Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
2017, volume 102, livre 4, pp. 194-204
Youth Scientific Conference "Kliment's Days", Sofia 2016

COMPARATIVE ANALYSIS OF CYTOTOXICITY OF CHLOROFORM EXTRACTS FROM *LAMIUM ALBUM* L. AND MORPHOLOGICAL ALTERATIONS IN MAMMALIAN CELLS

RALITSA VELEVA[1], VESELINA MOSKOVA-DOUMANOVA [1],
DANIELA DRAGOLOVA[2], JORDAN DOUMANOV[3],
TANYA TOPOUZOVA-HRISTOVA[1]*

1 – Department of Cytology, Histology and Embryology, Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria
2 – Department of Plant Physiology, Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria
3 – Department of Biochemistry, Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria
**Corresponding author: topouzova@biofac.iuni-sofia.bg*

Keywords: *Lamium album* L., cytotoxicity, chloroform extracts

Abstract: *Lamium album* L. is a plant commonly used in traditional medicine because of its anti-inflammatory, astringent, antiseptic and antispasmodic activities. There are differences in the composition of secondary metabolites in plants grown in their natural habitats (*in vivo*), micropropagated plants (*in vitro*) and after their subsequent cultivation in a natural environment (*ex vitro*). In this study we aim to compare the activity of chloroform Soxhlet extracts from *Lamium album* plants, collected from a wild population, cultivated *in vitro* and adapted *ex vitro* on A549 human cancer cell line and the non-cancer kidney epithelial cells MDCKII. We have performed cell cytotoxicity test (crystal violet assay) and membrane permeability test (trypan blue exclusion assay) as well as morphological analysis of changes induced by the extracts.

Upon treatment with extract *ex vitro* a large percentage of cells with damaged membranes at 24 hours has been observed. After 48 hours the total number of cells has been reduced, but the cells with damaged membranes have a relatively small rate. For comparison, 48 hours after treatment with *in vitro* extract, 100% damaged A549 cells has been observed.

INTRODUCTION

Medicinal plants are appreciated for their beneficial impact on human health. *Lamium album* (white dead-nettle) is widely used in folk medicine because of its properties – antioxidant, antispasmodic, anti-inflammatory and astringent. It also possess bacteriostatic and antibiotic activities and prevents haemorrhagia (from the lungs and uterus). It is used for treatment of urinary bladder and kidney problems (Alipieva et al., 2003, Yordanova et al., 2014). Some of the health – related beneficial activities of extracts of *L. album* have been scientifically proven. *In vitro* investigations have indicated antiviral, antimicrobial, antioxidant, anticancer, cytoprotective, wound healing and other pharmacological effects (Xu F, 2008, Chipeva et al., 2013, Yordanova et al., 2014). Extracts from white dead nettle are found to exhibit anti-HCV entry activity and significant inhibition of HSV-1 and HSV-2 replication (Zhang et al., 2009, Todorov et al., 2013). Cytoprotective effect of purified ethanol extracts of *L. album* plants has been demonstrated against potassium dichromate-induced toxicity in hepatoblastoma Hep G2 cell model (Pereira et al., 2013). The available *in vitro* investigations are still insufficient to fully determine the pharmacological effects of different extracts from *L. album*.

Extensive use of herbs could endanger the existence of their populations in nature. Preservation of medicinal plant could be done by *in vitro* propagation to avoid depletion from their natural habitat. There are differences in the composition of secondary metabolites in plants grown in their natural habitats (denoted as "*in vivo"*), micropropagated plants (denoted as "*in vitro"*) and after their subsequent cultivation in a natural environment (denoted as "*ex vitro"*) (Kapchina-Toteva et al., 2014). It is important to examine how these differences affect the biological activity of plant extracts. In this study we aim to compare the activity of chloroform Soxhlet extracts from *L. album* cultivated diversely.

MATERIALS AND METHODS

All reagents and chemicals were supplied by Sigma-Aldrich (FOT, Sofia, Bulgaria) unless otherwise stated.

