COMPARATIVE ANALYSIS OF BIOLOGICAL ACTIVITY OF EXTRACTS FROM CULTIVATED AND WILD PLANTS *LAMIUM ALBUM L.*

R. Veleva [*], V. Moskova-Doumanova*, J. Doumanov[**], V. Kapchina-Toteva[***], T. Topouzova-Hristova*

** Department of Cytology, histology and embryology,*
*** Department of Biochemistry,*
**** Department of Plant physiology, Biological faculty, Sofia University „St. Kl. Ohridski", bul. "Dragan Tzankov" 8, 1164 Sofia, Bulgaria*
Corresponding author: topouzova@biofac.uni-sofia.bg

ABSTRACT

Lamium album L. (white dead nettle) is a plant widely used both in traditional and in folk medicine. In the scientific literature there is evidence of its broad-spectrum activity as astringent and anti-inflammatory agent, as well as a bacteriostatic and antispazmotik. This remarkable therapeutic effect was due to the wide variety of biologically active secondary metabolites of the plant. Mass collection of plants for commercial and medical purposes would jeopardize its natural populations. This requires comparison of the biological activity of extracts of plants cultured *in vitro* with those collected from natural deposits.

In our previous studies we have demonstrated the potential antitumour activity of extracts of *Lamium album* L. On cell cultires. In this study, our attention was focused on the effect of extract from plants grown under different conditions on the cellular genome, the mitochondrial activity or the adhesion of normal and carcinoma cells. Our results indicate that, despite the poorer secondary metabolite composition, *in vitro* cultivated plants showed better antitumor activity.

Key words: *Lamium album L., in vitro, extracts, anticancer, A549, HeLa, MDCK2*

Introduction

Herbal remedies are preferred because of their natural origin. In many cases, they are more accessible and more affordable for the body than conventional medications. The plants are used in the preparation of ointments, syrups, infusions, teas, as well as to make compresses and inhalations. Their action is due mainly to the secondary metabolites synthesized by plants. Secondary metabolites are a heterogeneous group of biologically active organic compounds. They are not directly involved in vital cellular biochemical processes, but perform important ecological functions (repellents, attractants, etc.) and also participates in the defense mechanisms against oxidative stress.

Recently, many studies of biological activity of *Lamium* sp. extracts with different solvents are published. There is evidences that the *Lamium album* extracts have significant antioxidant activity, and does not interfere with normal human cells [Valyova, M., et al., 2011.]. Methanol, ethyl acetate and heptane extract of *Lamium album* have non-toxic effect on a cell culture of normal human skin fibroblasts in a concentration of 25-225 μg/ml. Upon treatment with the methanol extract is registered remarkable effect of scavenging free radicals and the MTT assay showed that mitochondrial activity reached a peak at 75 mg/ml concentration of the extract [Paduch R., Matysik G. et al, 2008]. Purified ethanol extracts of *Lamium album* significantly removes ROS under conditions of oxidative stress in liver cells HepG2 [Pereira OR et al, 2013]. Chloroform and the combined extracts significantly affect cell adhesion, and cell cycle of tumor cells [Moskova-Doumanova et al., 2011]. Chloroform and the combined extracts significantly alter the cell morphology of the tumor cells, but did not affect normal. [Topouzova-Hristova et al., 2012].

Antimicrobial activity of extracts of Lamium album was Detected [Chipeva VA et al., 2013]. The methanol and ethanol extracts of *in vitro* propagated plants, have a broader spectrum of

antimicrobial activity than that of the wild plant (*in vivo)*. The opposite trend was observed in the chloroform extracts. [Chipeva VA et al., 2013]. The chloroform extracts of Lamium album L. also have antiviral activity. The extracts from *in vitro* plants possess an inhibitory effect on the replication of HSV type 1 and type 2 in MDBK cells. The extracts of wild plants also inactivate the extracellular form of HSV-1. [Shishkov S., 2013, Todorov, D., et al, 2013]

Artificial synthesis of the biologically active substance is a complicated and energy-consuming process, so preparation of herbal remedies requires retrieval from the plant material by extraction with various solvents. In order to maintain the populations of major medical plant species, *in vitro* cultivated plants can be used [Balaraju, K., et al., 2008]. Very often the process of *in vitro* propagation influenced the composition of secondary metabolites in the plant. For this reason it is important to track the differences and to compare the biological activity of extracts from cultivated plants and those grown in natural habitats. The goal of this study is to compare biological activity and potential antitumor activity of extracts of white dead nettle (*Lamium album* L.) from the field in the Lozen Mountain (described as *in vivo* plants) and *in vitro* cultured plants.

