Original Article

The Effect of *Lamium album* Extract on Cultivated Human Corneal Epithelial Cells (10.014 pRSV-T)

Roman Paduch[1], PhD; Anna Woźniak[2], MD

[1]*Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Lublin, Poland*
[2]*Department of General Ophthalmology, Medical University of Lublin, Lublin, Poland*

Abstract

Purpose: To evaluate the effect of *Lamium album* extract on human corneal epithelial cells (10.014 pRSV-T cell line) cultured *in vitro*.
Methods: Normal human corneal epithelial cells were incubated with ethanol, ethyl acetate and heptane extracts from *Lamium album*. Their effect on cells was evaluated by neutral red (NR) uptake and MTT assays for cytotoxicity, ELISA for immunomodulation, Griess method for nitric oxide levels, DPPH assay for free radicals scavenging activity. A blank control consisted only of culture medium.
Results: In NR and MTT assays, *Lamium album* extracts did not affect cell viability (80% at 125 μg/ml concentration). Ethanol was the least toxic extract (cell viability over 88%) and expressed the most potent reactive oxygen species (ROS) scavenging action. It was 19.88 ± 0.87% higher than controls representing a reduction corresponding to 7.136 μg/ml of trolox. Heptane extract revealed no ROS scavenging activity. All extracts decreased NO production by cells. The most active extract was ethanol (8 μg/ml) which reduced NO level to 0.242 μM (75% decrease compared to control). Extracts influenced pro-inflammatory (IL-1, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines levels reducing all of them in general. The strongest reduction in tested cytokines level was observed by the heptane extract. On the other hand, the ethanol extract induced mainly TNF-α level in a concentration dependent manner.
Conclusion: Selected *Lamium album* extracts influence human corneal epithelial cells. Generally, while not toxic, they modulate pro-inflammatory and anti-inflammatory cytokines levels, and decrease NO release by cells; moreover, ethanol and ethyl acetate extracts reduce ROS levels.

Keywords: Cytotoxicity; Human Corneal Epithelial Cells; Immunomodulation; *Lamium Album* Extracts; Reactive Oxygen Species Scavenging

J Ophthalmic Vis Res 2015; 10 (3): 229-237.

Correspondence to:
Roman Paduch, PhD. Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland.
E-mail: rpaduch@poczta.umcs.lublin.pl

Received: 07-10-2013 Accepted: 25-12-2013

Access this article online

Quick Response Code:

Website: www.jovr.org

DOI: 10.4103/2008-322X.170349

INTRODUCTION

The human cornea, the outer transparent part of the eye, is constantly exposed to the external environment,[1,2] and as a highly specialized organ requires specific conditions to function properly and provide ideal vision.[3] Continuous exposure of the corneal epithelium

How to cite this article: Paduch R, Woźniak A. The effect of *Lamium album* extract on cultivated human corneal epithelial cells (10.014 pRSV-T). J Ophthalmic Vis Res 2015;10:229-37.

to stressors including mechanical injury, chemical and biological toxins, bacterial and fungal infections, and radiations such as ultraviolet light, may adversely affect eye function and even lead to loss of vision.[2] Therefore, any inflammation and damage to the corneal epithelial lining should be quickly but gently eliminated to maintain corneal transparency. Corneal epithelial wound healing and integrity chiefly depend on cell proliferation from healthy areas.[3] Moreover, paracrine interactions among cells and the influence of microenvironmental elements such as cytokines, growth factors or reactive oxygen species (ROS) are similarly of high significance. Ocular diseases should be treated in the most delicate way and meanwhile in a quickly and effective manner. In general, ophthalmological medications are administered topically and besides chemical drugs, at times single herb or herbal mixtures are used as effective ingredients in eye drops. Polyherbal eye drops (Itone™), a mixture of nineteen natural ingredients is an example that possesses anti-inflammatory, antioxidant and anti-angiogenic properties which can be potentially used in neovascular or inflammatory eye diseases in human.[4] Moreover, in some cases, β-1,3-glucan isolated from *Euglena gracilis* suppressed acute inflammation and induced wound healing by inducing corneal epithelial cell migration.[5] Some limitations associated with commercially used drugs may be circumvented by the multiple action of herbal or natural medicines.[6] The beneficial effects of botanical compounds on major eye diseases including age-related macular degeneration (AMD), glaucoma, cataract, retinal disorders such as retinitis pigmentosa or diabetic retinopathy have recently been examined.[7] Biochemical properties of plant extracts and health benefits after their application have persuaded scientists and ophthalmologists to assess and use them increasingly in medical protocols.

Lamium album (white deadnettle) is a perennial flowering plant in the Lamiaceae used in folk medicine widely as a hemostatic, anti-inflammatory, antispasmodic or immunoprotective agent and even for treatment of trauma.[8,9] This broad spectrum of pharmacological activity is the result of biologically active substances such as flavonoids, saponins, phenolic acids, iridoids, terpenes, mucilages, polysaccharides and tannins.[9,10] Due to the different biological features of these substances, potential ophthalmological formulations should be tested to specify their activity and complications both *in vitro* and *in vivo*.[11] Lamiaceae family plant extracts are currently tested as formulations reducing intraocular pressure and are used in traditional medicine for treatment of eye and skin infections.[12,13]

The present study was conducted to evaluate the cytotoxic, anti-inflammatory and anti-oxidant effects of ethanol, ethyl acetate and heptane extracts of *Lamium album* on cultivated human corneal epithelial cells (10.014 pRSV-T cell line).