Plant material

L. album plants were collected from their natural habitat in the Lozen Mountain, Sofia, Bulgaria. The voucher specimen SO 105183 was deposited in the Herbarium of Sofia University "St. Kliment Ohridski".

For *in vitro* cultivation, mono-nodal 1–2 cm stem segments from the above-ground material of the plants grown in their natural habitats were cultivated on basal Murashige and Skoog medium (MS) (Murashige & Skoog, 1962) containing 3% (w/v) sucrose and 7 g/L agar without any supplement of growth regulators. (Dimitrova et al., 2010, Kapchina-Toteva et al., 2014). For further *ex vitro* acclimation, the *in vitro*-grown plantlets were removed from the culture

tubes and transferred into plastic pots containing a mixture from sterile soil and peat. A one-month acclimation was maintained in a growth chamber. Next, the acclimated plants were transferred to greenhouse for another month followed by transfer to normal garden soil in the field of Lozen mountain, near Sofia. After one year of acclimation to the field conditions, newly formed fully expanded leaves of *ex vitro* plants were harvested. The *in situ* (wild) and *ex vitro* plants were grown simultaneously (Kapchina-Toteva et al., 2014).

Soxhlet extraction

Samples of 3 g from powdered above-ground material of whole *L. album* plants were extracted by Soxhlet extraction with 30 mL chloroform for 8 h until fully colourless and then the same plant materials were used for second extraction with methanol. Solvents were removed by rotary evaporation and drying (Valyova et al., 2011).

The extracts were dissolved in 250 μL dimethylsulfoxide (DMSO). Final concentration of extracts in medium was presented in mg/mL.

Cell cultures

We have used the non-cancer canine kidney epithelial cells (MDCK II) and human lung carcinoma cell line (A549) for this research. Cells were grown in 25 cm^2 'CELLSTAR®' flasks, at standard conditions in humidified atmosphere with 5% CO_2, at 37 °C, in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) antibiotic–antimycotic solution (penicillin 100 U/mL, streptomycin 100 μg/mL and amphotericin B 0.25 μg/mL). Cells were plated on Nunk™ 96-well plates and 24-well plates depending on the tests to be performed. Treatment was carried out for 24 and 48 h with plant extracts diluted in the culture medium at final concentration of 1 mg/mL.

Morphological and cell viability tests

Crystal violet assay

For determination of cytotoxicity we have treated cells for 24 and 48 hours with extracts from plants grown in their natural habitats (denoted below as "*in vivo* extracts") and *ex vitro* (denoted below as "*ex vitro* extracts") at concentrations 1mg/mL. The cytotoxic activity of the extracts was determined by crystal violet assay. On the 24th/48th hour after treatment the cell monolayers were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde solution in PBS for 20 min. Plates were washed with distilled water and 1% crystal violet solution was added to every well for 20 min at room temperature. The samples were washed, air dried and the protein-bound dye (which is corresponding to the cell number) was solubilized with 10 % acetic acid.

The optical density of each sample was measured spectrophotometrically at $\lambda = 570$ nm, by Epoch Microplate Spectrophotometer with the Gen5 Data Analysis software. Two types of controls were performed: 1) cells incubated at the same conditions without extracts and 2) with added DMSO at the highest concentration

we have used to dissolve the extracts. The values were calculated as per cent of control 1 (cells incubated at the same conditions, but without extracts) and IC_{50} was determined where it is applicable. IC_{50} values were estimated with Microsoft Excel, using mean values of triplicate experiments.

Trypan blue exclusion test

Loss of the integrity of plasma membrane can be demonstrated using polar dyes, such as trypan blue, which are excluded by an intact membrane. After incubation, the samples were washed with PBS and stained *in situ* with 200 µL 0.4% trypan blue in PBS per well. After washing the samples were observed under inverted microscope (XDS-2A, China) supplied with a digital camera (DV-130, China) and several microphotographs of each well were taken.

