ANTICANCER EFFECT OF PLANT EXTRACTS FROM *LAMIUM ALBUM* L. BY INDUCTION OF CELL DEATH IN VITRO

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ABSTRACT

Lamium album L. (white dead nettle) is a medicinal plant widely used in traditional and folk medicine as potent astringent, anti-inflammatory, bacteriostatic and anti-haemorrhagic agent. In our study we check the *in vitro* anticancer effect of *Lamium album* L. extracts from plants, harvested in the Losen mountain, Bulgaria and micropropagated plant specimens on cell lines with cancerous and normal origin. Our results showed a promising anticancer effect of extracts from *Lamium album* L. either natural or grown by micropropagation technique. The most powerful effect was obtained by treatment with combined methanol/chloroform extracts at concentration 2.5 mg/ml, while the application only of methanol extract (2.5 mg/ml) displayed weaker effect. This effect need more detailed further investigation.

The use of medicinal herbs in treating or preventing different complaints has rejuvenated interest in folk medicine practices, especially those transcendent across generations. *Lamium album* L. (white dead nettle) is a medicinal plant widely used in traditional and folk medicine as potent astringent and anti-haemorrhagic in reproductive tract agent [Yalcin and Kaya, 2006]. Flos extract is used as expectorant and to treat some respiratory conditions, especially with catarrh and bronchitis [Blumenthal M 1998; van Wyk and Wink, 2004; Bartram, 1998]. Beside its anti-inflammatory effect, some of active constituents of *Lamium album* have bacteriostatic and antispasmodic effects [Paduch et al., 2007]. This remarkable therapeutic effect is due to the great variety of biologically active components of the plant, which are not well studied yet. Studies on variety of medicinal plants have shown that active anti-inflammatory ingredients in water extracts include many natural chemicals such as phenols, alkaloids, glycosides, and carbohydrates. Black tea polyphenols have been demonstrated to suppress proliferation and induce apoptosis in a variety of cancer cell lines by modulating various molecular targets [Kundu et al., 2005]. Most probably the tannins in *Lamium album* are responsible for the positive effects in gynaecological use; saponins give the beneficial effects from the tea and other clinical effects of the plant extracts are due to the great variety of essential oils and glycosides [Talhouk et al., 200; Yalcin and Kaya, 2006]. However, the mechanism of action and the components involved in these effects have not been identified clearly.

The micropropagation technique is of high importance for the conservation, rapid multiplication, genetic improvement, and pharmaceutical application of medicinal plants. Interest in medicinal plants cultivation is determined by the possibility of obtaining *in vitro* secondary metabolites and their application in phytotherapy and pharmaceutical industry, especially for limited plant resources.

In our study we check the *in vitro* anticancer effect of *Lamium album* L. extracts from plants, harvested in the Losen mountain, Bulgaria and micropropagated plant specimens on cell lines with cancerous and normal origin. Our results showed a promising anticancer effect of total extracts from *Lamium album* L. either natural or grown by micropropagation technique. This effect need more detailed further investigation.

Material and methods

Plant material

Explants were collected from mature plants of *Lamium album* L. harvested in the Lozen mountain, near Sofia, Bulgaria. The voucher specimen 105183 has been deposited in the Herbarium of the Department of Botany, Faculty of Biology, Sofia University. The explants were cut into 1.0 to 2.0 cm nodal segments and used for induction of multiple shoots. Explants were washed thoroughly under running tap water for 30 min, sterilized with 0,1 % $HgCl_2$ (w/v) for 8 min, and washed three times with sterilized distiller water. Under aseptic conditions, explants were inoculated on basal MS [Murashige and Skoog, 1962] medium containing 3% (w/v) sucrose and 7g.l^{-1} agar without any supplementation of growth regulators. Plants were *in vitro* cultivated under controlled environmental conditions (16h light / 8h dark, 60 μmol. m^{-2}. s^{-1} photosynthetic photon flux density, Philips TLD-33, temperature 25ºC and 60–70% relative air humidity, RH). *In vitro* propagated *Lamium album* L. plants were collected and air-dried after four weeks cultivation. The plant materials were dried in the shade and ground in the grinder.

Preparation of plant extracts by Soxhlet extraction

Samples of 3 g of *in vivo*, *in vitro* powdered aerial plants of *L. album* L. were extracted by Soxhlet extraction with 30 ml chloroform for 8 h until full colourless and then the same plant materials were used for second extraction with methanol. Solvents were removed by rotary evaporation and dryness. Extracts were concentrated, dried and kept in the dark at 4˚ C for further experiments. Prior to usage, dried extracts were dissolved in 1ml DMSO and sonicated for complete dissolving. Final concentration was estimated as mg of dried extracts in ml of culture medium.

