. The method was validated for linearity (correlation coefficient, r=0.9985), precision (% RSD=1.4–3.5%) and accuracy (recovery values range 98.4-103.1%).

In phenolic acid analyze multiplay gradient development technique (MGD) was used. In the first step the mixture of heptane + dichloromethane + izoprophyl ether + formic acid + water (2.3 ml + 0.3 ml + 2 ml + 0.1 ml + 1 ml, v/v/v/v/v) as the mobile phase was used and developed two times at a distance of 8.5cm. In the second step the plates were developed five times over the same distance with mobile phase composed of heptane + dichloromethane + izoprophyl ether + formic acid (2.8 ml + 0.4 ml + 2 ml + 0.1 ml + 1 ml,

v/v/v/v/v). HPTLC plates with silica gel and fluorescence indicator were used. Spots were visualized in UV light at λ=254 nm. In HPTLC investigations of phenolic acids, validation parameters were calculated: caffeic acid - linearity (correlation coefficient, r = 0.97842), precision (% RSD = 0.7–1.9%) and accuracy (recovery values range 97.2-103.2%); vanillic acid - linearity (correlation coefficient, r = 0.98543), precision (% RSD = 0.8–1.5%) and accuracy (recovery values range 98.1-104.2%).

Quantitative analysis of phenolic acids also were carried out by HPLC in reversed phase system on chromatograph LaChrom with diode array detector (Merck, Darmstadt, Germany). Zorbax SB C18 steel column 250 mm x 4.6 mm filled with adsorbent with particle diameter 5 µm was used. 20 µl of each solution was injected. Elution was performed at 1ml/min flow- rate and at temperature of 25ºC. For the analysis of the compounds, the following mobile phase was used: methanol/water/formic acid (25 : 75 : 0.05, v/v/v). The method for caffeic acid was validated



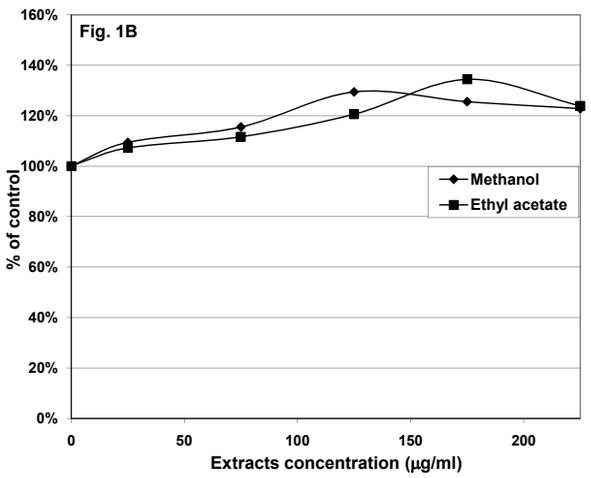


Fig. 1. The effect of 24 h treatment of HSF cells ($1x10^5$ cells/ml) with methanol and ethyl acetate extracts of *Lamium album*. The MTT assay (A) and Neutral Red assay (B). The results of the viability tests are expressed as a percentage of the control arbitrarily set to 100%. The figure shows an average of three independent, representative experiments.

for linearity (correlation coefficient, r = 0.9975), precision (% RSD = 0.8–1.8%) and accuracy (recovery values range 99.1-102.1%).

The method for vanillic acid was validated for linearity (correlation coefficient, r = 0.996494) precision (% RSD = 0.65–1.2%) and accuracy (recovery values range 98.2-101.9%).

Standardization of Plant Material

Chemical identification of plant material is an important element of their quality studies. Phenolic compounds and phytosteroids (triterpenes are in this group) are commonly used chemical markers in phytopharmaceutical analyses.

All chromatographic sets were chosen experimentally.

In preliminary chromatographic tests the most typical compounds of the mentioned groups were found, e.g. caffeic acid and ursolic acid. Their concentrations were appropriate to determine in each investigated extract of *Lamium album*. Standards of determined compounds are commercially available and this method of standardization can be easily employed in routine laboratory tests.

Aqueous methanol and ethyl acetate were used for extraction of flos of Lamii albi. The extracts were clean enough for chromatographic analysis of triterpenes. It was possible because the flowers of this plant are not very ample with chlorophyll. Isolation of caffeic acid is described in the chromatographic analysis part.

The described standardization method allows fast analysis of flos of Lamii albi and is not expensive (small amounts of solutions, cheap equipment, possibility of simultaneous analysis of many samples), which enables common use in laboratories.

Biological Experiments

Establishment of HSF Cell Culture

Freshly excised fragments of human skin were washed two times 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 37ºC in a humidified 5% CO_2/95% air incubator. Outgrowths of skin fibroblasts were separated and cultured. For experiments, HSF cells obtained from two 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 37ºC in a humidified atmosphere with 5% CO_2.

For experiments, the total number of cells was estimated by counting in haemocytometer. A 100 µl of cell suspension ($2x10^4$ cells/ml for cells proliferation activity tests or $1x10^5$ 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 new ones containing 2% FBS and appropriate plant extract concentrations in the 25–225 µg/ml range were 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). A blank control culture medium with 2% FBS was used.

MTT Assay

Sensitivity of cells to *Lamium album* extract activity was determined by a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (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 *Lamium album* 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 37ºC 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 shacked for 20 min at room temperature and the extracted dye absorbance was spectrophotometrically measured at 540 nm.

DPPH· Free Radical Scavenging Test

Free radical scavenging activity of methanol and ethyl acetate extracts of *Lamium album* was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH·) assay. This method is based on the ability of antioxidants to reduce the stable dark violet radical DPPH· (Sigma) to the yellow colored 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 (25–225 µg/ml) and standards. Trolox (Sigma) at increasing concentrations (1–50 µg/ml) was used as a reference for free radical scavenging activity. After 2, 5, 10, 15 and 20 min. of incubation at room temperature, the absorbance of the solution was measured at 515 nm. The lower 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:

$$\text{DPPH}\cdot \text{ scavenging effect (\%)} = = [(X_{control} - X_{extract}/X_{control}) \times 100]$$

... where: $X_{control}$ is the absorbance of the control and $X_{extract}$ is the absorbance in the presence of plant extract [12, 13].