The assessment of cell morphology

Was obtained through crystal violet staining. Prior to staining, the cells were treated with the plant extracts and fixed with 4% formaldehyde solution. Plates were washed with distilled water and 1% crystal violet solution was added to every well for 20 min at room temperature. After washing, the cells were observed under inverted microscope and micrographs were taken.

RESULTS AND DISCUSSION

Cytotoxicity of plant extracts

The influence of the extraction solvent (chloroform) on the cells is negligible, because the resulting extracts were dried completely. In the controls with DMSO in maximal used concentration (as the intermediate solvent of dried extracts) reduction in cell growth was less than 10 %. Further we have compared the activity of chloroform Soxhlet extracts obtained from *L. album* plants, collected from a wild population and from acclimated *ex vitro* plants during 24 and 48 hours of treatment. The *in vivo* extract didn't show significant cytotoxic activity on the non-cancerous cell line. Even on the 48th hour of treatment only 10-15% decrease in viability was detected as compared to 24th hour for both tested cell lines – there were near 75% viable MDCK II cells and under 50% A549 (Fig. 1).

Similar results were obtained from treatment for 24 hours with *ex vitro* extracts. After one day of application of both cell lines were slightly affected (viability respectively 70.55 % for A549 and 65.25% for MDCK II cells, Fig. 1). After 48 hours the treatment led to considerable change in the vitality of the normal cells (only 30% survived), while for the cancerous cells this index was up to 55%.

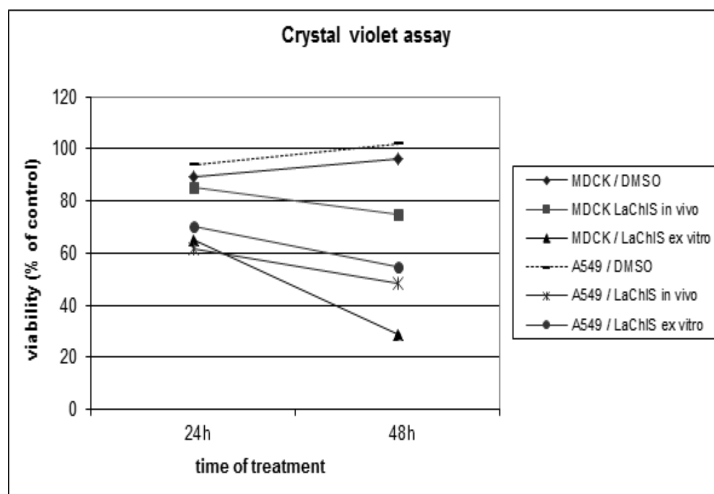


Fig. 1. Cytotoxicity of chloroform Soxhlet extracts obtained from *L. album* plants, grown *in vivo* in their natural habitat (LaChlS *in vivo*) and from acclimated *ex vitro* plants (LaChlS *ex vitro*) on MDCK II and A 549 cells. Concentrations of extracts are 1mg/mL. Cytotoxicity was estimated by crystal violet assay and measured at OD 570nm.

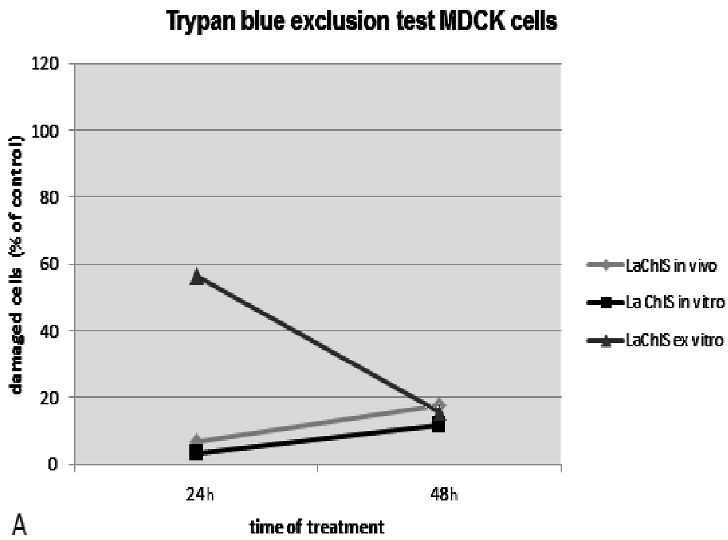
Membrane permeability assay.