METHODS

Plant Material

A voucher specimen of *Lamium album* is deposited at the Department of Pharmaceutical Botany in the Medical University of Lublin, Poland. The strict plant material was obtained in summer 2010 from the botanical garden of Maria Curie-Skłodowska University in Lublin, Poland. The plant was identified by Professor T. Krzaczek (Medical University of Lublin). After collection, the plants were washed and dried at 40°C.

Extract Preparation

The materials for testing were ethanol, ethyl acetate and heptane extracts, derived from the White Deadnettle (*Lamium album* L. [Lamiaceae]). Dried and pulverized material was weighed in portions of five gram, and then exhaustive extraction was carried out in an ultrasonic bath (Bandelin, Sonorex, Germany) (U, 230 V 50/50 Hz; –1.2 A, P, 120/480W; *f*, 35 kHz; temp, 20-80°C). Raw material underwent fourfold extraction under the following conditions:

- I - 15 min extraction using 100 ml of ethanol 90% in 45°C
- II - 15 min extraction using 100 ml of ethanol 90%
- III - 15 min extraction with 70 ml of ethanol 90%
- IV - 15 min extraction with 70 ml of ethanol 90%.

Each time the extract was decanted from above the raw material. After the extraction process, all four solutions were combined, then evaporated to dryness in a rotary vacuum evaporator IKA RV 05-ST 1 (IKA-Werke, Staufen, Germany) at 35°C. The resultant dry residue was used in biological research tests. The procedure of preparing two other extracts, ethyl acetate and heptane, from white deadnettle proceeded similarly but using 100% solutions.

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 concentration of applied plant extract did not exceed 0.25% with no influence on 10.014 pRSV-T cell viability in culture medium, consistent with our previous tests.[14,15]

Cell Culture

Normal human corneal epithelial cell line 10.014 pRSV-T (ATCC No. CRL-11515) was used for the experiments. The cells were cultured as monolayers in 25 cm^2 culture flasks (Nunc. Roskilde, Denmark) coated

with PureCol™ ultrapure collagen (INAMED Biomaterials, Fremont, CA, USA) at 3.1 mg/ml concentration. Cell lines were maintained in defined keratinocyte-serum free medium (K-SFM) (Gibco, Gibco™, Paisley, UK) supplemented with 75 µg/ml endothelial cell growth factor (ECGF) (Sigma-Aldrich Co., St. Louis, MO, USA), 0.05 mg/ml bovine pituitary extract (BPE) (Gibco, Gibco™, Paisley, UK), 500 ng/ml hydrocortisone (Sigma-Aldrich Co., St. Louis, MO, USA) and 0.0005 mg/ml bovine insulin (Gibco, Gibco™, Paisley, UK) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) (Sigma-Aldrich Co., St. Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO_2.

Incubation of Cells with *Lamium album* Extracts

The total number of cells for the experiments was estimated by counting them in a Thomas hemocytometer. A dose of 100 µl of cell suspension (1×10^5 cells/ml) was added to the wells of 96-well flat-bottomed microtitre plates (MTT and NR methods). After 24 hours of incubation, the medium was discarded and a new one was added, with appropriate concentrations of extracts. Serving as controls and also incubated for 24 hours, untreated cells were cultured in 100 µl of medium with a total cell number equivalent to that of in the sample wells. A blank control consisted only of culture medium.

Incubation was performed for 24 hours, and cytotoxicity and anti-proliferative activity of the extracts was estimated using spectrophotometric methods, i.e. MTT and NR assays.

Neutral Red (NR) Uptake Assay

NR cytotoxicity assay is based on the uptake and lysosomal accumulation of the supravital dye, neutral red. Dead or damaged cells do not take up the dye.[16] Cells were grown in 96-well multiplates in 100 µl of culture medium (K-SFM) with supplements and *Lamium album* extracts at three doses of 25, 75 and 125 µg/ml and standards. Subsequently, the medium was discarded and 0.4% NR (Sigma-Aldrich Co., St. Louis, MO, USA) solution medium was added to each well. The plate was incubated for 3h at 37°C in a humidified 5% CO_2/95% air incubator. After incubation, the dye-containing medium was removed, the cells were 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 shaken for 20 min at room temperature and the extracted dye absorbance was measured using a spectrophotometer at 540 nm using a microplate reader (Molecular Devices Corp., Emax, Menlo Park, CA, USA).

MTT Assay

Cells sensitivity to *Lamium album* extracts was determined in a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann's study.[17] MTT test is based on conversion of yellow tetrazolium salt by viable cells to purple crystals of formazan. The reaction is catalyzed by mitochondrial succinyl dehydrogenase.