Labeling 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 three times in PBS, exposed to Triton X-100 (0.2%, v/v) solution for 5 min. and rinsed three times with PBS. 0.5 ml PBS containing tetramethyl-rhodamine-isothiocyanate-phalloidin (TRITC-phalloidin, 1 µg/ml) (Sigma) was added to each well and incubation in the dark at 37ºC/5% CO_2 for 30 min was accomplished. Cell observation was conducted under a fluorescence microscope (Olympus, BX51). Quantitative analysis of fluorescent images was performed by an 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 37ºC, 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 three times. The results were expressed as means ± S.D and the difference was tested by Student's t-test. P-values lower than 0.05 were considered statistically significant.

Results

Plant extracts contain many substances demonstrating free radical scavenging, anti-proliferative or cytotoxic features. The cytotoxic and anti-proliferative activity of methanol and ethyl acetate extracts of *Lamium album* were measured using NR uptake and MTT tests. The experiments with HSF cells seeded at a density of $2x10^4$ cells/ml were led for 72 h (results read every 24 h) (anti-proliferative activity analysis) and with density of $1x10^5$ cells/ml conducted for only 24 h (cytotoxicity analysis).

Toxic and Anti-Proliferative Activity of the Extracts (NR and MTT Assays)

Figs. 1A and 1B show the toxicity of extracts on HSF cells. We observed no cytotoxicity of both used extracts. The MTT test showed that for extract doses ranging from 25–225 μg/ml the succinyl and mitochondrial dehydrogenase activity was not inhibited. The enzyme activity slightly increased to the maximal value at 225 μg/ml of extract. For methanol and ethyl acetate extracts it was 142% ± 4 and 127% ± 3 of the control value, respectively (Fig. 1A). In the NR test, methanol and ethyl acetate extracts increased the dye uptake by HSF cells to maximal value 129% ± 7 at 125 μg/ml and 134% ± 7 at 175 μg/ml, respectively (Fig. 1B).

Figs. 2A-C and 3A-C show the anti-proliferative activity of extracts on HSF cells. The MTT assay revealed that methanol extract did not inhibit cell proliferation during 72 h of incubation. At concentrations up to 225 μg/ml, the succinyl dehydrogenase activity remained above control values. Ethyl acetate extract began to inhibit cell proliferation after 48 h of incubation only at 225 μg/ml (62% ± 14 of the control). After 72 h decreased enzyme activity was observed for all used extract concentrations. The lowest value was 33% ± 13 of the control at 225 μg/ml (Figs. 2A-C).

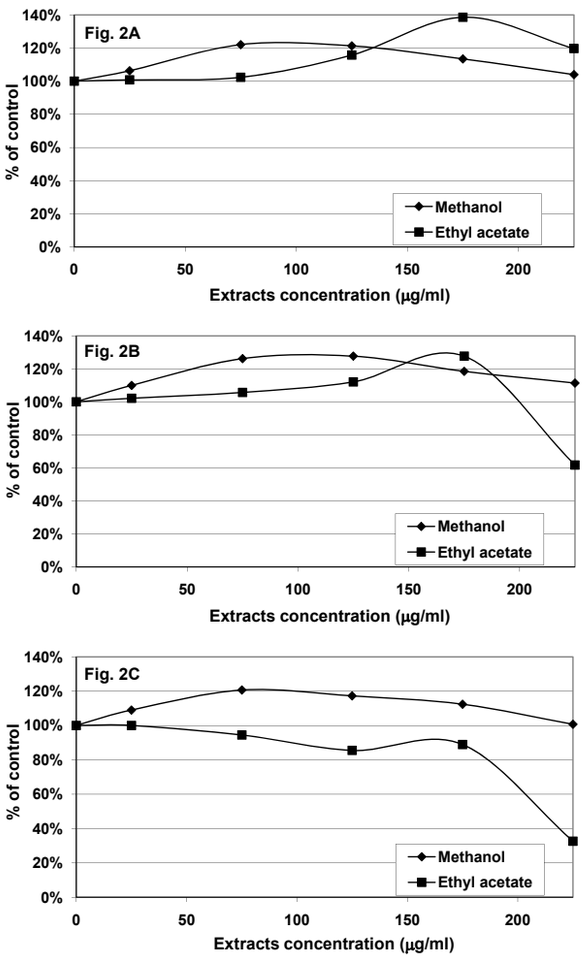


Fig. 2. Antiproliferative, dose-dependent effect of the methanol and ethyl acetate extracts of *Lamium album* on HSF cells ($2x10^4$ cells/ml) after 24 h (A), 48 h (B) and 72 h (C) of growth. The MTT assay. The results of the viability tests are expressed as a percentage of the control arbitrarily set to 100%. The figure shows average of three independent, representative experiments.

