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

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Abstract: The genus *Lamium (Lamiaceae)* comprises of about 40 species. *Lamium album* L. possesses anti-inflammatory, astringent and anti-septic activity and is widely utilized in several of the treatments. In our *in vitro* study, water extracts received by lyophilization extraction as well as ethanol extracts obtained by thermostat extraction from *in vivo* and *in vitro* propagated plants were tested for antiviral activity.

The water extracts inhibited the replication of Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in MDBK cells significantly without apparent cytotoxicity. The 50% effective concentration (EC_{50}) of the water *in vitro* extract was 1130 μg/ml for HSV-1 and 990 μg/ml for HSV-2. The EC_{50} of the water *in vivo* extract was 940 μg/ml and 1970 μg/ml, respectively. The replication of both viral types was suppressed over 95% of the *in vitro* extract applied in maximal nontoxic concentrations. The ethanol extracts shown no antiviral effect. The extract applied in MTC inactivated the entry of HSV-1 above 60% (Δlog0,5).

INTRODUCTION

Herpes simplex viruses type 1 and type 2 are important widespread human pathogens (Khan et al., 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 appearance 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 good alternative for overcoming these problems is the use of natural products which have several major advantages over the currently applied chemotherapeutics. Firstly, medicinal plant extracts are more readily assimilated by the body due to their natural origin and have fewer side effects. Moreover, development of resistant strains of such antivirals is hampered due to their complex chemical structure and often to their multi-stage mode of action (Mukhtar et al. 2008).

The genus *Lamium* comprises of about 40 species of annual and perennial herbaceous plants distributed in Europe, Asia and Africa. *L. album* is known for its rich content of flavonoids and glucosides (quercetin and tiliroside), and phenylethanoid - verbascoside (Budzianowski and Skrzypczak, 1995). Extracts from the plant exhibit anti-inflammatory, astringent and antiseptic activity (Staneva et al. 1982). Anti herpetic properties were observed for methanol and chloroform extracts (Shishkov et al. 2008, Todorov et al. 2013). The extracts affected several stages of the herpes virus life cycle – adsorption, penetration and the first two stages of the viral replication cycle. Chloroform extracts of the plant in a concentration of 1000 μg/ml blocked adsorption and penetration of the extracellular form of the virus up to 90%. The replication of both HSV type 1 and type 2, was inhibited completely after application of the same concentration. The concentrations at which 50% inhibition of viral replication was observed were around 600 μg/ml for both extracts and viral strains. The isomer lamiridosin A/B present in the aqueous extract of the flowering tops of *L. album* (100 μg/ml) was found to inhibit significantly Hepatitis C virus entry (Zhang et al. 2009). When tested in a cell line *L. album* extracts also exhibited anticancer properties. At a concentration of 5000 μg/ml, the methanol extract from the plant demonstrated the strongest effect (Moskova-Doumanova et al. 2011).

MATERIALS AND METHODS

Plant material. Aboveground material was collected from mature of *L. album* L. plants 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. *In vitro* cultures were induced from sterilized mono-nodal stem segments of the mature growing wild plant. After sterilization, the plants were propagated under controlled environmental conditions *in vitro* (Dimitrova et al., 2010), and after four weeks cultivation they were collected and air-dried. For the *in vivo* variants, mature plant material was harvested from the natural habitats and dried in the shade for grinding.

Samples of 2 g from *in vivo* and *in vitro* air-dried *L. album* L. plants were extracted by the method described by Schinella et al. (2002) - for 24 h at 40^{o}C using

the following solvents - methanol and water. After filtration the raw materials were extracted twice in the same conditions. The solvents were removed under vacuum evaporator, and the extracts were concentrated, dried and kept in dark at 4°C.

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 the 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 (Mosmann, 1983).

Cytopathogenic effect (CPE) reduction assay. Experiments were performed in multicycle growth conditions. Triplicate confluent cell monolayers 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 the full destruction of the cell monolayer in the viral control. The concentration inhibiting viral CPE with 50% (IC_{50}) in comparison to the virus control was estimated from plots of the data (Serkedjieva, 1996.). The selectivity index (SI) was calculated as CC_{50} to IC_{50} 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 at the 48th hour of culturing by the method of Reed and Muench (Reed and Muench, 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.

Effect on the adsorption of extracellular virus. Equal volumes of viral stock containing $10^{5,5}$ $CCID_{50}$/ml and media with MTC of the appropriate extract and viral control of equal volumes of viral stock containing $10^{5,5}$ $CCID_{50}$/ml and media were applied on confluent cell monolayers for 15', 30', 45', 60' and 120 minutes. After the particular time interval has passed the medium with the virus was removed and the monolayer was washed three times. At the 48th hour, all samples and controls were frozen and titrated to determine the infectious titer of the virus by the method of Reed and Muench (Yarmolinski et al., 2009, Reed and Muench, 1938).

