

COMPARATIVE ANTI-HERPES EFFECTS OF THE CHLOROFORM *IN VITRO* AND *IN VIVO* EXTRACTS, DERIVED FROM *LAMIUM ALBUM* L.

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Abstract

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Lamium album L. is a valuable medicinal plant which possesses astringent, spasmolytic, anti-inflammatory, antibiotic and bacteriostatic properties. In our study, chloroform extracts derived by Soxhlet extraction from *in vivo* and *in vitro* propagated plants were tested for antiviral activity. The extracts inhibited significantly the replication of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in MDBK cells, without apparent cytotoxicity. The extracts showed strong antiviral effect. The 50% inhibitory concentrations (IC₅₀) of the chloroform *in vitro* extract (CS) were 552 µg.mL⁻¹ and 487 µg.mL⁻¹, respectively. The IC₅₀ of the chloroform *in vivo* extract (CES) were 668 µg.mL⁻¹ and 780 µg.mL⁻¹, respectively. Viral replication was suppressed over 90% when both chloroform extracts were applied at maximal tolerated concentrations (MTC). The data suggested that extracts derived from wild plants have stronger inhibitory effects than *in vitro* extracts. The chloroform *in vivo* extract CES showed strong virucidal effect. The extract applied in MTC inactivated the extracellular HSV-1 after 5 minutes of contact at a rate exceeding 90% (Δlog1.5).

Key words: *in vitro* and *in vivo* extracts, *Lamium album*, herpes simplex virus, viral inhibitor, virucidal effect

Abbreviations: CS – chloroform *in vitro* extract, CES – chloroform *in vivo* extract

Introduction

Herpes simplex viruses type 1 and type 2 are important widespread human pathogens (Khan, 2005; Xu et al., 2006). Antiviral chemotherapy is a standard practice in the management of herpesvirus infections in humans, and currently there are about 11 licensed anti-herpetic drugs available (De Clercq et al., 2006). The most commonly used ones are the nucleoside analog acyclovir, its derivatives and cidofovir (Elion, 1993). These drugs have been well established for over two decades, however, continuous therapy leads to the development of resistant strains (Bacon et al., 2003). Current data indicate the existence of

mutant clinical strains, with cross-resistance and double-crossed resistance against these antiviral drugs (Sarasini et al., 1995). That is why the search for new therapeutic agents is an ongoing process. A special attention is focused on compounds with natural origin. The advantages of this kind of compounds over the synthetic drugs are that the occurrence of resistant strains against their action is delayed due to their complex chemical structure and their lower cytotoxicity. Many natural active compounds have been identified worldwide (Harvey, 2000; Newman, 2007; Istatkova et al., 2012). Therefore, natural products, including traditional medicinal plants, are promising potential sources of new effective antiviral drugs.

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Lamium album L. (*Lamiaceae* family) is widely used in folk medicine. It possesses a wide spectrum of therapeutic activities: anti-inflammatory, astringent, antiseptic, antibiotic, spasmolytic and anti-proliferative, which are related to the variety of biologically active substances (Paduch et al., 2007). The aqueous extract of flowering tops of *Lamium album* was found to express significantly inhibition of hepatitis C virus entry *in vitro* (Zhang et al., 2009). The use of the micropropagation is of high importance for rapid multiplication and biosynthesis of compounds with pharmaceutical importance. By controlling the environmental parameters and the components of the culture medium, it is possible to modify the metabolite content to obtain high-producing secondary metabolites genotypes (Kirakosyan et al., 2004; Arencibia et al., 2008).

Materials and Methods

Plant material. *Lamium album* was collected from the natural habitat in the Lozen Mountain, Sofia, Bulgaria and multiplied *in vitro* by mono-nodal stem segments (Dimitrova et al., 2011).

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 of antiviral activity.

Viruses and cells. Herpes simplex virus type 1, strain Vic, (HSV-1) and type 2, strain BA, (HSV-2) were supplied by NCIPD, Bulgaria. The cell line MDBK (Madin-Darby Bovine Kidney) was supplied by National Cultural Cell Bank.

Cytotoxicity assay. The cytotoxicity was determined by microscopic examination of the cell morphology in treated and untreated cultures. The maximum concentration, which did not alter the morphology of the cells, was recognized as maximum tolerable concentration (MTC) (Montanha et al., 2004). In another experiment, the cell viability was determined by the ability of the cells to cleave the tetrazolium salt MTT (Sigma-Aldrich, USA) through the mitochondrial enzyme succinate dehydrogenase which gives a formazan blue product, following the procedure described earlier (Mossman, 1983).

Cytopathogenic effect (CPE) reduction assay. Experiments were performed in multicycle growth conditions. Triplicate confluent cell monolayers in distributed in 96-well microplates were infected with 320 CCID_{50/0.1ml} of the virus. After 1 hour adsorption at room temperature the investigated extracts were added to the monolayers in the respective dilutions. The viral cytopathic effect was determined by the four-cross system when there was full destruction of the cell monolayer in the viral control. The concentration inhibiting

viral CPE by 50% (IC₅₀) with respect to the virus control was estimated from plots of the data (Serkedjieva, 1996.). The selectivity index (SI) was calculated as CC₅₀ to IC₅₀ ratios.

Effect on the extracellular virus (virucidal effect). Equal volumes of viral stock containing 10^{5.5} CCID_{50/ml} and media with MTC of the appropriate extract were mixed and incubated at 37°C for 5, 10, 15, 30, 60, 120 and 360 min. The samples were frozen and thawed. Infectious virus titers were calculated on the 48th hour of culturing by the method of Reed and Muench (Reed et al., 1938). The virucidal effect was determined by the reduction of the infectious virus titer of each sample as compared to that of the relevant viral control – equal volumes of viral stock and medium incubated as described above.