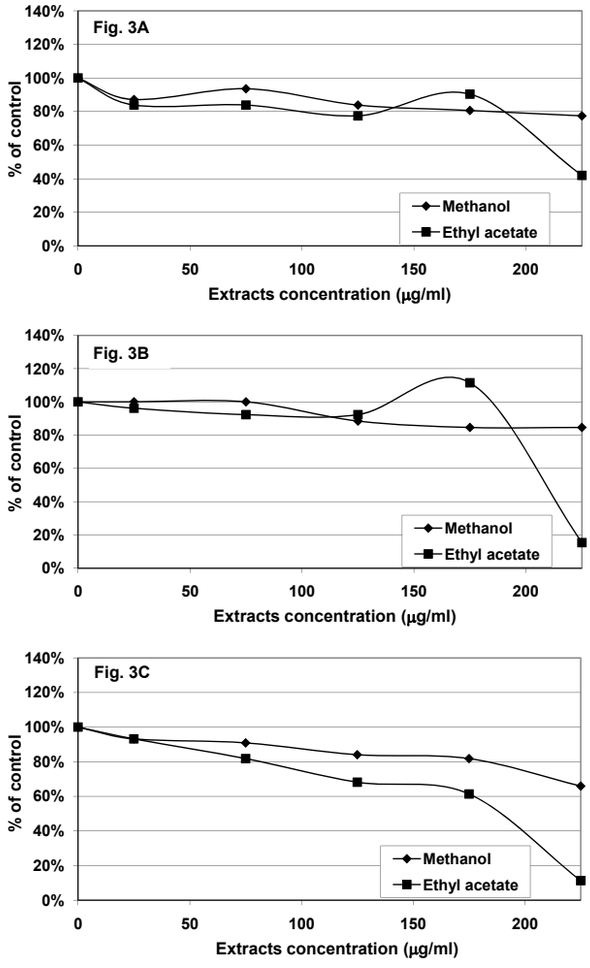


Fig. 3. Antiproliferative, dose-dependent effect of the methanol and ethyl acetate extracts of *Lamium album* on HSF cells ($2x10^4$ cells/ml) after 24 h (A), 48 h (B) and 72 h (C) of growth. The Neutral Red assay. The results of the viability tests are expressed as a percentage of the control arbitrarily set to 100%. The figure shows average of three independent, representative experiments.

Table 1. The concentration of *Lamium album* extracts that inhibited viability of human skin fibroblasts (HSF) by 25% (IC_{25}).

Extract	Time of incubation (hours)	IC_{25} (μg/ml)	
		MTT	**NR**
Methanol	24	376	283
	48	488	441
	72	341	195
Ethyl acetate	24	341	190
	48	215	194
	72	188	101

NR uptake assay showed that both methanol and ethyl acetate extracts reduced cell proliferation as early as 24 h of incubation. Loss of cell viability was notable already at such a low concentration as 25 μg/ml. The inhibitory activity increased systematically both with higher extract concentrations and time of incubation. The lowest value was 11% ± 4 of the control after 72 h of culture with 225 μg/ml of ethyl acetate extract (Figs. 3A-C).

In Table 1, obtained and calculated extracts concentrations that inhibited cell viability of 25% (IC_{25}) are shown.

Free Radical Scavenging Activity of the Extracts (DPPH Assay)

Many plants possess good free radical scavenging capacities. In DPPH test, free radical scavenging activity of methanol and ethyl acetate extracts of *Lamium album*, compared to trolox under the same experimental conditions was analyzed. Trolox concentration, which gave 50% inhibition (IC_{50}), was 33 ± 0.2 μg/ml. IC_{25} value was 14 ± 0.2 μg/ml. Ethyl acetate extract did not exhibit DPPH· reduction activity. Methanol extract revealed free radical scavenging properties and had an average IC_{50} of 465.9 ± 4.4 μg/ml. The average IC_{25} value was 215.8 ± 4.4 μg/ml. Methanol extract scavenging activity at concentrations of 33 μg/ml was 3.75 times lower (13.4% decrease of control) than trolox reference at the same concentration (trolox IC_{50} value). This means that essential free radical scavenging activity occurs at low concentrations of methanol extract. The DPPH· scavenging activity was evaluated after 2, 5, 10, 15, 20 and 30 min. of incubation with extracts. The results measured at these points were not significantly different. Table 2 shows the capability of *Lamium album* extracts to scavenge the DPPH· radical. The results represent the percentage of reduced radical compared to control (0% of reduction).

Organization of the Cytoskeleton

F-actin cytoskeletal protein organization was analyzed using TRITC-phalloidine fluorescent staining. Control cells are presented in Fig. 4. Methanol extract at low concentration (25 μg/ml) did not influence cytoskeleton structure (Fig. 5A). At 125 μg/ml (Fig. 5B), and 225 μg/ml (Fig. 5C) cell shapes became elongated and microfilament amounts were lower compared to control. Ethyl acetate extract had no influence on actin filament composition at 25 μg/ml (Fig. 6A), and 125 μg/ml (Fig. 6B) concentrations. However, at 225 μg/ml cells contracted and began to lose close touch with themselves, but the F-actin filament organizations remained similar to control cells (Fig. 6C).

Evaluation of Cell Proliferation by AgNOR Staining

In control cells there were many interphase AgNOR deposits (Fig. 7). After incubation with methanol extract (≥75 μg/ml) many argyrophilic NOR proteins and vacuolization of cells was observed (Figs. 8A and 8B). The cells after incubation with ethyl acetate extract (75 μg/ml) (Fig. 9A) were characterized by larger solitary interphase NORs than control. 125 μg/ml concentrations led to synthesize a great number of smaller nucleolin (Fig. 9B) when compared to the sample with 75 μg/ml and control.

HPTLC Analysis

In both extracts, the pharmacologically active compounds were investigated by HPTLC combined with the densitometry and HPLC methods. Flavonoids, phenolic acids, iridoids and triterpenes were determined. Large amounts of flavonoids, in the form of glycosides and aglycones, were present in the methanol extract. In ethyl acetate extract there were only small amounts of flavonoids, mainly in the form of aglycones. Phenolic acids were present mainly in the methanol extract. There were also found protocatechuic, chlorogenic, vanillic, caffeic, coumaric and ferrulic acids. Triterpenes were present in both extracts. Ursolic acid and β-amyrin were identified. Densitometric analysis revealed only trace amounts of vanillic and caffeic

Table 2. DPPH scavenging effect (%). The % of reduced DPPH· radical by *Lamium album* extracts is compared to the control (0% of reduction).