Materials and Methods

Plant material. Above-ground material was collected from mature plants from *Lamium album* L. harvested in the Lozen mountain near Sofia, Bulgaria. *In vitro* cultures were induced from sterilized mono-nodal stem segments of the mature growing wild plant. The plants were propagated under controlled environmental conditions *in vitro* [Dimitrova et al., 2010].

The aerial parts of *in vivo* and *in vitro* cultivated *L. album* L. were extracted by Soxhlet [Valyova et al., 2011] and used for investigation on the genome of cancer and normal cultured mammalian cells. Dried extracts were diluted in up to 500 μl DMSO and sonicated with ultrasound for completed dissolving. Final concentration was estimated as mg of dried extracts in ml of culture medium.

Cell cultures. Human lung cell line A549 and cervical cancer cell line HeLa were purchased from Bulgarian National Bank for Industrial Microorganisms and Cell Cultures. The normal renal epithelial cell line MDCK2 was used as control for assessment the anticancer effect of plant extracts. Cells were cultivated following the procedure described earlier [Moskova-Doumanova et al., 2012].

Cell viability test and determination of EC_{50}. The viability of cells was assessed by MTT assay, which is based on the reduction of MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of intact cells to a purple formazan product. The MTT test was performed as previously described [Moskova-Doumanova et al., 2012].

Single cell gel electrophoresis (comet assay). The slides were coated with 1% agarose (Sigma) in distilled water, by immersing vertically for 2 sec., dried until became totally transparent. At the 24 and 48 h after treatment, single cell gel electrophoresis was performed according to Singh et al. (1988) and Fairbairn et al. (1995), as described (Daza et al., 2002). Cells were analysed using ImageJ software. Two parameters were estimated for each comet: (1) integral fluorescence, which is proportional to the DNA content in the tail or head and (2) tail length. The amount of damaged DNA in each cell was calculated on the basis of the fluorescence in the tail as a percent of integral fluorescence in the comet.

Test for cell adhesion. Cells were resuspended at $1x10^4$ cell/ml in cultural media, with different concentration and combination of extracts and seeded in 24 wells cultural plates „Nunk™". On every 60 min cells from one well per concentration and combination of extracts were collected and number of unattached cells was determined by counting on haemocytometer. Results were determined as a percent of cells seeded in the well.

Assessment of membrane permeability with a modified trypan blue exclusion assay (TBE assay). After treatment with plant extracts in concentration 1 mg/ml, the samples were

washed three times with PBS to remove unadherent cells, and stained *in vivo* with 200 µl 0.8% trypan blue in PBS per well. Negative control was alveolar cells cultivated in the same plate without extracts, but with appropriate amount of DMSO (not above 2 %). As a positive control for membrane permeabilisation, A549 cells permeabilised 5 min with 0.5% Tween 20 were used. Each sample was observed under Olympus inverted microscope and several microphotographs of each well were taken immediately after staining.

Results and discussion

Sixteen phenolic compounds in different amounts were detected in methanolic extracts from *in vivo* and *in vitro* plants by HPLC-UV. Secondary metabolites in plants cultured *in vitro* were significantly less, in terms of quantity and composition. The methanol extract of in vivo plants was a very rich source of chlorogenic, sinapic, ferulic, protocatechuic and rosmarinic acids, and hesperidine. Chloroform extracts accumulated considerably less phenolic acids (data not shown).

MTT test revealed slight, statistically insignificantly higher activity in wild plants. For A549 carcinoma cells, IC50 concentrations of plant extracts after 24 h treatment were between 2 - 4 mg/ml (wild plants) and 3-3.8 mg/ml (*in vitro* propagated plants). After 48 h treatment cancer cells were more sensitive and IC50 (wild plants) were between 0.5 and 0.8 mg/ml for chloroform and methanol extracts respectively. In this case, in vitro propagated plants showed less activity: IC50 was calculated as 0.8 and 0.9 mg/ml for chloroform and methanol extracts respectively. Cytotoxic effect on HeLa carcinoma cells was observes only after 48 h treatment with IC50 values 1.65 and 1.00 mg/ml of methanol and chloroform extracts respectively for both plant sources. For MDCK2 cells, treatment for 24 h results in week cytotoxic effect of chloroform extracts only (above 2,5 mg/ml) and all samples showed activation of mitochondrial activity after 48 hours treatment.