Cells grown in 96-well multiplates in 100 µl of culture medium were incubated for three hours with MTT solution (5 mg/ml, 25 µl/well) (Sigma-Aldrich Co., St. Louis, MO, USA). The yellow tetrazolium salt was metabolized by viable cells to purple formazan crystals by mitochondrial succinyl dehydrogenase. The crystals were immediately solubilized in 10% sodium dodecyl sulfate (SDS) in 0.01M HCl mixture. The product was quantified spectrophotometrically by absorbance measurement at 570 nm wavelengths using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

DPPH Free Radical Scavenging Test

Free radical scavenging activity 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-Aldrich Co., St. Louis, MO, USA) to the yellow colored diphenyl-picrylhydrazine. Briefly, 100 µl of DPPH solution (0.2 mg/ml in ethanol) was added to 100 µl of extracts at three concentrations, 25, 75 and 125 µg/ml and standards. Trolox (Sigma-Aldrich Co., St. Louis, MO, USA) at increasing concentrations (1-50 µg/ml) was used as a standard for free radical scavenging activity. After 20 minutes incubation at room temperature, the absorbance of the solution was measured at 515 nm. The lower the absorbance, the higher the free radical scavenging activity of the extracts. The activity of each extract was determined by comparing its absorbance with that of a control solution (reagents without extract) and standards.

The capability to scavenge DPPH radical was calculated by the following formula: DPPH 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 extracts.[14]

Nitric Oxide (NO) Measurement

Nitrate, a stable end product of NO, was determined in culture supernatants by a spectrophotometric method based on the Griess reaction. The level of nitrite reflects NO production.[18] In the current study, corneal epithelial cells were incubated for 24 hours with two concentrations of *Lamium album*, 8 and 20 µg/ml, and then culture supernatants were collected. Afterwards, 100 µl of the supernatant was plated in 96-well flat-bottomed plates in triplicate and incubated with 100 µl of Griess reagent (1% sulphanilamide/0.1% N-(1-naphthyl)

ethylenediamine dihydrochloride) (Sigma-Aldrich Co., St. Louis, MO, USA) in 3% H_3PO_4 (POCH, Gliwice, Poland) at room temperature for 10 min. The optical density was measured at 550 nm using a microplate reader (Molecular Devices Corp., Emax, Menlo Park, CA, USA). A standard curve was achieved using 0.5-25 μM sodium nitrite ($NaNO_2$) for calibration.

ELISA Assay

Levels of human IL-1β, IL-6 and TNF-α were tested immuno-enzymatically (ELISA) in culture supernatants using commercially available kits (Diaclone, Besançon cedex, France) according to the manufacturer's instruction. The optical density at 450 nm with the correction wavelength of 570 nm of each ELISA sample was determined using a microplate reader. IL-1β, IL-6 and TNF-α concentrations were calculated on the basis of a standard curve with detection limits of 7 pg/ml, 2 pg/ml and 8 pg/ml, respectively.

Labeling of Cytoskeleton F-actin

Phalloidin staining is a useful means for investigating the distribution of F-actin in cells.[19] Cells were incubated in 4-well Lab-Tek chamber slides (Nunc. Roskilde, Denmark) in 1 ml culture medium with plant extracts. After incubation, the cells were rinsed with K-SFM medium and exposed to paraformaldehyde 10% (v/v) solution for 20 min, rinsed three times in phosphate buffered saline (PBS), exposed to Triton X-100 0.2% (v/v) (Sigma-Aldrich Co., St. Louis, MO, USA) 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-Aldrich Co., St. Louis, MO, USA) was incubated to each well in the dark at 37°C/5% CO_2 for 30 min. Cell observation was conducted under a fluorescence microscope (Olympus, BX51, Olympus Optical Co. Ltd., Tokyo, Japan). Quantitative analysis of fluorescent images was performed by an analysis imaging software system.

Statistical Analysis

Results are presented as mean±(standard deviation) SD from three experiments. In each experiment, 4-8 replicates were performed. Data were analyzed using one-way analysis of variance (ANOVA) with Dunnett *post hoc* test. Differences of P values ≤0.01 were considered as statistically significant.

RESULTS

Cytotoxicity and Anti-proliferative Activity of *Lamium album* Extracts

Neutral Red (NR) uptake and MTT assays were performed to respectively analyze cytotoxic and proliferation inhibitory effects of *Lamium album* extracts on human corneal 10.014 pRSV-T epithelial cells after 24 hours of incubation. The cells were seeded at 1×10^5 cells/ml density.

The NR uptake and MTT assays showed that the ethanol extract was the least toxic and thus the most promising preparation for possible clinical application. Viability of corneal epithelial cells did not decrease below 88% at the applied concentrations. The remaining extracts progressively reduced corneal epithelial cell viability, the levels of which were lower than 80% at the highest concentration (125 μg/ml) depending on the method of analysis [Figure 1a and b].

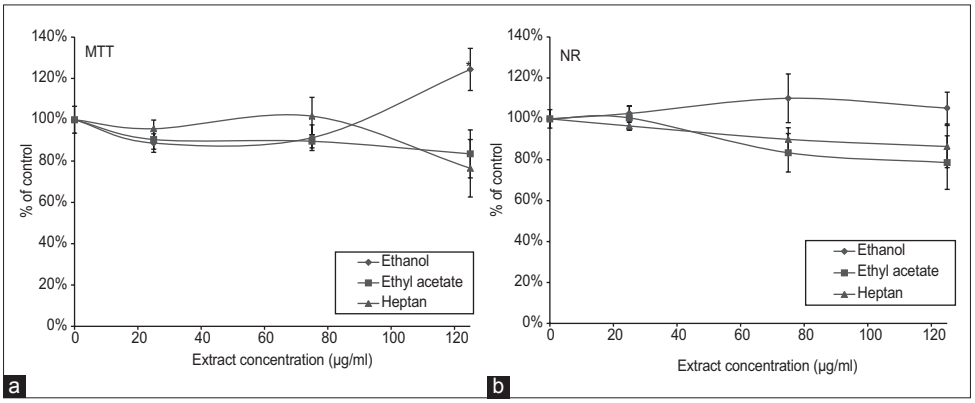

Figure 1. The effect of 24 hour incubation of 25, 75 and 125 μg/ml of ethanol, ethyl acetate and heptane *Lamium album* extracts on human corneal epithelial cells (line 10.014 pRSV-T) as compared to a non-treated culture control. The MTT assay (a) and neutral Red assay (NR). (b) The results are presented as a percentage of the controls, arbitrarily set to 100% showing an average of three independent experiments. Bars show mean±standard deviation (n=3). *$P \leq 0.01$. In each of three experiments, 8 replicates were performed.