Extract concentration (μg/ml) / Extract	25	75	125	175	225
Methanol	14.55±0.6	7.3±0.48	13.56±0.48	20.11±0.5	28.96±1.26
Ethyl acetate	0	0	0	0	0.96±0.8

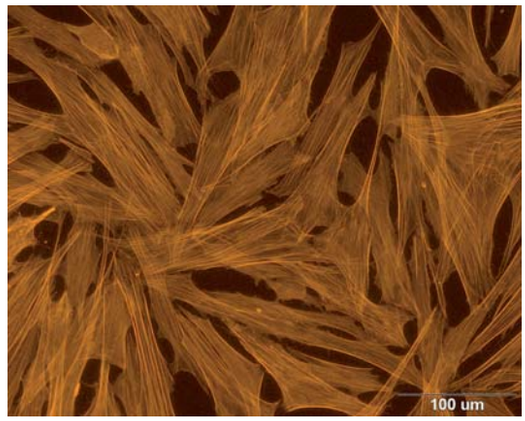
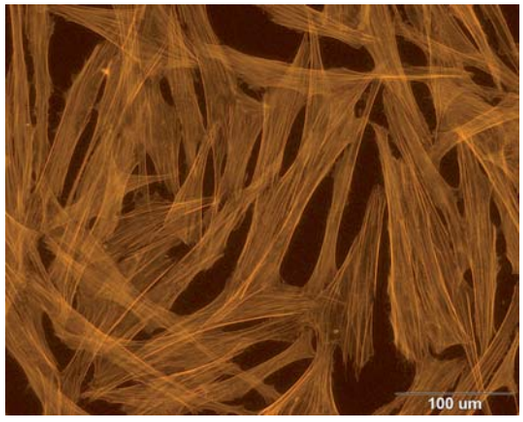


Fig. 4. Cytoskeleton organization in HSF cells. The control sample. TRITC-phalloidin fluorescent staining. Bar 100 μm.

Fig. 5A



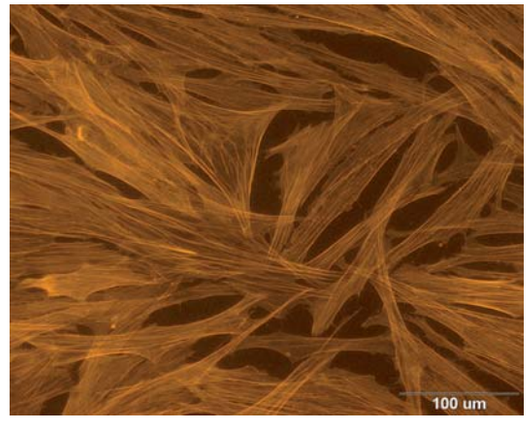
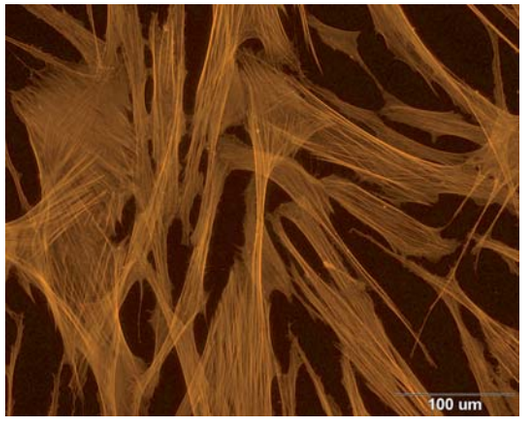


Fig. 6A

Fig. 5B



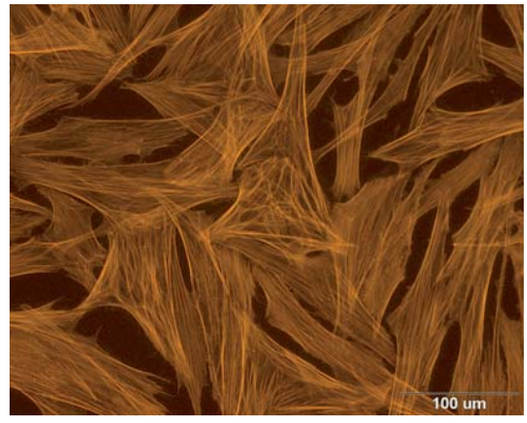
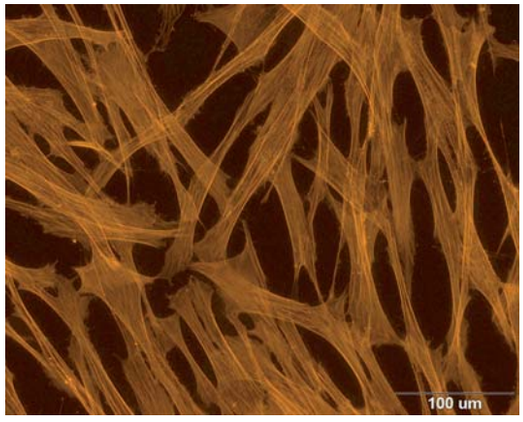


Fig. 6B

Fig. 5C



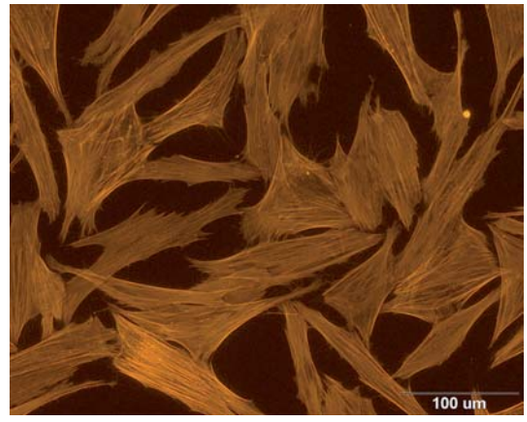


Fig. 6C

Figs. 5 and 6. Cytoskeleton organization in HSF cells after 24 h of incubation with methanol (5A-C) and ethyl acetate (6A- C) extracts of *Lamium album*. The employed extract concentrations were: 25 μg/ml (A); 125 μg/ml (B) and 225 μg/ml (C). The TRITC-phalloidin fluorescent staining. Bar 100 μm.