Cell cultures and treatment

P cells (human fetal lung-derived diploid cells) and A549 (human lung adenocarcinoma, ATCC® Number: CCL-185™) were kindly provided by the National bank for industrial microorganisms and cell cultures (Sofia, Bulgaria). The tumor cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % Fetal Bovine Serum (FBS) and antibiotic-antimycotic solution (BioWittaker, Cambrex BioScience, Belgium) at 37ºC in humidified atmosphere with 5% CO_2. The A549 cells were additionally grown in the same medium but containing 10 % Newborn Calf Serum (NCS). The P cells were cultured at the same conditions, but the medium was supplemented with 10 % NCS. All the cells were grown to 90-95 % confluence. Treatment was carried out for 24 hours with chloroform and methanol extracts diluted in culture medium at final concentration of 2.5 mg/ml, alone or in combination.

Fluorescent staining for detection of changes in membrane permeability

Changes of membrane permeability were estimated according M. Ormerod [M. Ormerod, 1994] with minor modifications. The cells, cultured on sterile coverslides and treated with diluted plant extracts were stained in vivo with the mixture of fluorochromes 5 μg/ml Proidium iodide, 2 μg/ml Hoechst 33342 and 2 μg/ml fluorescein diacetate for 5 min and were fixed with phosphate buffered formalin for 20 min in darkness. Washed slides were mounted with glycerol and observed under fluorescent microscope Nikon EZ-C1.

Giemsa staining for evaluation of cell morphology

The cells, cultured on sterile coverslides were treated with diluted plant extracts for 24 h. The samples were fixed with phosphate buffered formalin for 20 min and stained with Giemsa (BioOptca, Italy) for 5 min, dehydrated and mounted with Canada balsam. Cell morphology was observed under light microscope Olympus CX21

Results and discussion

Methanol, chloroform and combined extracts of *Lamium album* L. (Lamiaceae) in final concentration of 2.5 mg/ml for 2-24 h were tested for *in vitro* anti-cancer effect on human non-

small cell lung carcinoma (A549 cells) and normal human fetal lung-derived diploid cells (P). Our results showed insignificant changes of monolayer integrity of both cell lines after treatment with either methanol or chloroform extracts (data not shown).

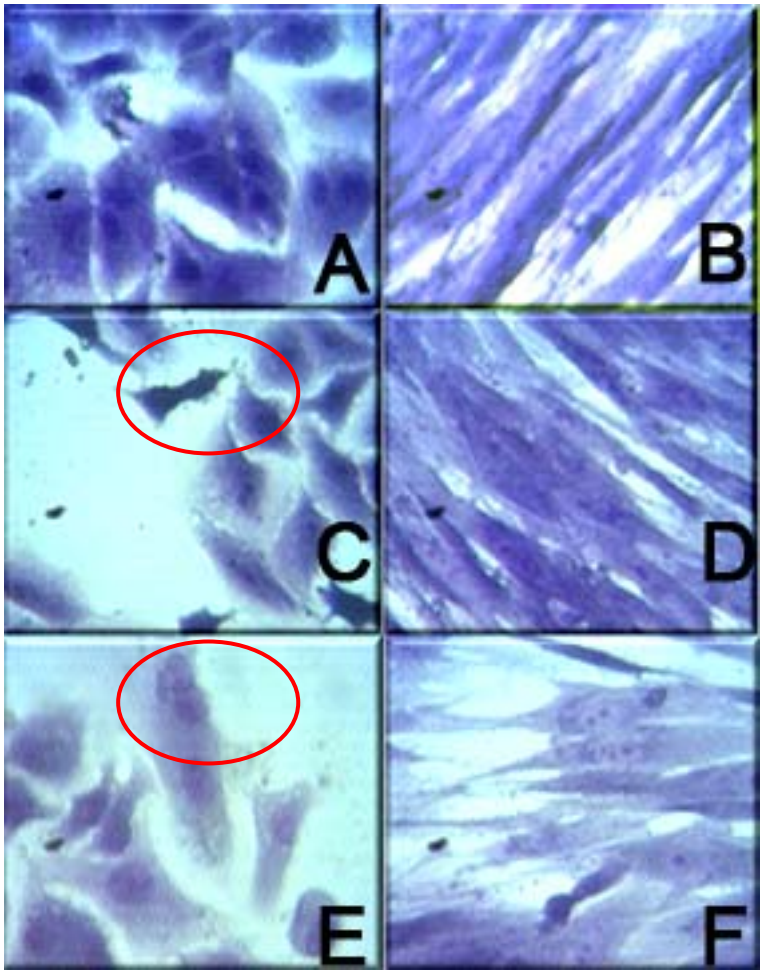

Fig. 1 Giemsa staining for visualization of changes in cell morphology.
Left - A549 cells: Control (A) and after treatment with combined methanol/chloroform extracts (C, E). Right - P cells: Control (B) and after treatment with combined methanol/chloroform extracts (D, F). Cells with impaired morphology are marked with ovals.

The morphology of non-cancer embryonic P cells was not affected significantly and they kept relatively high confluence of the cellular monolayer after treatment with sole or combined extracts (Fig. 1 B, D, F). Similar results of stimulation of human skin fibroblasts proliferation by the heptane extract of Lamii albi flos were obtained by other groups [Paduch et al. 2007]. In contrast, we observed decreasing in number of the cancer A549 cells after treatment with combined extracts at concentration of 2.5 mg/ml (Fig. 1 A, C, E). Many cells displayed morphological changes such as nuclear fragmentation (Fig. 1E), decreased volume of cytoplasm (probably due to the dehydratation, see Fig. 1C), impairment of cell attachment and disruptions in cellular monolayer. The observed morphological changes were similar using plant extracts either from micropropagated or collected from natural habitat samples.