We have performed trypan blue exclusion test on cells, treated with *in vivo* and *ex vitro* extracts. The results from this experiment were compared with data from our previous study on *in vitro* extracts (Veleva et al., 2015).

The *ex vitro* extract caused extensive damage on cell membranes of both cell lines. After 24h treatment more than half of cells in population were impaired (about 56% of MDCK II cells and 65% of A549 cells). At 48 h the rate of cells with damaged membranes was relatively small (near 16% for MDCK II and about 6% for A549). It is obvious that cells underwent distinct adaptation toward the *ex vitro* extract (Fig. 2, 3).

The extract obtained from the plants grown in their natural habitats showed weak activity towards the cell membranes of the both tested cell lines. Results indicated that less than 20% of the cells had compromised membrane integrity (Fig. 2, 3).

The results obtained with extracts from *in vitro* propagated plants displayed distinct difference in the way it affected membranes of the cancerous and non-cancerous cells. For MDCK II cell line the impaired cells were only 10% and for A549 – close to 100% (Fig. 2, 3).



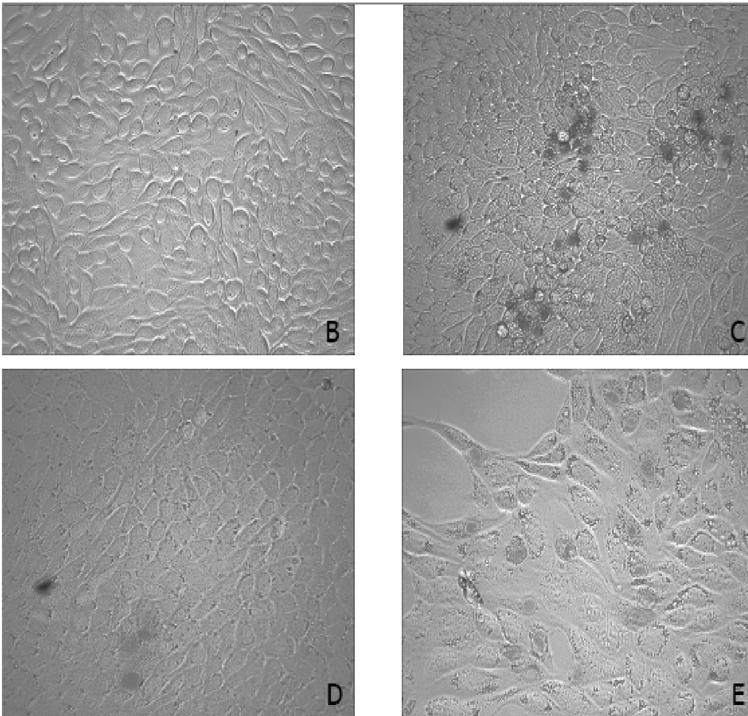


Fig. 2. Membrane permeability test with trypan blue exclusion in MDCKII cells. *A)* – quantitative analysis. (B-E) – Typical morphology of cells: untreated control (B) and treated with chloroform Soxhlet extracts obtained from: *in vitro* (C), *in vivo* (D), and *ex vitro* (E) propagated *L. album* L. plants. Concentration of all extracts is 1mg/mL