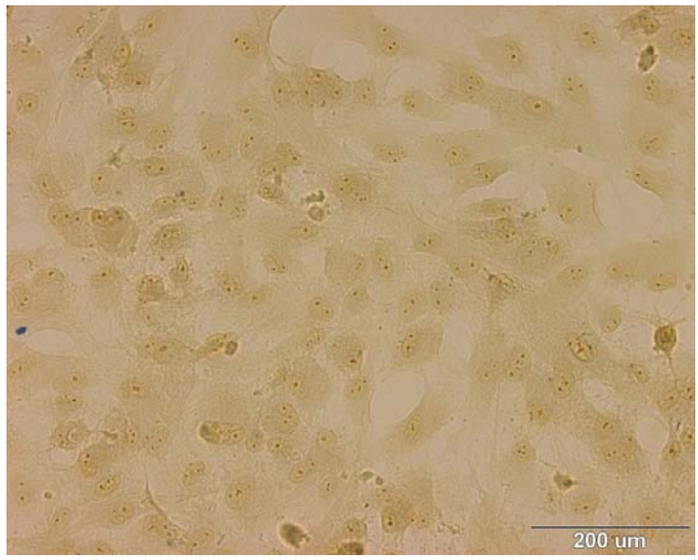


Fig. 7. The nuclei of HSF cells silver-stained for the AgNOR proteins. The control sample. Bar 200 µm.

Fig. 8A

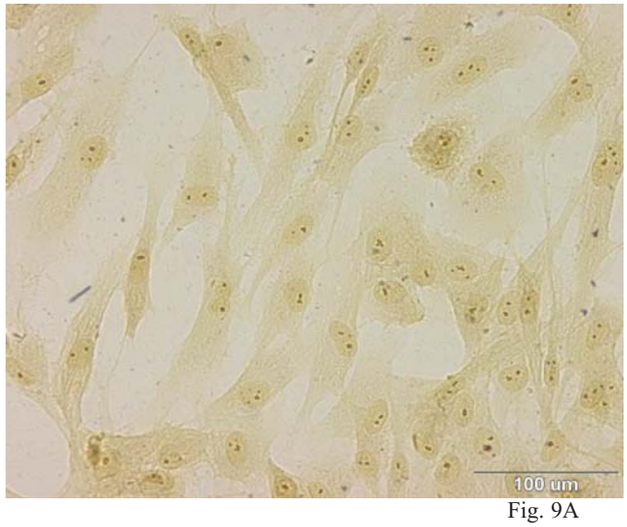


Fig. 9A

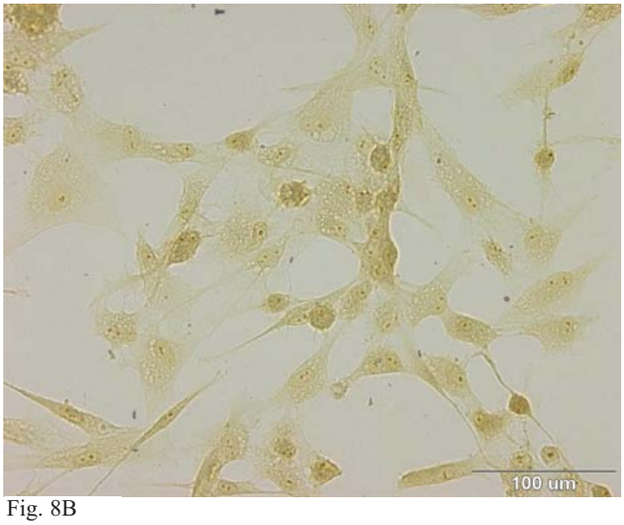


Fig. 8B

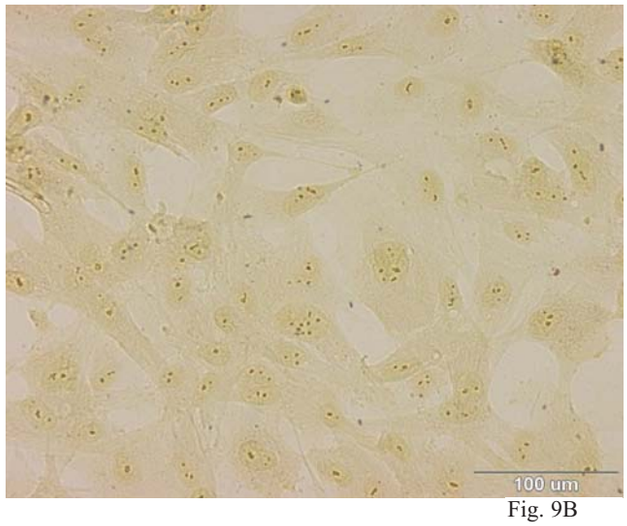


Fig. 9B

Figs. 8 and 9. The silver-stained NOR proteins of HSF cells after 24 h of incubation with methanol (8A-B) and ethyl acetate (9A-B) extracts of *Lamium album*. The employed extract concentrations were: 75 µg/ml (A) and 125 µg/ml (B). Bar 100 µm.