Free Radical Scavenging Action of the Extracts Using DPPH Test

The reactive oxygen species (ROS) reducing effect of *Lamium album* is presented in Table 1. The analysis was performed using stable radical (DPPH) reduced by *Lamium* active compounds and thereafter the effect was read by a spectrophotometer. The greatest ROS scavenging action was observed for the ethanol extract. The effect was concentration dependent and was 19.88 ± 0.87% greater than the control at the highest analyzed concentration of 125 µg/ml. The effect of the ethyl acetate extract was approximately 2.5 times weaker than that of the ethanol extract (8.03 ± 1.0% as compared to the control) at an extract concentration of 125 µg/ml. The heptane extract of *Lamium album* revealed no ROS scavenging action.

Nitric Oxide (NO) Production

The control level of NO was 0.973 µM [Figure 2]. Two concentrations of Lamium extracts including 8 and 20 µg/ml were used. After spectrophotometric analysis of nitrate, a stable end product of NO, it was found that all extracts led to a decrease in NO level produced by corneal epithelial cells during 24 hours. The most active extract was 8 µg/ml ethanol which reduced the radical level by 75% as compared to non-treated controls. On the other hand, the higher concentration of 20 µg/ml decreased NO level by about 6%. However, the ethyl acetate extract showed a reverse effect in which applying a higher concentration led to a stronger reducing effect of NO. For 8 µg/ml, NO was reduced by about 25%, while for 20 µg/ml by about 55%, as compared to controls. Heptane extract reduced NO levels by about 40% for both tested extract concentrations.

Table 1. Reduced free radical scavenging activity (%) by *Lamium album* extracts in three different concentrations as compared to the control (0% of reduction)

Extract	Extract concentration (µg/ml)	Percentage of reduction as compared to control (0% of reduction)	Reduction value, corresponding to the following trolox concentration (µg ml)
Ethanol	25	0	0
	75	7.7±0.6	2.618
	125	19.88±0.87	7.136
Ethyl acetate	25	0	0
	75	2.21±1.0	0.575
	125	8.03±1.0	2.726
Heptane	25	0	0
	75	0	0
	125	0	0

ELISA Tests for Pro-inflammatory Cytokines (IL-1β, IL-6, TNF-α) Levels

The level of IL-1β released by control, non-treated 10.014 pRSV-T cells was 18.81 pg/ml. The addition of *Lamium album* extracts decreased the cytokine level. The strongest effect was produced by heptane extract when IL-1β level was reduced to 11.81 pg/ml and 13.06 pg/ml after employing 8 µg/ml and 20 µg/ml of the extract, respectively. On average, IL-1β was 66% of the control level. Both ethanol and ethyl acetate extracts reduced IL-1β levels approximately by 79% of the controls [Figure 3a].

IL-6

The control level of IL-6 was 17.06 pg/ml. After addition of *Lamium album* extracts, in the case of ethanol and ethyl acetate extracts, the lower concentrations inhibited IL-6 production stronger. However, though the extracts generally reduced IL-6 level, heptane with 8 µg/ml concentration stimulated this cytokine production to a level of 20.27 pg/ml (18.9% above the control) [Figure 3b].

TNF-α

Non-treated human corneal epithelial cells released 17.46 pg/ml of TNF-α accepted as a control level. Ethanol extract of *Lamium album* induced TNF-α production by these cells in a concentration dependent manner. At 20 µg/ml concentration, TNF-α level was 34.86 pg/ml, i.e. two times higher than the control. On the other hand,

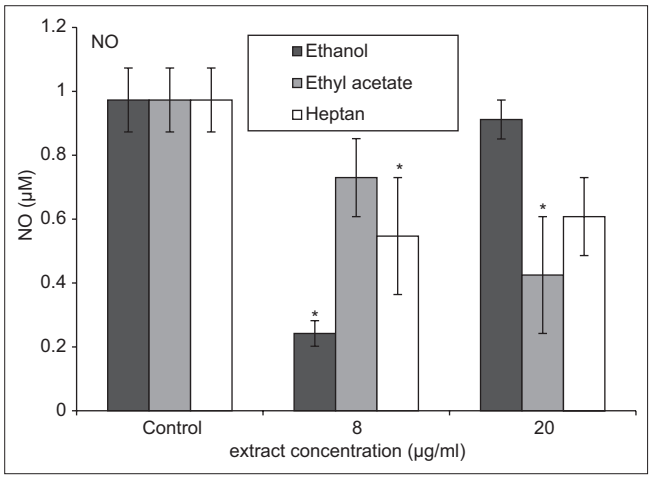

Figure 2. Nitric oxide (NO) secretion in the culture of 10.014 pRSV-T normal human corneal epithelial cells after 24 hours of incubation with 8 and 20 µg/ml of ethanol, ethyl acetate and heptane extracts of *Lamium album* compared to a non-treated culture control. Analysis was performed using the Griess method. Columns and bars show mean±standard deviation (n=3). *$P \leq 0.01$ in each of three experiments, 6 replicates were performed.