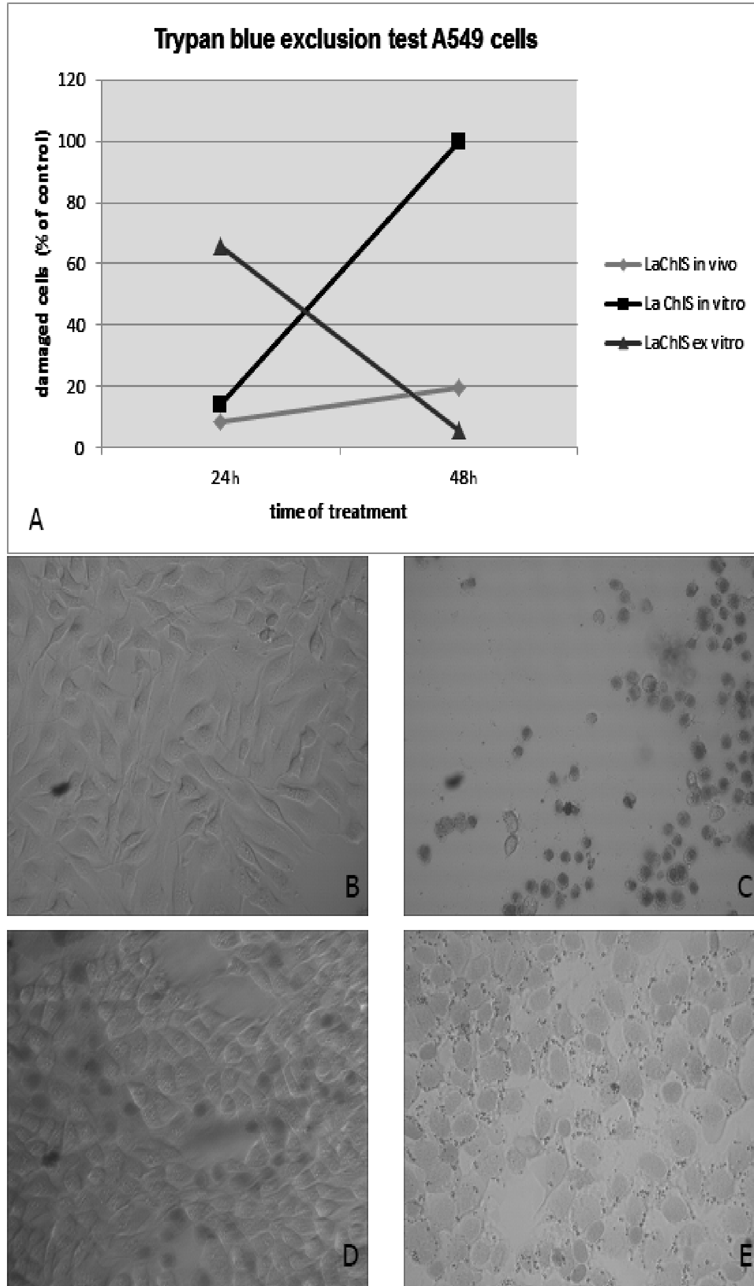


Fig. 3. Membrane permeability test with trypan blue exclusion in A549 cells. A) – quantitative analysis. (B-E) – Typical morphology of cells: untreated control (B) and treated with chloroform Soxhlet extracts obtained from: *in vitro* (C), *in vivo* (D), and *ex vitro* (E) propagated *L. album* L. plants. Concentration of all extracts is 1mg/mL

Analysis of cell morphology

The morphology of the cell lines used for this study is generally epithelial. Untreated MDCK II cells are organized in tightly formed monolayer, which corresponds to the presence of many adherent junctions and tight junctions, specific for them (Dukes et al., 2011). A 549 cells has a cobblestone epithelial morphology (Fig. 4).