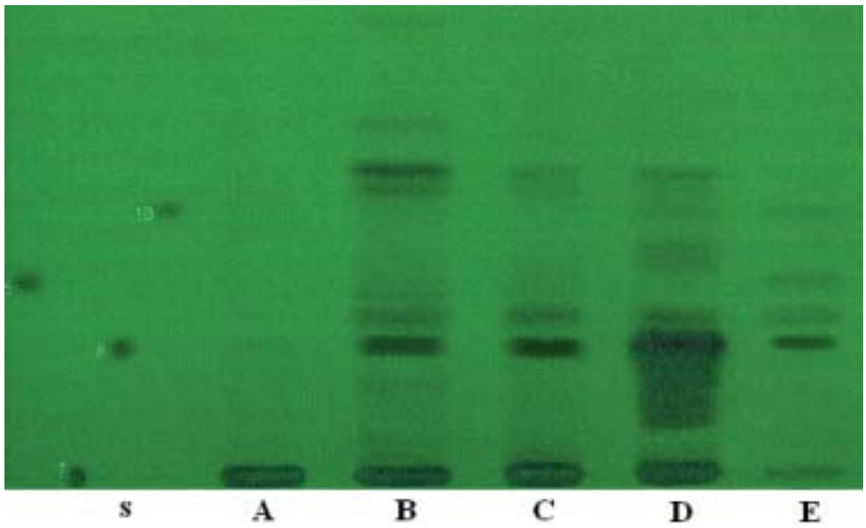


Fig. 10. The chromatogram of *Lamii albi* extracts. A - eathyl acetate extract, B - methanolic extract, C - acidic hydrolysis, D - alcalic hydrolysis, E - standard mixture, s - caffeic acid standard.
The photograph of the plate in UV light at λ = 254 nm.

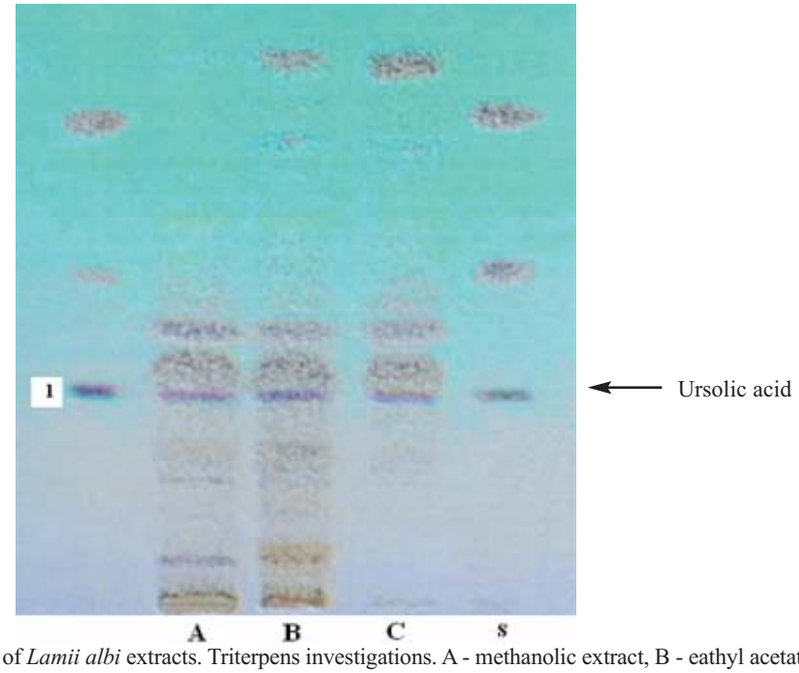


Fig. 11. The chromatogram of *Lamii albi* extracts. Triterpens investigations. A - methanolic extract, B - eathyl acetate extract, C - heptane extract, s - standard mixture, l-ursolic acid standard.
The photograph of the plate after derivatization (anisaldehyde-sulfuric acid reagent).

acids in ethyl acetate extracts. In methanol one of the amounts was detectable and estimated at 6.7 μg/g and 0.46 μg/g of stock dry mass, respectively. Moreover, ursolic acid was analyzed. In methanol extract 0.37 μg/g and in ethyl acetate 4.63 μg/g amounts were obtained.

Discussion

Many plants have been widely examined to identify new and effective substances with cytotoxic and/or antioxidant properties. In this work, methanol and ethyl acetate extracts of *Lamium album* have been analyzed. The study has been performed from the point of extract toxicity against human normal skin fibroblasts and free radical scavenging activity. Cytotoxicity screening models provide important preliminary data to select plant extracts that demonstrate features with potential usefulness in modern medicine. Viability and proliferation activity of HSF cells exposured to *Lamium album* extracts have been studied using MTT and NR assays. Two different cytotoxicity tests enabled us to obtain results based on different specificity profile assays employed. The MTT is based on the enzymatic conversion of MTT by the succinate dehydrogenase in the mitochondria of living cells. The NR method is a

colorymetric assay measuring the uptake of the dye by functional lysosomes [14]. The studied plant extracts did not exhibit toxic properties against HSF cells. It resulted from the non-toxic or low toxic features of substances composing the plant samples. As was shown in our previous study [5], *Lamium album* extracts consist of different amounts of the phenolic acids, flavonoids, triterpenes and iridoids. These substances exert low toxicity against non-proliferating cells (contact inhibition of normal cell proliferation in high density culture). In proliferation activity analysis, the results of NR and MTT tests were comparable. However, in the NR assay higher anti-proliferative activity of the extracts were shown. In contrast to NR, in MTT test plant extracts did not affect succinate dehydrogenase activity. Inhibition of cell proliferation was observed for ethyl acetate extract but not untill 72 h of incubation. Therefore, the differences in detection levels of anti-proliferative activity between these two methods may be associated with a profound effect of plant extracts on lysosomes without affecting cell mitochondria. This specific effect could account for the lower HSF cell viability with NR but not MTT assay. However, any anti-proliferative effect may be associated with flavonoid quercetin and triterpene ursolic acid present in the extracts. These substances are known to inhibit proliferation of human normal and tumor cells by blocking cell cycle progression in the G1 phase and triggering apoptosis [15, 16, 17]. Ethyl acetate extract limited HSF cell proliferation more effectively than the methanol one, because it contained significantly higher quantities of, e.g., ursolic acid.