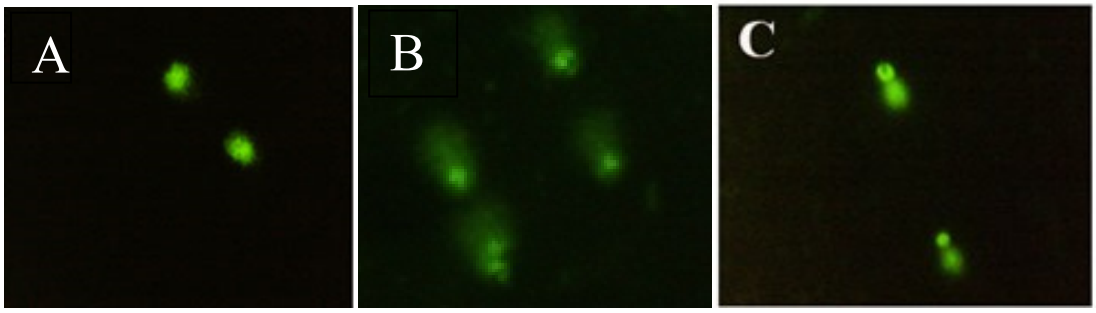

Fig.1 Typical comet assay pictures after treatment with plant extracts. A – MDCK2; B – A549 and C – HeLa cells. SibrGreen staining, magnification 200 x.

The MTT results did not revealed any significant advantages of wild or *in vitro* grown plants. Our next step was to investigate the genotoxic effect of the extracts and to compare DNA integrity using alcaline comet assay. Mean 70% DNA damage in A549 cells, 50% in HeLa and no significant DNA damage in MDCK2 cells was detected. All plant extracts again displayed similar activity. Typical comets are shown on fig. 1

The ability of cells to adhere is essential for the execution of their specific functions, and on the other hand is important to the assessment of the invasiveness of cancer cells. In the control (cells, cultivated in the absence of extracts), six hours were enough for all the cells to be attached to the surface. Cells, cultivated in the presence of different extracts had a diminished capacity to attach at a new location, compared to the untreated cells (Tabl.1). While methanol extracts affects both cancer and non-cancer cells, the chloroform extracts displayed clear anticancer activity. For cancer cells, *in vitro* propagated plants possessed stronger effect.

Extract	Cancer cells	Normal cells
Methanol *in vivo*	Slow dawn, about 20% unattached cells	Weak impact on the attachment dynamic, about 20% unattached cells
Methanol *in vitro*	Slow dawn, about 35% unattached cells	Weak impact on the attachment dynamic, less than 10% unattached cells
Chloroform *in vivo*	Suppressed in about 25% of cells	Unchanged
Chloroform *in vitro*	Suppressed in about 30% of cells	Unchanged

Tabl. 1 Effect of plant extracts on adhesive properties of cancer (A549 and HeLa) and non-cancer (MDCK2) cells.

The attachment ability of cells is strongly related the cell periphery and membrane properties. We decided to check out membrane integrity in the presence of plant extracts by TBE test. Surprisingly, chloroform extracts from *in vitro* propagated plants showed a potential antitumor activity and impaired significantly the membranes of cancer cells without affecting non-cancerous. This effect was observed only during prolonged treatment (48 h), while shorter treatments did not show statistically significant differences in the potency of wild and cultivated plants. Antitumor activity of various phytochemicals was previously discussed [Lee, K., A. Bode and Z. Dong, 2011]. It is possible chloroform extracts to have such antitumor phytochemical, but it is necessary to make further study with highly purified fractions and complete chemical analysis of this extract.

Conclusions

Secondary metabolites in plants cultured in vitro are significantly less, in terms of quantity and composition. Although the differences in the composition were obvious, no significant differences were observed in the response of the mitochondrial activity in cancerous and non-cancerous cells. Accordingly there were no significant differences in the genotoxic effect of extracts of wild and cultivated plants.

The chloroform extracts of *in vitro* cultivated plant damaging the membranes of tumor cells, without substantially affecting the non-cancer. Extracts of in vitro cultivated plant to suppress 5-15% greater initial cell adhesion of cancer cells. Methanol extracts also affect the adhesion of non-cancerous cells while chloroform extracts did not sowed significant influence on the cell adhesion processes.

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