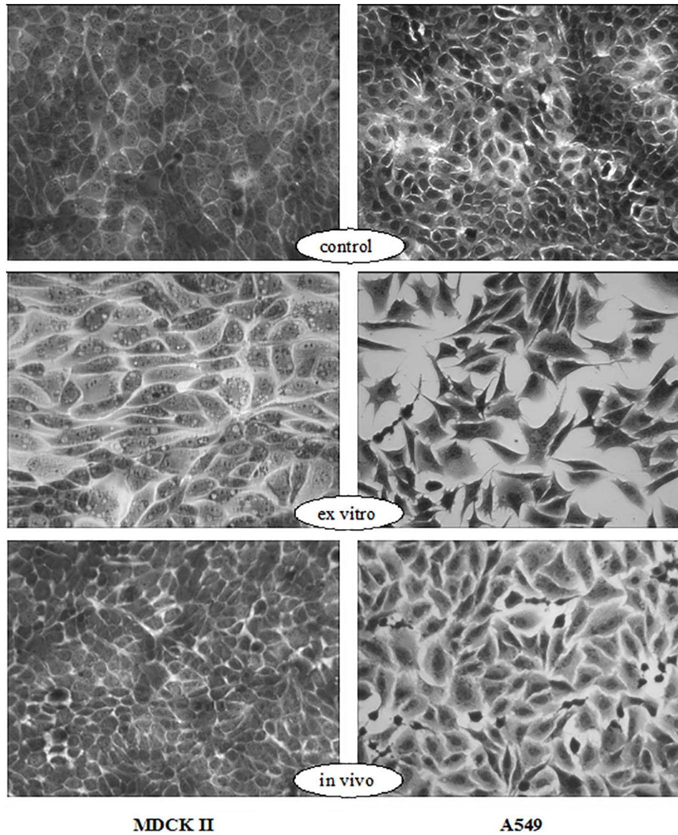


Fig 4. Morphology of MDCK II and A 549 cells. Crystal violet staining, magnification 100x. Untreated controls and cells after treatments with extracts.

Treated MDCK II cells appear more loosely spread with thin line of space between cells. Their morphology was considerably disturbed after application of *ex vitro* extract. The induced alterations included many large vesicles in light, transparent and granular cytoplasm. There were much milder changes in morphology after treatment with *in vivo* extract. The monolayers seemed confluent, with slightly dilated intercellular contacts. These results are consistent

with low cytotoxicity observed by Crystal violet assay, where only 5 to 25% reduction of cell number was detected (Fig. 4). The observed changes intensified with time.

Cancerous A549 cells were more sensitive to studied extracts and their morphological changes were more obvious. Treatment with *ex vitro* extracts led to formation of long and thin cytoplasmic outgrowths. Granular cytoplasm, elongated and shrunk cells were also observed. Extracts obtained from *plants grown in their natural habitats* caused considerably less alterations in morphology and the granular cytoplasm is the most prominent of them (Fig. 4).

Environmental conditions affect the primary plant metabolism and initiate appropriate transition in the content of secondary metabolites as well. A comparative investigation on primary and secondary metabolism in fully expanded *L. album*. leaves grown in *in situ*, *in vitro*, and *ex vitro* conditions has been performed previously (Kapchina-Toteva et al., 2014). It is demonstrated that extracts from *in vitro* grown plants have low total phenolic and flavonoid content and reduced radical scavenging activity (Valyova et al., 2011). The chloroform fraction of *ex vitro* plants has particularly low phenolic content. Phenolic compounds have potent antioxidant activity, with beneficial protective effect for cells. Shortage of phenolic compounds in the *ex vitro* extracts could be the cause of their greater toxicity towards MDCKII cells. *In vitro* extract affected mostly the membranes of cancer cells. This distinct cytotoxic effect on tumor cells could be explained by the higher content of ferulic and sinapic acid, both of which have anticancer properties (Peng et al., 2013; Senawong et al., 2013; Eroğlu et al., 2015; Fong et al., 2016). Ferulic acid is twofold less in *in vivo* extract and is not present in the *ex vitro* one. Certain substances with anticancer activity like sitosterol acetate and squalene (Ding et al., 2009, Hema et al., 2011) were found exclusively in the extracts obtained from *in vitro* propagated plants. Another substance with antitumor properties that prevails in the same extracts is α- tocopherol (Hema et al., 2011; Hollander-Czytko et al., 2005).