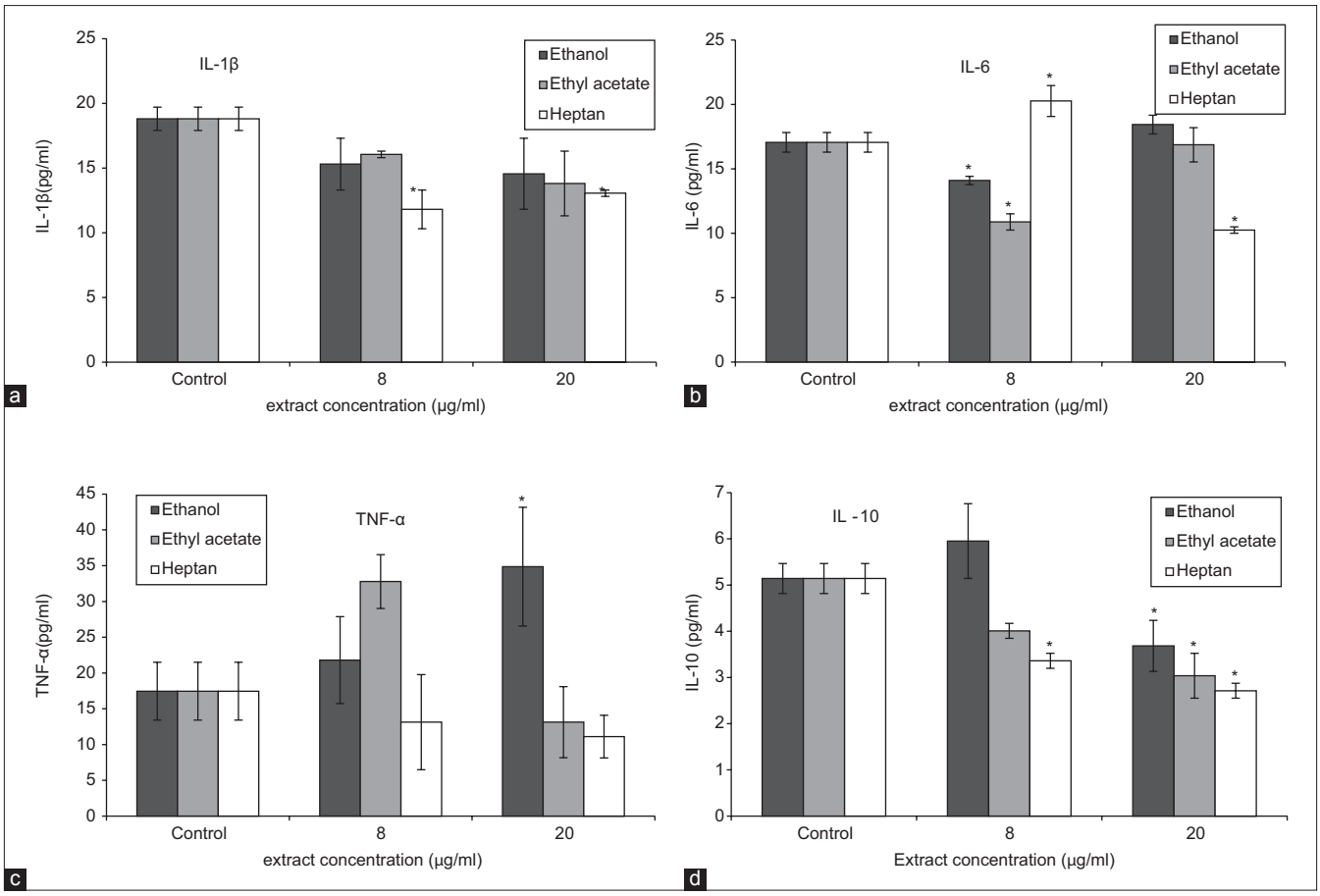

Figure 3. IL-1β (a), IL-6 (b), TNF-α (c) and IL-10 (d) secretion in culture of 10.014 pRSV-T normal human corneal epithelial cells after 24 hours of incubation with 8 and 20 μg/ml of ethanol, ethyl acetate and heptane extracts of *Lamium album* compared to a non-treated control culture. ELISA test. *$P \leq 0.01$ - in each of three experiments, 4 replicates were performed.

the ethyl acetate extract decreased TNF-α production in a concentration dependent manner. However, the lower extract concentration (8 μg/ml) induced cytokine release to a level of 32.78 pg/ml (87.7% above the control) while the 20 μg/ml concentration decreased its production to a level of 13.13 pg/ml (25% lower than the control). Heptane extract in both applied concentrations inhibited TNF-α release [Figure 3c].

Anti-inflammatory Cytokine (IL-10) Level

The level of IL-10, produced by non-treated corneal epithelial cells (as the control level), was 5.142 pg/ml. The administration of higher extract concentrations resulted in less IL-10 production. However, when the ethanol extract with 8 μg/ml concentration was added, the level of the cytokine was 15.8% higher (5.952 pg/ml) as compared to the control [Figure 3d].