Loss of integrity of the plasma membrane can be demonstrated either using polar dyes, such as propidium iodide (PI, red), which are excluded by an intact membrane, or fluorescent dyes such as fluorescein diacetate (FDA, green) which are retained in the cell only if the membrane is intact. FDA is uncharged and diffuses into cells. In the cytosole, it is converted to fluorescein by the

endogenous esterase activity, and being charged is retained in a viable cell. The viable cells are fluorescein positive, PI negatve and the dead cell fluorescein negative, PI positive [M. Ormerod, 1994]. The simultaneously application of both fluorochromes can help us to distinguish live and death cells in situ.

We stained A549 carcinoma cells with the mix of PI and FDA in order to evaluate the membrane integrity after treatment for 24 h with methanol, chloroform and combined extracts of *Lamium album* L. in final concentration of 2.5 mg/ml (Fig. 2).

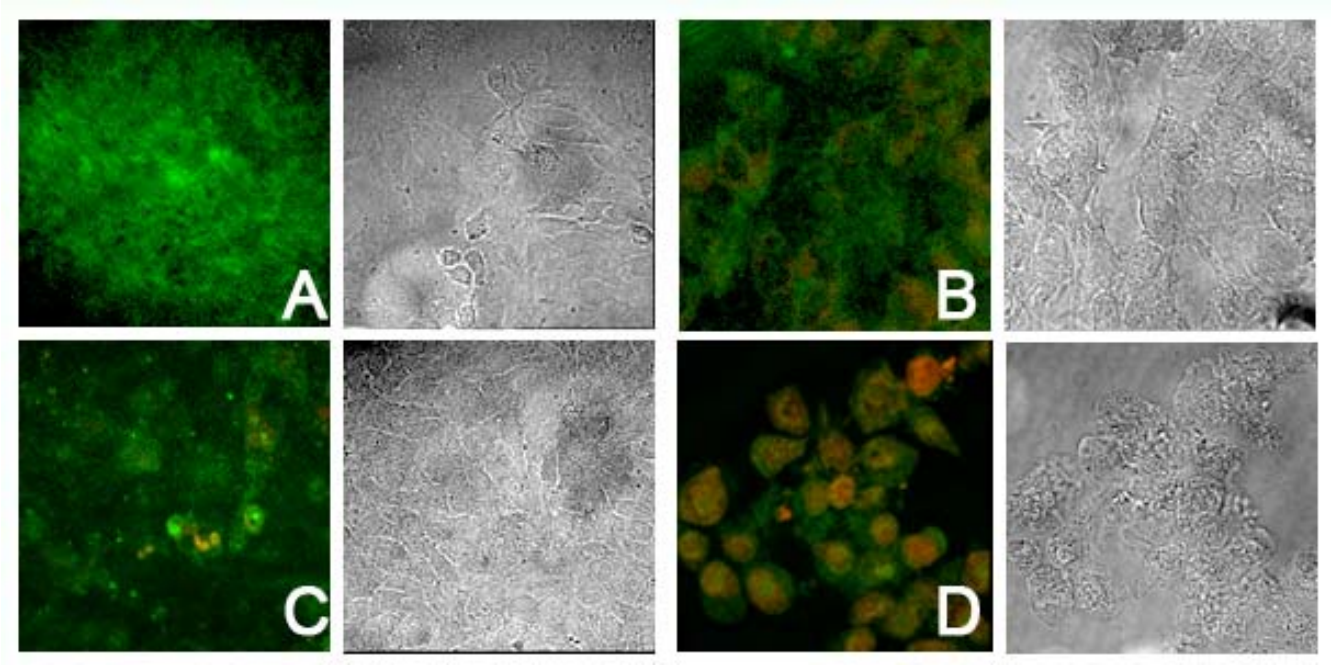

Fig. 2. Fluorescent staining (left) and DIC (right) of A549 for evaluation of cell vitality.
Control (A) and after treatment with chloroform (B), methanol (C) and combined extracts (D).

This experiment confirmed the extensive cell loss after treatment with combined extracts (Fig. 2D). Most of the cells were PI positive, indicating progression of cell death with disturbances of membrane integrity. Some of the cells had considerable chromatin clustering and nuclear fragmentation. Treatment with methanol extracts alone did not affect cellular monolayer, but significantly decrease FDA signal, indicating diminished enzyme activity in cytosole (Fig. 2C). Chloroform extracts had stronger effect, thinning out the cellular monolayer, but FDA signal slightly decrease comparing with the control cells (Fig. 2 B).

Based on all the obtained results we can conclude that the extracts of *Lamium album* L. possess potential anti-tumor effect and inhibit the development of tumor cells. This effect does not occur during treatment of normal embryonic cells, indicating a selective action of part of the herbal ingredients.

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