It is also thought that inhibition of mitochondrial respiration induces active oxygen-related cell death. Anti-proliferative activity of tested extracts may also be a result of stimulation of the reactive oxygen species (ROS) production within mitochondria. Mitochondria-generated ROS can damage not only mitochondrial components (enzymes, membranes), and therefore inhibiting respiratory tract activity, but may efflux the organellum and affect other cell compartments, e.g. lysosomes. ROS are continuously produced during normal physiological processes. There is a balance between the generation and inactivation of ROS by the antioxidant systems in an organism [12]. Under pathological events, ROS is overproduced and results in alterations and loss of structural and functional architecture in the cell membrane. It leads directly to cytotoxicity or indirectly to genotoxicity and other abnormalities [18]. In the presented study, MTT cytotoxicity analysis revealed that mitochondrial activity reached a peak at 75 µg/ml of methanol extract. At the same concentration the extract showed the lowest DPPH$^{\cdot}$ scavenging activity. At this point there is probably a balance between stimulated cell metabolism, expressed by mitochondrial respiratory tract activity, and trapping the excess mitochondria-released radicals stimulated by the extract. Therefore, the extract at this concentration did not inhibit electron transport in mitochondria but the radical reduction activity was enough not to admit its efflux and affect the structure of cell components. Moreover, this concentration acted gently, stimulating cell metabolism and quenching the overproduced injurious free radicals. The higher the extract concentrations the more effective free radicals scavenging activity, but thereby active extract components interfered in cell biochemical pathways, especially respiratory tract. In consequence, there was reduced cell metabolism and cell proliferation. However, oxygen is an important element for aerobic life. On the other hand, organisms are often exposed to endogenous and exogenous factors generating excess reactive oxygen species, resulting in homeostatic imbalance and many diseases [18]. Therefore, there is a considerable interest in finding effective antioxidants, advisable from natural sources. Plants produce a variety of antioxidants, preventing damage caused by ROS. Phenolic compounds are the most active and among them flavonoids are the most important group [19]. The antioxidant activity of phenolic compounds relies mainly on their redox properties [2]. However, many antioxidant behaviors can be attributed to these compounds. They can trap free radicals directly or scavenge them through reactions, including enzymatic inhibition or ferrous chelating effects [3, 12, 20]. Among phenolics, caffeic acid is considered a superior antioxidant [12]. It was found that caffeic acid scavenges effectively intracellular ROS and DPPH$^{\cdot}$ radical and thus prevented lipid peroxidation. Moreover, this compound has been demonstrated to modulate cellular response on oxidative challenge [21]. In our tests caffeic acid and also vanillic acid were present in methanol extract, where the radical scavenging activity was observed. However, these phenolics were not detectable in ethyl acetate extract, and no reduction of the radical was found.

The nucleus plays an important role in the regulation of cell proliferation and protein synthesis [22]. Therefore, staining of the argyrophilic non-histone proteins, localized in the nucleolar organizer region, is an essential step in the rate of cell proliferation analysis. These proteins are associated with ribosomal genes and the number of these silver-stained nucleolar organizer region (AgNORs) proteins is strictly related to rRNA transcriptional activity and in consequence with rapidity of cell proliferation. It has been shown that the shorter the cell doubling time, the greater amount of small, interphase AgNORs. Nucleoli of resting cells are characterized by large solitary interphase NORs [23, 24]. On that basis it may be speculated that in short time of exposure, methanol and ethyl acetate extracts of *Lamium album* may initiate HSF cell proliferation. However, the exact mechanism of this action needs further testing and explanation.

Cell shape is closely related to F-actin cytoskeleton organization. Changes in cytoskeletal composition influence many cellular processes such as cell shape, cell attachment, proliferation, motility, cell polarity, intracellular trafficking of organelles, wound healing and viability [25]. Junctional complexes linking adjacent cells to the cytoskeleton provide molecular support for the transfer of mechanical signals across the cell surface. Moreover, the cytoskeleton is coupled by adhesion molecules to the extracellular matrix elements or neighboring cells and participates in signal transduction [26]. Substances present in ethyl acetate extract of *Lamium album*, at higher concentrations and prolonged

exposure, influenced cell-cell adhesion complexes. Therefore, it could disturb cellular signaling utilizing F-actin filaments and in consequence led to observed decrease in close touch with neighboring cells and their viability. Methanol extract, however, influenced cytoskeleton organization and F-actin filament polymerization, but it did not considerably disturb biochemical signaling pathways and therefore cell viability was higher than in ethyl acetate samples. This is only one possible explanation but active components of plant extracts may also influence other mechanisms closely associated with cytoskeleton organization and in consequence cell viability. Plant compounds or their metabolites may also change cell architecture by indirect mechanisms and factors, e.g. components of inflammatory state (cytokines, growth factors, and inflammatory proteins). Wound healing is a progressive process that reestablishes the integrity of damaged tissue. This cascade of healing events has been shown to be accelerated by several natural, particularly plant products [27]. Both et al., reported that, e.g. ursolic acid increases both the ceramide content of cultured normal human epidermal keratynocytes and the collagen content of cultured normal human dermal fibroblasts [28]. These exemplary effects of ursolic acid on skin physiology are very important not only with reference to anti-wrinkling or anti-aging processes but also wound healing of damaged skin layer. Moreover, Di Mambro et al. demonstrated that flavonoids may provide protective effects against UV radiation [19]. Therefore, application of flavonoids in topical formulations may protect skin from oxidative injury and cancer. In our experiments, methanol extract of *Lamium album* was non-toxic and expressed antioxidative activity in short time of incubation with normal human skin fibroblasts. The extract also had a free radical scavenging activity. Therefore, after careful elaboration of exposure time and specimen concentration the *Lamium album* extracts could be taken into consideration to be used as wound healing and skin protective formulations.

Acknowledgments

Our research was supported by a grant from the Ministry of Scientific Research and Information Technology, KBN No. 2PO5F05028.