Cytoskeletal F-actin Filament Organization

F-actin filaments were analyzed using TRITC-phalloidine fluorescent staining method after 24 hours incubation of 10.014 pRSV-T cells with 50 μg/ml of *Lamium album* extracts. Control cells are presented in Figure 4a. After incubation of cells with the ethanol extract there were no cytoskeletal changes [Figure 4b]. Ethyl acetate [Figure 4c] and heptane extracts [Figure 4d] revealed slight intercellular adhesion violations as compared to controls.

DISCUSSION

In the current study, the beneficial effects of *Lamium album* extracts were evaluated as a possible natural protective agent for human corneal epithelial cells. All the tests were done *in vitro* which are considered as preliminary but continually developing settings. They also reflect the structure of the original tissue and may replace isolated animal corneas for analysis of drugs or natural compounds activity.[20] The *in vitro* culture of corneal epithelial cells enables one not only to estimate their sensitivity to common substances presenting in and contacting the eye surface, but also to establish expression of genes, released substances or general function under defined conditions. Moreover, cell culture is an accepted substitute for animal models, thus its application reduces the number of

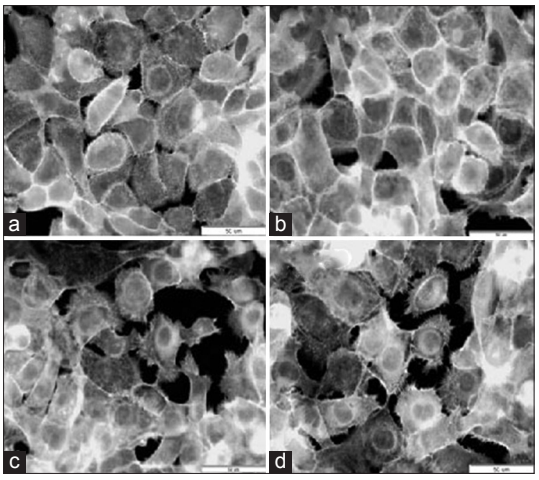

Figure 4. Cytoskeleton organization and reciprocal direct interactions of 10.014 pRSV-T normal human corneal epithelial cells. Control (a), after incubation with 50 µg/ml of ethanol (b), ethyl acetate (c) and heptane extracts (d). TRITC-phalloidin fluorescent staining. Magnification ×400. Bar 50 µm. In each of the three experiments, 4 independent images were obtained.

animal experiments which is an important aspect of cell culture.[21] Regarding drug discovery and analysis, cell cultures are an advantageous means for identification of chemical and natural substances and evaluation of their pharmacokinetic properties. Before registration or transition into further stages of examination, every drug or natural compound has to undergo numerous toxicological tests.[22] In the present study, the ethanol extract of *Lamium album* was shown to be non-toxic to human corneal epithelial cells at concentrations up to 125 µg/ml. This obviously results from the composition of the extract. According to our previous studies, ethanol extract mainly contains polar flavonoids in the form of glycosides, phenolic acids or iridoides.[23] Protocatechuic and caffeic acids in plant extracts, both free and bound as glycosides and ester derivatives, have been determined by HPLC and by HPTLC combined with densitometry and comparable results were obtained although the HPLC results were higher. Although traditional isocratic planar chromatography on silica was not successful, multiple gradient developments enabled satisfactory separation and quantification of the phenolic acids by densitometry.[23] In turn, in ethyl acetate extracts, small amounts of flavonoids in form of aglycones or phenolic acids were identified. HPTLC combined with densitometry was used to determine the content of oleanolic acid in acetone extract from the *Lamii albi flos* L. The plant extract was separated on Si 60 HPTLC plates and defined using two densitometric methods: Measurement of fluorescence and absorbance. The content of oleanolic acid was calculated. In a measurement of absorbance, in 1 g of dry *Lamii albi flos* L., 1458 µg of oleanolic acid was gauged (0.147%) and using the fluorescence method, in 1 g of the plant material, 1516 µg of oleanolic acid was determined (0.149%).[24] Heptane extract of *Lamium album* especially had a high concentration of triterpenes.[14] The biological effect of natural compounds strongly depends not only on their concentration but also on the cell type they influence, their mode of cellular membrane interactions or time of intracellular uptake.[15,25] We showed that polar compounds of *Lamium album* maintain cells intact, or as verified by MTT assay, stimulate cellular mitochondrial metabolism. On the other hand, according to Ward et al[11] substances affecting viability of corneal epithelial cells exert their cytotoxic effect by metabolic activity up-regulation, consistent with our findings in an MTT assay, or influence and damage functional cellular junctions (tight or intermediate) and hence disruption of transcellular and pericellular permeability [Figure 4a and b]. Moreover, when cellular membranes become permeable, they allow easier penetration of plant components during continuous exposure, as an example tetrazolium salt (MTT) dye, into the cells; this is likely to be an explanation for the effect of heptane, and to a lesser degree, ethyl acetate extracts on human corneal epithelial cells viability.