RESULTS AND DISCUSSION

Cytotoxic activity. The extracts of *L. album* were applied at concentrations ranging from 5000 to 500 µg/ml and both MTC and CC_{50} were evaluated simultaneously. The preliminary data suggested that the ethanol extracts altered stronger the cell morphology, while the water extracts caused less significant alterations. The MTC value of both *in vitro* and *in vivo* ethanol extracts ethanol was 1000 µg/ml. For water extracts the value was 2000 µg/ml (Table 1). The results obtained by the CC_{50} assay confirmed lower toxicity of the water extracts. The difference between the CC_{50} values of the ethanol extracts was small – 1732 µg/ml for one obtained from *in vitro* cultivated plant and 1803 µg/ml for native one. For water extracts the values are 2620 µg/ml and 5000 µg/ml, respectively (Figure 1). The reported higher cytotoxicity of the *in vitro* extracts was probably due to the composition of the medium utilized for propagation.

Table 1. The Antiherpes effect of extracts, derived from *L. album* L.

Extract	MTC /µg/ml/	CC_{50} /µg/ml-/	HSV-1		HSV-2	
			ID_{50} /µg/ml-/	SI	ID_{50} /µg/ml/	SI
Ethanol *in vitro*	1000	1732	no effect	no effect	no effect	no effect
Ethanol *in vivo*	1000	1803	no effect	no effect	no effect	no effect
Water *in vitro*	2000	2620	1130	2,32	990	2,65
Water *in vivo*	2000	5000	940	5,32	1970	2,54

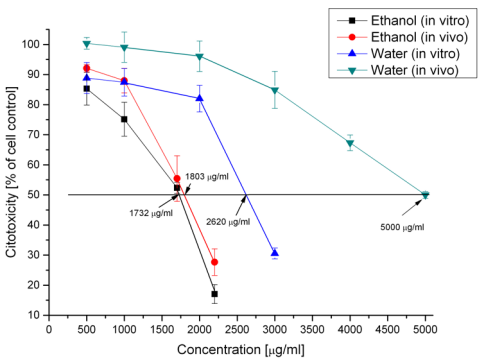

Figure 1. Comparative cytotoxicity effect of the extracts over MDBK cell.

Antiviral activity. The data showed dose-dependent inhibition of the replication of HSV-1 and HSV-2 from the both water extracts. When applied in MTC the *in vitro* extract inhibited almost completely the replication of HSV-1/2 – over 95%. The other extract applied in MTC inhibited less the replication of both viruses. The values of IC_{50} against HSV-1 of the investigated water extracts were similar – the IC_{50} of the *in vitro* extract was 1130 µg/ml and the IC_{50} of the *in vivo* extract was 940 µg/ml (Table 1). The influence of the investigated extracts on the replication of HSV-2 was similar (Figure 2b). 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 to both viruses. The values of SI of the investigated extracts for both viruses did not show a significant difference around 2.5. The only exception is native the water extract with a SI of 5,32 against HSV-1.

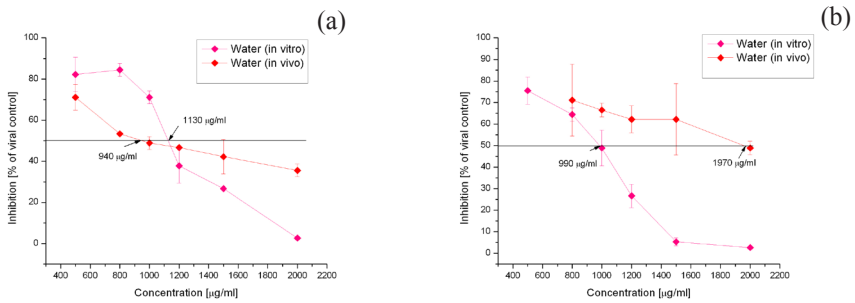

Figure 2. Comparative effect of the extracts against HSV-1 (a) and HSV-2 (b) replication in MDBK cell.

The inactivation of the adsorption of HSV-1 by the in vitro water extract exhibited effect after 15 minutes of contact (Figure 3). The influence decreased with the duration of the incubation period and the inactivation reached 0% after 60 minutes of contact.

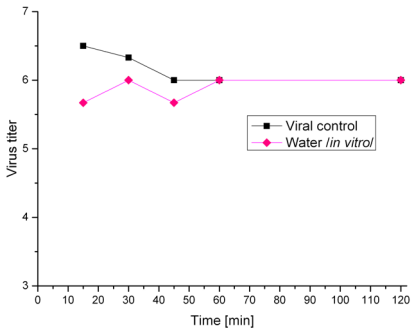

Figure 3. Inactivation on the viral adsorption of HSV-1 by the *in vitro* water extract.

CONCLUSION

The present work revealed that the water extracts derived from *Lamium album* L. propagated *in vivo* and *in vitro* possess antiviral capacity. The water *in vitro* extract expressed strong inhibitory effect against the replication of HSV type 1 and type 2 in MDBK cells, inhibiting over 95% viral replication when applied in the MTC. In addition to that the same extract inactivated also the adsorption of HSV-1 virions. The difference in activity between ethanol extracts and previously described methanol and chloroform extracts (Todorov et al., 2013) can be due to differences in the affinity of active molecules towards the extraction agent. The results showed that *L. album* 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*, fractionation of crude extracts and identification of biologically active compounds.

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