References

1. LEE J.-Y., HWANG W.-I., LIM S.-T. Antioxidant and anticancer activities of organic extracts from *Platycodon grandiflorum* A. De Candolle roots. J. Ethnopharmacol., **93**, 409, **2004**.
2. ŠTĚRBOVÁ D., MATĚJIČEK D., VLČEK J., KUBÁŇ V. Combined microwave-assisted isolation and solid-phase purification procedures prior to the chromatographic determination of phanolic compounds in plant materials. Anal. Chim. Acta., **513**, 435, **2004**.
3. DORMAN H.J.D., PELTOKETO A., HILTUNEN R., TIKKANEN M.J. Characterization of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. Food Chem., **83**, 255, **2003**.
4. JU E.M., LEE S.E., HWANG H.J., KIM J.H. Antioxidant and anticancer activity of extract from *Betula platyphylla var. japonica.* Life Sci., **74**, 1013, **2004**.
5. PADUCH R., WÓJCIAK-KOSIOR M., MATYSIK G. Investigation of biological activity of *Lamii albi* Flos extracts. J. Ethnopharmacol., **110**, 69, **2007**.
6. KOSAR M., DORMAN H.J.D., BACHMAYER O., BASER K.H.C., HILTUNEN R. An improved on-line HPLC-DPPH method for the screening of free radical scavenging compounds in water extracts of Lamiaceae plants. Chem. Nat. Comp., **39**, 161, **2003**.
7. CHUDNICKA A., MATYSIK G. Research of enzymatic activities of fresh juice and water infusions from dry herbs. J. Ethnopharmacol., **99**, 281, **2005**.
8. KOWALSKI R., WOLSKI T. TLC and HPLC analysis of the phenolic acids in *Silphium perfoliatum* L. leaves, inflorescences and rhizomes. J. Planar Chromatogr., **3**, 230, **2003**.
9. ŚWIĄTEK L., DOMBROWICZ E., ZADERNOWSKI R. Zawartość fenolokwasów w korzeniach goryczki żółtej (*Gentiana lutea* L.). Herba Pol., **34**, 15, **1988** [In Polish].
10. JORK H., FUNK W., FISCHER W., WIMMER H.H. Thin-layer chromatography, reagents and detection methods, vol.1a, VCH Publisher, New York, **1990**.
11. WAGNER H., BLADT S. Plant drug analysis. Springer-Verlag, Berlin, Heidelberg, New York, **1996**.
12. GÜLÇIN İ. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology, **217**, 213, **2006**.
13. GÜLÇIN İ. Antioxidant and antiradical activities of L-carnitine. Life Sci., **78**, 803, **2006**.
14. FOTAKIS G., TIMBRELL J.A. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol. Lett., **160**, 171, **2006**.
15. HSU Y.-L., KUO P.-L., LIN Ch.-Ch. Proloferative inhibition, cell-cycle dysregulation, and induction of apoptosis by ursolic acid in human non-small cell lung cancer A549 cells. Life Sci., **75**, 2303, **2004**.
16. JAKUBOWICZ - GIL J., PADUCH R., PIERSIAK T., GŁOWNIAK K., GAWRON A., KANDEFER-SZERSZEŃ M. The effect of quercetin on pro-apoptotic activity of cisplatin in Hela cells. Biochem. Pharmacol., **69**, 1343, **2005**.
17. MA CH.-M., CAI S.-Q., CUI J.-R., WANG R.-Q., TU P.-F., HATTORI M., DANESHTALAB M. The cytotoxic activity of ursolic acid derivatives. Eur. J. Med. Chem., **40**, 582, **2005**.
18. TRIATHI R., MOHAN H., KAMAT J.P. Modulation of oxidative damage by natural products. Food Chem., **100**, 81, **2007**.
19. DI MAMBRO V.M., FONSECA M.J.V. Assays of physical stability and antioxidant activity of a topical formulation added with different plant extracts. J. Pharm. Biomed. Anal., **37**, 287, **2005**.
20. TROUILLAS P., CALLISTE C.-A., ALLAIS D.-P., SIMON A., MARFAK A., DELAGE CH., DUROUX J.-L. Antioxidant, anti-inflammatory and antiproliferative properties of sixteen water plant extracts used in the Limousin countryside as herbal teas. Food Chem., **80**, 399, **2003**.
21. KANG K.A., LEE K.W., ZHANG R., PIAO M., CHAE S., KIM K.N., JEON Y.J. PARK D.B., YOU H.J., KIM J.S. HYUN J.W. Caffeic acid protects hydrogen peroxide induced cell damage in WI-38 human lung fibroblast cells. Biol. Pharm. Bull., **29**, 1820, **2006**.
22. REBOLLEDO GODOY A.P.R., REBOLLEDO GODOY M.R., MEISSNER C., OEHMICHEN M. Proliferative activity of epidermal basal cells after wounding. AgNOR counts compared with bromodeoxyuridine reactivity in rats. Exp. Toxicol. Pathol., **53**, 65, **2001**.

23. DERENZINI M. The AgNORs. Micron, **31**, 117, **2000**.
24. GIUFFRÈ G., CARUSO R.A., BARRESI G., TUCCARI G. Prognostic significance of standardized AgNOR analysis in early and advanced gastric carcinomas. Virchows Arch., **433**, 261, **1998**.
25. KOULOUKOUSSA M., ALEPOROU-MARINOU V., ANGELOPOULOU B., TROUGAKOS I.P., PANAGOPOULOU E., KITTAS CHR., MARINOS E. Phorbol myristate acetate induces changes on F-actin and vinculin content in immature rat Sertoli cells. Tissue Cell., **36**, 149, **2004**.
26. MARTIN M., SIMON-ASSMANN P., KEDINGER M., MARTIN M., MANGEAT P., REAL F.X., FABRE M. DCC regulates cell adhesion in human colon cancer derived HT-29 cells and associates with ezrin. Eur. J. Cell Biol., **85**, 769, **2006**.
27. PANCHATCHARAM M., MIRIYALA S., GAYATHRI V.S., SUGUNA L. Curcumin improves wound healing by modulating collagen and decreasing reactive oxygen species. Mol. Cell Biochem., **290**, 87, **2006**.
28. BOTH D.M., GOODTZOVA K., YAROSH D.B., BROWN D.A. Liposome-encapsulated ursolic acid increases ceramides and collagen in human skin cells. Arch. Dermatol. Res., **293**, 569, **2002**.