Eukaryotic cells rapidly repair wounds on their plasma membrane. Resealing is Ca^{2+}-dependent, and involves exocytosis followed by massive endocytosis (Castro-Gomes et al., 2016). Endocytosis of lesions is followed by intracellular degradation. Characterization of injury-induced endosomes have revealed a role for caveolae, sphingolipid-enriched plasma membrane invaginations that internalize toxin pores and are abundant in mechanically stressed cells (Andrews et al., 2014). In our results we observed many vesicles in cells after application of *ex vitro* extract and high percentage of impaired membranes as well. On 48h of treatment these vesicles were even more abundant, but the amount/number of damaged cells lowered drastically. We suspect that cells had recovered their membranes in the manner as set out above.

Our previous studies have demonstrated differences in behavior of cancerous and non-cancerous cells treated with methanol and chloroform extracts from plants grown in their natural habitats and *in vitro* propagated plants. The most considerable changes were observed in cancer cells incubated with chloroform *in vitro* extracts and potential anticancer properties were suggested. These findings are consistent with literature data for the activity of the compounds found in

the corresponding extracts. The present study showed that chloroform extracts obtained from *Lamium album* L. plants after *ex vitro* acclimation didn't show significant distinction in the response of cancerous and non-cancerous cells.

REFERENCES

1. Alipieva K, Evstatieva L, Handjieva N, Popov S, 2003. Comparative analysis of the composition of flower volatiles from *Lamium L*. species and *Lamiastrum galeobdolon Heist. ex Fabr*. Z Naturforsch C. 58:779–82.
2. Andrews NW, Corrotte M, Castro-Gomes T. 2015. Above the fray: Surface remodelling by secreted lysosomal enzymes leads to endocytosis-mediated plasma membrane repair. Semin Cell Dev Biol; 45: 10–17.
3. Andrews NW, Almeida PE, Corrotte M, 2014. Damage control: cellular mechanisms of plasma membrane repair. Trends Cell Biol. 24(12):734-42. Castro-Gomes T, Corrotte M, Tam C, Andrews NW. 2016. Plasma Membrane Repair Is Regulated Extracellularly by Proteases Released from Lysosomes. PLoS One. 11(3): e0152583.
4. Chipeva VA, Petrova DC, Geneva ME, Dimitrova MA, Moncheva PA, Kapchina-Toteva VM., 2013. Antimicrobial activity of extracts from in vivo and in vitro propagated *Lamium album* L. plants. Afr J Tradit Complement Altern Med. 10(6):559-62. eCollection 2013.
5. Dimitrova M, Zh. Yordanova, D. Dragolova, V. Kapchina-Toteva, 2010. Influence of indole butyric acid on the micropropagation of *Lamium album* L. Biotechnol. and Biotechnol. Equip. Special edition, 42-44.
6. Ding Y, Nguyen H, Kim S, Kim H, Kim Y, 2009. The regulation of inflammatory cytokine secretion in macrophage cell line by the chemical constituents of *Rhus sylvestris* Bioorganic & Medicinal Chemistry Letters 19; 3607–3610.
7. Dukes JD, Whitley P, Chalmers AD, 2011. "The MDCK variety pack: choosing the right strain." BMC Cell Biol 12: 43.
8. Eroğlu C, Seçme M, Bağcı G, Dodurga Y. 2015. Assessment of the anticancer mechanism of ferulic acid via cell cycle and apoptotic pathways in human prostate cancer cell lines. Tumour Biol. 36(12):9437-46.
9. Fong Y, Tang CC, Hu HT, Fang HY, Chen BH, Wu CY, Yuan SS, Wang HD, Chen YC, Teng YN, Chiu CC. 2016. Inhibitory effect of *trans*-ferulic acid on proliferation and migration of human lung cancer cells accompanied with increased endogenous reactive oxygen species and β-catenin instability. Chin Med. 11: 45. eCollection 2016.
10. Hema R, Kumaravel S, Alagusundaram K, 2011. GC-MS Study on the bioactive components and anti-cancer activities of *Solanum surattense*. Cancer Biology 1 (1), 13-17.
11. Hollander-Czytko H, Grabowski J, Sandorf I, Weckermann K, Weiler W. 2005 Tocopherol content and activities of tyrosine aminotransferase and cystine lyase in Arabidopsis under stress conditions, J. Plant Physiol. 162, 767-770.