The toxic effects of oxidative processes are of high importance. The cornea, as an external epithelial surface of the eye, is often exposed to high-energy light or radiation generating potentially damaging reactive oxygen species (ROS).[26] Therefore, antioxidants may prevent ocular injury. One possible method for such prevention involves employing plant compounds in the form of eye drops. In our study, we found that ethanol compounds, and to a smaller degree ethyl acetate extracts, express stable radical (DPPH) reducing effects. These observations are in accordance with Matkowski and Piotrowska's[27] results showing that polar methanolic extracts of *Lamium album* possess ROS scavenging and antioxidant properties. Their activity is possibly related to the presence of flavonoids and polyphenolic compounds in these extracts which have proven antioxidant activity.[28] Heptane extract was deprived of these compounds and this could be the probable explanation for lack of ROS scavenging effect with the heptane. Reduction of DPPH clarifies the antioxidant ability of analyzed substances. However, many physiological functions as well as pathological conditions are related to the presence of the short-lived but very reactive nitric oxide (NO) molecule. It may interact with ROS forming cytotoxic compounds (RNOS).[29] Thus, the antioxidant activity of plant compounds should be analyzed on the basis of both ROS reduction and NO changes. We showed that all tested extracts of *Lamium album* decreased NO levels and it could be interpreted, in a defined framework, as an advantageous effect limiting the appearance of additional toxic substances, besides ROS, in the cornea. ROS and RNOS compounds are often involved in the inflammatory stage of many diseases including eye disorders.

Ocular inflammation is related to elevated expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), and not to changed or decreased anti-inflammatory cytokines such as IL-10. The present study revealed that *Lamium album* extracts change cytokine production by human corneal epithelial cells. The level of the produced cytokines depended on both the type and concentration of the extract. Therefore, special care is required in interpretation of our results and possible application in eye inflammatory disease and healing, considering the fact that not only pro-inflammatory, but also anti-inflammatory cytokines may be decreased by *Lamium album* extracts. As a result, the possible anti-inflammatory effect of the extracts may be not as significant as estimated by inhibition of pro-inflammatory cytokines. However, *Lamium album* extracts possess immunomodulatory activity which could be utilized in maintaining ocular health by possible supplementation with carefully and well-chosen plant components.

The use of different extracts concentrations for two groups of experiments, which are cytoxicity with free radical scavenging activity and analysis of selected factors (NO and cytokines) production by cells was intended. We showed that at relatively high concentration extracts (25 to 125 μg/ml), no significant cytotoxic effects appeared. On the other hand, low concentration extracts (8 μg/ml and 20 μg/ml) were only used when specific substances secreted by cells were analyzed. The analysis ensured us that during the experiment; we did not influence cell density, viability or metabolism. It further demonstrated that small amounts of active plant ingredients may significantly regulate NO production and exhibit anti-inflammatory activity but this could not be observed when higher concentrations of the extracts were used.

Chemical formulations are currently not sufficient to fully guarantee eye protection against various harmful environmental influences. Therefore, new solutions are required enabling effective but gentle protection. One possibility is the use of pharmaceuticals originating from herbal sources. Meanwhile, there is a growing demand for natural medicinal herbs which are believed to be less toxic than manufactured substaces.[30]

In summary, the ethanol extract of *Lamium album*, differently from extracts of ethyl acetate and heptane, is not toxic for human corneal epithelial cells *in vitro.* In addition, being a relatively suitable ROS scavenger, it may protect cells against the toxic effect of these chemically reactive molecules. Moreover, it exerts immunomodulatory activity by changing pro-and anti-inflammatory cytokines level. Therefore, it may be possible that, after a series of additional tests followed by *in vivo* trials, selected *Lamium album* extracts be introduced into practice as supplements or even medicine for treatment of ocular diseases.

Financial Support and Sponsorship

Nil.

Conflicts of Interest

There are no conflicts of interest.