All data points represent an average of three independent assays.

Results and Discussion

Cytotoxic activity. The extracts of *L. album* were applied at concentrations ranging from 3 to 0.6 mg/mL⁻¹ and both MTC and CC₅₀ were evaluated simultaneously. The preliminary data suggested that the native extract (CES) altered slightly the cell morphology, while the *in vitro* extract caused alterations that are more significant. The MTC values of both extracts CES and CS were 1.2 and 1.0 mg/mL⁻¹ respectively (Table 1). The results obtained by the MTC assay confirmed lower toxicity of the native extract. The difference between the CC₅₀ values of the chloroform extracts was small – 1.75 mg.mL⁻¹ for CES and 1.57 mg.mL⁻¹ for CS. The reported higher cytotoxicity of the *in vitro* extracts was probably due to the composition of the medium utilized for propagation.

Table 1
Anti-herpes effects of the chloroform extracts, derived from *Lamium album*

Extract	MTC /µg/ mL ⁻¹ /	CC50 /µg/ mL ⁻¹ /	HSV-1		HSV-2	
			ID50 / µg/mL ⁻¹ /	SI	ID50 / µg/mL ⁻¹ /	SI
Chloro- phorm <i>in</i> <i>vitro</i> - CS	1000	1570	552	2.84	487	3.22
Chloro- phorm <i>in</i> <i>vivo</i> - CES	1200	1750	668	2.62	780	2.24

Antiviral activity. The data showed dose-dependent inhibition of the replication of HSV-1 and HSV-2. CS was the more effective inhibitor (Figure 1). Applied in MTC, CS inhibited almost completely the replication of HSV-1 – 97% (Δlog1.7). The other extract applied in MTC inhibited HSV-

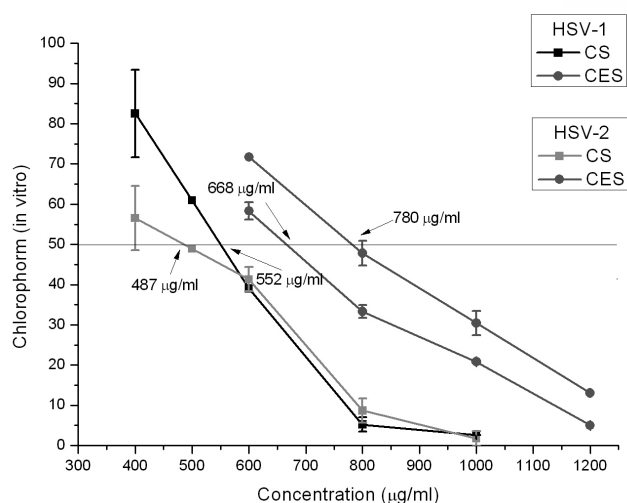


Fig. 1. Comparative effect of the extracts against HSV replication in MDBK cell

1 replication in the same mode. The values of IC_{50} of the investigated chloroform extracts were similar – the IC_{50} of CS was $552\mu\text{g}\cdot\text{mL}^{-1}$ and the IC_{50} of CES was $668\mu\text{g}\cdot\text{mL}^{-1}$ (Table 1). The influence of the investigated extracts on the replication of HSV-2 was similar (Figure 1). The different results for the inhibitory effect of the extracts against HSV-1 and HSV-2 may be due to minor differences in replication; different regulatory proteins (ICP group) and different activity of some viral enzymes of both viruses. The values of SI of the investigated extracts for both viruses did not show a significant difference – 2.24 and 3.22.

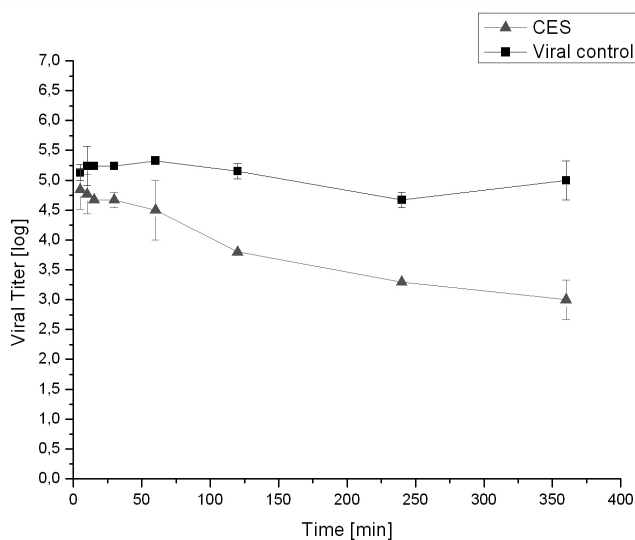


Fig. 2. Inactivation of the extracellular HSV-1 by the native chloroform extract

The inactivation on the extra cellular HSV-1 by the native chloroform extract CES exhibited after 5 min of contact (Figure 2). The influence increased with the duration of incubation period and the inactivation reached above 90% ($\Delta\log = 1$) at 60 min of contact. The extract from propagated plant did not affect the HSV-1 virions.

Conclusion

The present work revealed that the chloroform extracts derived from *Lamium album* L. propagated *in vivo* and *in vitro* showed antiviral capacity. The chloroform *in vitro* extract CS expressed strong inhibitory effect against the replication of HSV type 1 and type 2 in MDBK cells. The *in vivo* extract inactivated also the extra cellular HSV-1. The results showed that *L. album* L. could be an interesting source of natural antiviral substances with potential use in medicine. However, further investigations are needed for modulation of the synthesis of secondary metabolites in *in vitro* cultivated plants of *L. album* L., fractionation of crude extracts and identification of biologically active compounds.

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