12. Kapchina-Toteva V, Dimitrova M, Stefanova M, Koleva D, Stefanov D, Kostov K, Yordanova Zh, Zhiponova M. 2014. Adaptive changes in photosynthetic performance and secondary metabolites during white dead nettle micropropagation. J. Plant Physiol. 171 (15), 1344–1353.
13. Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bioassays Physiol. Plant., 15, 473-497
14. Peng CC, Chyau CC, Wang HE, Chang CH, Chen KC, Chou KY, Peng RY. 2013. Cytotoxicity of ferulic acid on T24 cell line differentiated by different microenvironments. Biomed Res Int. 2013:579859.
15. Pereira OR, Macias RIR, Perez MJ, Marin JJG, Cardoso SM, 2013. Protective effects of phenolic constituents from Cytisus multiflorus, *Lamium album* L. and *Thymus citriodorus* on liver cells. J Funct Foods 5:1170–1179
16. Senawong T, Misuna S, Khaopha S, Nuchadomrong S, Sawatsitang P, Phaosiri C, Surapaitoon A, Sripa B, 2013. Histone deacetylase (HDAC) inhibitory and antiproliferative activities of phenolic-rich extracts derived from the rhizome of *Hydnophytum formicarum Jack*.: sinapinic acid acts as HDAC inhibitor. BMC Complement Altern Med. 13:232.
17. Todorov D, Dimitrova M, Shishkova K, Yordanova Zh, Kapchina-Toteva V, Shishkov S, 2013. Comparative anti-herpes effects of the chlorophorm in vitro and in vivo extracts, derived from *Lamium album* L. Bulg J Agric Sci 19:190–193
18. Topouzova-Hristova T., V. Moskova-Doumanova, M. Keremidarska, J. Doumanov, G. Miteva, B. Petkova, V. Kapchina-Toteva. 2012. Anticancer effect of plant extracts from *Lamium album* L. by induction of cell death in vitro. Science and Technologies Vol. II, No. 1, 27-31
19. Valyova M., M. Dimitrova, Y. Ganeva, V. Kapchina-Toteva, Zh. Yordanova, 2011. Evaluation of antioxidant and free radical scavenging potential of *Lamium album* L. growing in Bulgaria. J. Pharmacy Research, 4 (4):945–947.
20. Veleva R, Petkova B, Moskova-Doumanova V, Doumanov J, Dimitrova M, Koleva P, Mladenova K, Petrova S, Yordanova Z, Kapchina-Toteva V, Topouzova-Hristova T. 2015. Changes in the functional characteristics of tumor and normal cells after treatment with extracts of white dead-nettle. Biotechnol Biotechnol Equip. 29(1):181-188.
21. Veleva R., Moskova-Doumanova V., Doumanov J., Kapchina-Toteva V., Topouzova-Hristova T., 2014. Comparative analysis of biological activity of extracts from cultivated and wild plants *Lamium album* L., Science & Technologies. 4(1):122-126
22. Xu F, 2008 Chinese medicine e.g. for treating arthropathy, comprises broad cocklebur, vervain, condyle grass, motherwort, saxifrage, cactus, mulberry branch, white dead nettle, Boston ivy, Folium photiniae, water pepper and Chinese fever vine. In: Xu F, XUFF-Individual, p 10
23. Yordanova Z.P., Zhiponova M.K., Iakimova E.T., Dimitrova M.A., Kapchina-Toteva V.M. 2014. Revealing the reviving secret of the white dead nettle (*Lamium album* L.). Phytochemistry Reviews, 13(2): 375 – 389.
24. Zhang H, Rothwangl K, Mesecar AD, Sabahi A, Rong L, Fong HH, 2009. Lamiridosins, hepatitis C virus entry inhibitors from *Lamium album*. J Nat Prod 72:2158–2162