REFERENCES

1. Huhtala A, Salminen L, Tähti H, Uusitalo H. Corneal models for the toxicity testing of drugs and drug releasing materials. In: Ashammakhi N, editor. Topics in Mulitifunctional Biomaterials and Devices. Vol. 1. Finland: University of Oulund; 2008. p. 1-23. Available from: http://www.oulu.fi/spareparts/ebook_topics_multifunctional/abstracts/huhtala.pdf.
2. Mohan RR, Possin DE, Mohan RR, Sinha S, Wilson SE. Development of genetically engineered tet HPV16-E6/E7 transduced human corneal epithelial clones having tight regulation of proliferation and normal differentiation. *Exp Eye Res* 2003;77:395-407.
3. Huo Y, Qiu WY, Pan Q, Yao YF, Xing K, Lou MF. Reactive oxygen species (ROS) are essential mediators in epidermal growth factor (EGF)-stimulated corneal epithelial cell proliferation, adhesion, migration, and wound healing. *Exp Eye Res* 2009;89:876-886.
4. Velpandian T, Gupta P, Ravi AK, Sharma HP, Biswas NR. Evaluation of pharmacological activities and assessment of intraocular penetration of an ayurvedic polyherbal eye drop (Itone™) in experimental models. *BMC Complement Altern Med* 2013;13:1.
5. Choi JA, Oh TH, Choi JS, Chang DJ, Joo CK. Impact of ß-1,3-glucan isolated from *Euglena gracilis* on corneal epithelial cell migration and on wound healing in a rat alkali burn model. *Curr Eye Res* 2013;38:1207-1213.
6. Singh D, Chauhan N, Sawhney SS, Painuli RM. Biochemical characterization of triphala extracts for developing potential herbal drug formulation for ocular diseases. *Int J Pharm Pharm Sci* 2011;3 Suppl 5:516-523.
7. Huynh TP, Mann SN, Mandal NA. Botanical compounds: Effects on major eye diseases. *Evid Based Complement Alternat Med* 2013;2013:549174.
8. Alipieva KI, Taskova RM, Jensen SR, Handjieva NV. Iridoid glucosides from *Lamium album* and *Lamium maculatum* (*Lamiaceae*). *Biochem Syst Ecol* 2006;34:88-91.
9. Petukhova NM, Buryakina AV, Avenirova EL, Burakova MA, Drozhzhina EV. Studies of the biological activity of an oil extract of the snakeflower *Lamium album*. *Pharm Chem J* 2008;42:354-356.
10. Budzianowski J, Skrzypczak L. Phenylpropanoid esters from *Lamium album* flowers. *Phytochemistry* 1995;38:997-1001.
11. Ward SL, Walker TL, Dimitrijevich SD. Evaluation of chemically induced toxicity using an *in vitro* model of human corneal epithelium. *Toxicol In Vitro* 1997;11:121-139.
12. Hussien J, Hymete A, Rohloff J. Volatile constituents and biological activities of *Pycnostachys abyssinica* and *Pycnostachys eminii* extracts. *Pharm Biol* 2010;48:1384-1391.
13. Wagh VD, Wagh KV, Inamdar B, Samanta MK. The effect of forskolin ophthalmic inserts on intraocular pressure in rabbit eyes. *Int J Pharm Pharm Sci* 2009;2:146-155.
14. Paduch R, Matysik G, Wójciak-Kosior M, Kandefer-Szerszeń M, Skalska-Kamińska A, Nowak-Kryska M, et al *Lamium album* extracts express free radical scavenging and cytotoxic activities. *Pol J Environ Stud* 2008;17:569-580.
15. Paduch R, Wójciak-Kosior M, Matysik G. Investigation of biological activity of *Lamii albi flos* extracts. *J Ethnopharmacol* 2007;110:69-75.

16. Ganbold M, Barker J, Ma R, Jones L, Carew M. Cytotoxicity and bioavailability studies on a decoction of *Oldenlandia diffusa* and its fractions separated by HPLC. *J Ethnopharmacol* 2010;131:396-403.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
18. Muzitano MF, Bergonzi MC, De Melo GO, Lage CL, Bilia AR, Vincieri FF, et al Influence of cultivation conditions, season of collection and extraction method on the content of antileishmanial flavonoids from *Kalanchoe pinnata*. *J Ethnopharmacol* 2011;133:132-137.
19. Chang HR, Huang HP, Kao YL, Chen SL, Wu SW, Hung TW, et al The suppressive effect of Rho kinase inhibitor, Y-27632, on oncogenic Ras/RhoA induced invasion/migration of human bladder cancer TSGH cells. *Chem Biol Interact* 2010;183:172-180.
20. Reichl S. Cell culture models of the human cornea - A comparative evaluation of their usefulness to determine ocular drug absorption *in-vitro*. *J Pharm Pharmacol* 2008;60:299-307.
21. Toropainen E, Ranta VP, Talvitie A, Suhonen P, Urtti A. Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest Ophthalmol Vis Sci* 2001;42:2942-2948.
22. Tavaszi J, Budai P. The use of HET-CAM test in detecting the ocular irritation. *Commun Agric Appl Biol Sci* 2007;72:137-141.
23. Wójciak-Kosior M, Matysik G, Soczewiński E. High-performance thin-layer chromatography combined with densitometry for quantitative analysis of phenoloc acids in complex mixtures. *J Planar Chromatogr* 2006;19:21-26.
24. Wójciak-Kosior M, Krzaczek T, Matysik G, Skalska A. HPTLC-densitometric method of determination of oleanolic acid in the *Lamii albi flos*. *J Sep Sci* 2005;28:2139-2143.
25. Spencer JP, Abd-el-Mohsen MM, Rice-Evans C. Cellular uptake and metabolism of flavonoids and their metabolites: Implications for their bioactivity. *Arch Biochem Biophys* 2004;423:148-161.
26. Bartlett H, Eperjesi F. An ideal ocular nutritional supplement? *Ophthalmic Physiol Opt* 2004;24:339-349.
27. Matkowski A, Piotrowska M. Antioxidant and free radical scavenging activities of some medicinal plants from the *Lamiaceae*. *Fitoterapia* 2006;77:346-353.
28. Trouillas P, Calliste CA, Allais DP, Simon A, Marfak A, Delage CH, et al Antioxidant, anti-inflammatory and antiproliferative properties of sixteen water plant extracts used in the Limousin countryside as herbal teas. *Food Chem* 2003;80:399-407.
29. Paduch R, Kandefer-Szerszen M, Piersiak T. The importance of release of proinflammatory cytokines, ROS, and NO in different stages of colon carcinoma growth and metastasis after treatment with cytotoxic drugs. *Oncol Res* 2010;18:419-436.
30. Paduch R, Matysik G, Wójciak-Kosior M, Niedziela P. The biological activity of medicinal plant extract. In: Govil JN, Singh VK, Siddiqui NT, editors. Natural Products II of the Series Recent Progress in Medicinal Plants. Vol. 18. Houston, USA: Studium Press LLC; 2007. p. 127-144.